Attenuation of the Acute-Phase Response in Thermally Injured Rats by Cholesterol-Containing Cationic Liposomes Used as a Delivery System for Gene Therapy

Marc G. Jeschke, MD; Robert E. Barrow, PhD; J. Regino Perez-Polo, PhD; David N. Herndon, MD

Hypothesis: Cholesterol-containing cationic liposomes alone modulate the acute-phase response and cytokine expression in thermally injured rats and are an effective delivery system for gene therapy in trauma.

Setting: Laboratory.

Intervention: Fifty-six adult male Sprague-Dawley rats with a full-thickness scald burn covering 60% of total body surface area were randomly divided into 2 groups to receive either intravenous injections of cholesterol-containing cationic liposomes or saline (control).

Main Outcome Measures: Body weights, muscle and liver dry-wet weights, serum levels of constitutive hepatic proteins, acute-phase protein levels, and cytokine levels were determined at 1, 2, 5, and 7 days after thermal injury.

Results: Rats receiving cholesterol-containing cationic liposomes had less body weight loss, increased serum transferrin levels, and decreased serum α1-acid glycoprotein levels when compared with controls (P<.05). Serum interleukin 1β and tumor necrosis factor α levels were decreased in rats receiving liposomes at 1 and 2 days after burn compared with controls (P<.05).

Conclusions: These results suggest that cholesterol-containing cationic liposomes alone may have a beneficial effect in modulating the hypermetabolic response after burn injury by decreasing type 1 acute-phase proteins and the expression of the proinflammatory cytokines interleukin 1β and tumor necrosis factor α. Therefore, cholesterol-containing cationic liposomes appear to be suitable as a delivery system for gene therapy in trauma.

Arch Surg. 1999;134:1098-1102

GENE THERAPY is a promising approach for the treatment of various clinical disorders. The success of this approach depends on the selection of appropriate vectors for gene delivery. Viruses have been used as delivery vectors because of their specificity and tissue penetration via consecutive cell transfection. The disadvantages of viral vectors, such as viral infection–associated toxic effects, immunological compromise, and mutagenic or carcinogenic effects, restrict its potential use in humans. Recent modifications to incorporate cholesterol into cationic liposomes have increased their efficiency of transfection to levels previously achieved only with adenoviral constructs. Thus, liposomes have now become an efficient delivery system for gene therapy in the fields of oncology, neurology, and cardiology. The use of liposomal gene complexes in trauma is a new approach to improve clinical outcome and mortality. The suitability of liposomes as a delivery system for gene therapy in trauma has not been defined. We attempted to determine the suitability of liposomes as a delivery system for gene therapy by measuring the effects of liposomes on the hepatic acute-phase response and cytokine concentrations.

The hepatic acute-phase response represents a cascade of events characterized by the up-regulation of types 1 and 2 acute-phase proteins, mediated by proinflammatory cytokines, and the down-regulation of constitutive hepatic proteins. These events are initiated to restore homeostasis after trauma. In clinical studies, however, it has been shown that a sustained or increased acute-phase response may contribute to multiple-organ failure, hypermetabolism, morbidity, and mortality. Thus, the uncontrolled and prolonged action of proinflammatory cytokines and acute-phase proteins is potentially dangerous. An attenuation of the overexpression of proinflammatory and acute-phase proteins thus may be beneficial, whereas an increase may be detrimental.
MATERIALS AND METHODS

Fifty-six adult male Sprague-Dawley rats (weight, 350-375 g) were studied according to a blinded protocol. Rats were housed in wire-bottom cages and kept in a temperature-controlled room with a 12-hour light-dark cycle. All animals were acclimatized to their environment for 7 days before the start of the study. All received the same amount of a liquid diet (Sustacal; Mead Johnson Nutritionalals, Evansville, Ind.) and water ad libitum throughout the study.

Rats received full-thickness scald burns covering 60% of their total body surface area as previously described and were randomly divided into 2 groups to receive injections of (1) cholesterol-containing cationic liposomes (20 µL of liposomes in 180 µL of isotonic sodium chloride solution intravenously; n = 28) or (2) isotonic sodium chloride solution (control, 200 µL intravenously; n = 28).

