Development of the Liver- and Lobe-Selective Nonviral Gene Transfer following the Instillation of Naked Plasmid DNA Using Catheter on the Liver Surface in Mice

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Purpose. The present study has undertaken the liver- and lobe-selective nonviral gene transfer following the instillation of naked plasmid DNA (pDNA) using catheter on the liver surface in mice.
Methods. The polyethernylon catheter was inserted intraperitoneally through the abdominal wall and was retained on the surface of the liver right and left medial lobes. pDNA was administered through the catheter to the liver right and left medial lobes.
Results. The luciferase levels produced in the applied liver lobes at 6 h after liver surface instillation of pDNA were significantly higher than those produced in other liver lobes and other tissues assayed, and ranged from approximately 5 folds higher in other lobes to 20–30 folds higher in other tissues. Following liver surface instillation of pDNA at a time from 2 to 24 h or at a volume from 15 to 60 μl, the gene expressions of the applied liver lobes were always significantly higher than those of other liver lobes and other tissues.
Conclusion. We have demonstrated the liver- and lobe-selective gene transfection following the instillation of naked pDNA using catheter on the liver surface in mice.

KEY WORDS: gene therapy; liver; plasmid DNA; transfection; mice; luciferase.

INTRODUCTION

Although relatively low efficiency *in vivo* is the main limiting factor of nonviral gene transfer, plasmid DNA (pDNA) has advantages in its safety compared with viral vectors, which are liable to undergo mutation, thereby reacquiring the ability to produce infection. Recently, >50% of all current clinical gene therapy trials are for cancer treatment (1). To improve the therapeutic properties of gene therapy, a delivery system of pDNA must be developed to target the localized site of the organ. Naked pDNA is the simplest and safest nonviral gene transfection system, and accordingly various studies have been evaluated for *in vivo* gene transfer using naked pDNA (2). Although it has been reported that the liver- and site-selective gene transfection has been achieved by the electroporation (3) and gene gun (4), there is great concern about liver safety when any electrical or physical force is involved consequently the continuous administration of pDNA is limited. Taking these into consideration, safety against the organ as well as the site-selective gene transfection system must be developed to achieve efficient *in vivo* gene therapy for clinical application.

Previously, we reported on liver- and lobe-selective gene transfection following the instillation of pDNA to the liver surface, by cutting the abdomen of mice in an experimental model (5). In this novel transfection system via the instillation on the liver surface, non-invasion against the organ as well as liver site-selective gene transfection was achieved, and therefore a novel administration method of pDNA to the liver surface must be developed for the clinical application. Moreover, the damage in the experimental animals should be decreased as a condition of administration.

In this study, we tried the liver- and lobe-selective gene transfection after the instillation of pDNA using a catheter to the liver surface without cutting the abdominal wall in mice.

MATERIALS AND METHODS

Materials

All chemicals were of the highest purity available.

Construction and Preparation of pDNA (pCMV-Luc)

pCMV-Luc was constructed by subcloning the Hind III/XbaI firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the *E. coli* strain DH5α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

In Vivo Gene Expression Experiments

All animal procedures in this study conformed to the Guidelines for Animal Experimentation in Nagasaki University. Five-week-old ddY male mice (22.0~35.0 g) were anesthetized with sodium pentobarbital (40~60 mg/kg). As shown in Scheme 1, the middle abdominal skin was cut open. Then, the polyethernylon catheter (SURFLO® I.V. Catheter, Terumo, Tokyo, Japan) was inserted intraperitoneally through the abdominal wall and was retained on the surface of the liver right and left medial lobes. Also, the instillation point of pDNA at the surface of small intestine was about 2.5 cm below the instillation point at the liver surface. pDNA (15~90 μl) was administered through the catheter to the liver right and left medial lobes at doses of 5, 10, 15, 30, and 60 μg. The administration volume of pDNA was adjusted by micropipette (PIPETMAN®, GILSON, Villier-le Bel, France). Mice were kept lying on their back for 1 h, then they were freed in the cage. At appropriate time intervals, mice were sacrificed, and the liver, kidney, spleen, heart, and lung were removed. Then, the right and left medial lobes (applied liver lobes) was separated from other liver lobes. The tissues were washed twice with saline and homogenized with a lysis buffer. The lysis buffer consisted of 0.1 M Tris/HCl buffer (pH 7.8)

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containing 0.