To determine the time course of liposomal effects, liposomes were injected intravenously into the tail vein 0.5 hour after the thermal injury and changes were examined during a 7-day period. The content of the injected solution was unknown to the investigators. The liposomes were a cholesterol-containing cationic type, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMRIE-C Reagent; Life Technologies, Rockville, Md.), prepared with cholesterol membrane-filtered water. The doses used were 10% liposomes; this is the highest concentration used in DNA transfer experiments that does not have deleterious consequences on DNA solubility and is compatible with gene transfer paradigms. The volume of 200 µL is an amount that can be administered into the tail vein of a rat without causing deleterious side effects, such as cardiac arrest.

Body weights were measured at the same time each day. Rats were killed by decapitation 1, 2, 3, or 7 days after burn. Blood was collected into serum and plasma separators, spun at 1000g for 15 minutes, decanted, and frozen at −73°C. Liver and gastrocnemius muscle tissues were harvested, weighed, and sectioned and samples of each were dried at 60°C to a constant weight. The dry-wet weight ratios were used to estimate protein content.

Serum cholesterol and free fatty acids were measured on a nephelometer (Nephelometer 100; Behringwerke AG, Marburg, Germany).

Serum acute-phase proteins (haptoglobin and α,2-macroglobulin), constitutive hepatic proteins (total protein, transferrin, and albumin), and glucose were measured on a nephelometer (Behringwerke AG). Serum α,1-glycoprotein level was determined by enzyme-linked immunosorbent assay (Wako Chemicals Inc, Richmond, Va). The standard curve for rat α,1-glycoprotein concentrations was linear from 0 to 1500 pg/mL on a logarithmic scale.

Plasma TNF-α levels were determined by enzyme-linked immunosorbent assay (Endogen, Woburn, Mass). The standard curve used for the quantification of rat TNF-α was linear from 0 to 833 pg/mL on a logarithmic scale. Serum IL-1β levels were determined by enzyme-linked immunosorbent assay (Biosource Int, Camarillo, Calif); its standard curve used for the quantification of rat IL-1β was linear from 0 to 1500 pg/mL on a logarithmic scale. Serum IL-6 level was determined by bioassay by means of B9 cells (mouse hybridoma line) in log phase of growth treated with increasing serum concentrations. Cell proliferation in response to additional serum was measured spectrophotometrically by the attenuated mitochondrial metabolism of tetrazolium salt.

The study was reviewed and approved for humane animal treatment by the Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, assuring 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 Student’s t test with Bonferroni correction. Data are expressed as mean ± SEM. Significance was acceptance at P < .05.

There is evidence that cholesterol-containing cationic liposomes, a delivery system for gene therapy, can exert a beneficial effect during the hypermetabolic response by increasing body weight and muscle protein concentration. We therefore propose that cholesterol-containing cationic liposomes modulate constitutive hepatic proteins, acute-phase proteins, proinflammatory cytokines (interleukin [IL] 1β, IL-6, and tumor necrosis factor [TNF] α), and losses in body weight during the hypermetabolic response and are therefore an effective delivery system for gene therapy in trauma. To test this hypothesis, we measured the effect of cholesterol-containing cationic liposomes on the hepatic acute-phase response in thermally injured rats.

RESULTS

All rats survived the 60% total body surface area scald burn and experimental drugs with no evidence of deleterious side effects. The percentage change in total body weight increased in both groups during the first 2 days, followed by a decrease in body weight 2 days after burn. Rats receiving liposomes maintained their body weight better than the saline-treated controls (significant on days 3 and 4 at P = .01 and on day 5 at P = .02) (Figure 1). There were no differences in dry-wet ratios between liposome-treated and control animals for gastrocnemius muscle and liver.

Serum total protein concentration decreased in both groups after burn injury. Rats receiving liposomes showed an increase in serum total protein level 5 days after burn (liposomes, 56 ± 1 g/L, vs controls, 52 ± 1 g/L) (P = .03). Serum transferrin level decreased after burn injury to below normal in both groups. Those treated with liposomes showed a significant increase in serum transferrin level at 2 and 5 days after burn compared with controls (P = .004 and P = .02, respectively) (Figure 2). There were no significant differences in serum albumin level between groups. No significant difference in serum cholesterol, free fatty acid, and glucose levels could be demonstrated throughout the study period.

Type 1 acute-phase proteins, serum haptoglobin, and α,1-glycoprotein levels increased after thermal injury. Liposomes decreased serum α,1-glycoprotein lev-
els 1 and 5 days after burn (P = .002 and P < .001, respectively) (Figure 3). There were no significant differences in serum haptoglobin levels between rats receiving liposomes and controls. Type 2 acute-phase protein increased in both groups after burn by nearly 50%. There were no differences in serum α2-macroglobulin levels between liposome-treated animals and control animals. Levels of all measured cytokines increased after the thermal injury. Serum IL-1β level decreased during the first day after burn (P = .002) (Figure 4) and serum TNF-α level at 1 and 2 days after burn in rats receiving liposomes compared with controls (P = .01) (Figure 5). No change in serum IL-6 level was observed in liposome-treated rats compared with controls.

**COMMENT**

One major contributor to the hypermetabolism associated with a thermal injury is the increase in acute-phase proteins and cytokines. In this in vivo study, we showed that administration of cholesterol-containing cationic liposomes improved body weight and the expression of the constitutive hepatic proteins transferrin and total protein after burn compared with saline. We further showed that liposomes decrease serum levels of type 1 acute-phase proteins and the proinflammatory cytokines responsive to type 1 acute-phase proteins, serum TNF-α.
and IL-1β. It is likely that the decrease in IL-1β and TNF-α levels subsequently leads to a decrease in levels of these acute-phase proteins. No effect of liposomes on type 2 acute-phase proteins and the proinflammatory cytokine IL-6 could be shown, indicating a partial effect of liposomes on the hepatic acute-phase response.

Although the reasons for the beneficial effects associated with liposomes are not fully understood, they may result from the direct effect of the liposomal lipid moieties on damaged cell membranes or from an enhancement of the uptake of extracellular nutrients and the in situ encapsulation and protection of the endogenous growth factors and cytokines elaborated locally as part of the hypermetabolic response that is triggered by acute-phase proteins and cytokines. Proinflammatory cytokines, in particular TNF-α, inhibit protein synthesis and induce weight loss. After a thermal injury, as well as in sepsis, TNF-α serum levels increase along with sepsis-induced muscle proteolysis. Decreased serum TNF-α levels are associated with an improvement in net protein balance and a reduction in body weight loss in thermally injured pediatric patients. Thus, as shown in this study, a decrease in serum TNF-α level may preserve body weight and increase serum transferrin levels. Several studies have attempted to determine the mechanisms by which cationic liposomes exert an inhibitory effect on cytokine expression in vitro; however, the exact mechanisms are currently not defined. It seems likely that nuclear factor κB (NF-κB) plays an important role. The NF-κB is a transcription signal and is crucial in the development of the cellular immune and inflammatory response. Because of electrostatic interactions between cells and cationic liposomes, the liposomes bind to the receptor for oxidized low-density lipoproteins. Sambrano and Steinberg have shown that this competitive binding and subsequent activation of the oxidized low-density lipoprotein receptor indirectly suppress activation and/or binding of NF-κB to its cognate DNA site. Furthermore, it has been shown that cationic liposomes inhibit tyrosine phosphorylation of p41, a protein of the mitogen-activated protein kinase transcription factor family, which consecutively leads to a down-regulation of NF-κB. The inhibition of p41 and NF-κB inhibits the induction of inducible nitric oxide synthetase, which subsequently decreases nitric oxide expression. These changes in the signal pathway have been hypothesized to be responsible for the selective inhibition of TNF-α expression at the posttranscriptional level. Given that nitric oxide and inducible nitric oxide synthetase stimulate IL-1β expression, it seems likely that the inhibition of nitric oxide or inducible nitric oxide synthetase activity through cationic liposomes leads to decreased IL-1β expression.

The administration of cholesterol-containing cationic liposomes modulates the hypermetabolic response by affecting cytokine expression, although the effects of the injection are not likely to persist beyond 5 days. There were no differences in serum cytokine and protein level 7 days after burn between liposome-treated and control animals. Furthermore, serum transferrin decreased from day 5 to day 7 after burn in rats receiving liposomes, whereas control-treated animals showed an increase in serum transferrin levels from day 5 to day 7 after burn. In addition, serum haptoglobin level increased from day 5 to day 7 in the liposome-treated group, while serum haptoglobin level decreased during the same period in control animals.

In summary, cholesterol-cationic liposomes increased levels of constitutive hepatic proteins and decreased levels of type 1 acute-phase proteins, with associated decreases in IL-1β and TNF-α levels. Thus, cholesterol-cationic liposomes appear to be suitable as a delivery system for gene therapy in trauma, because liposomes favorably modulate the trauma-induced hypermetabolic response and do not display the cytotoxic effects typically associated with the use of other cationic liposomes in vivo. We therefore suggest that cholesterol-cationic liposomes should be used as a vector-delivery system for gene therapy in the trauma-induced hypermetabolic response.

This study was supported by grants from the Shriners Hospitals for Children, Galveston, Tex, and the Clayton Foundation for Research, Houston, Tex.

Corresponding author: Robert E. Barrow, PhD, Shriners Hospital for Children, 815 Market St, Galveston, TX 77550 (e-mail: RBarrow@sbi.utmb.edu).

### REFERENCES


IN OTHER AMA JOURNALS

INSTRUCTION

JAMA

Interpretation of Genetic Test Results for Hereditary Nonpolyposis Colorectal Cancer: Implications for Clinical Predisposition Testing

Sapna Syngal, MD, MPH; Edward A. Fox, PhD; Christine Li, MD; Marisa Dovidio, BS; Charis Eng, MD, PhD; Richard D. Kolodner, PhD; Judy E. Garber, MD, MPH

Context: Genetic testing for cancer predisposition is evolving from purely research applications to affecting clinical management.

Objective: To determine how often genetic test results for hereditary nonpolyposis colorectal cancer (HNPCC) can be definitively interpreted and used to guide clinical management.

Design: Case-series study conducted in 1996 to 1998 in which a complete sequence analysis of hMSH2 and hMLH1 coding sequences and flanking intronic regions was performed. Mutations were categorized as protein truncating and missense. In the case of missense alterations, additional analyses were performed in an effort to assess pathogenicity.

Setting and Participants: Families were identified by self-referral or health care provider referral to a cancer genetics program. Participants and kindreds were classified into 1 of 4 categories: (1) Amsterdam criteria for HNPCC, (2) modified Amsterdam criteria for HNPCC, (3) young age at onset, or (4) HNPCC-variant. In addition, each proband was classified according to the Bethesda guidelines for identification of individuals with HNPCC.

Main Outcome Measure: Alterations of hMSH2 and hMLH1 genes.

Results: Twenty-seven alterations of hMSH2 and hMLH1 were found in 24 of 70 families (34.3%). Of these, deleterious mutations that could be used with confidence in clinical management were identified in 25.7% (18/70) of families. The rates of definitive results for families fulfilling Amsterdam criteria, modified Amsterdam criteria, young age at onset, HNPCC-variant, and Bethesda guidelines were 27 (39.3%), 13 (18.2%), 12 (16.7%), 11 (15.8%), and 21 (30.4%), respectively. The prevalence of missense mutations, genetic heterogeneity of the syndrome, and limited availability of validated functional assays present a challenge in the interpretation of genetic test results of HNPCC families.

Conclusions: The identification of pathogenic mutations in a significant subset of families for whom the results may have marked clinical importance makes genetic testing an important option for HNPCC and HNPCC-like kindreds. However, for the majority of individuals in whom sequence analysis of hMSH2 and hMLH1 does not give a definitive result, intensive follow-up is still warranted. (1999;282:247-253) www.jama.com

Corresponding author and reprints: Judy E. Garber, MD, MPH, Dana Farber Cancer Institute, 44 Binney St, Smith 208, Boston, MA 02115 (e-mail: judy_garber@dfci.harvard.edu).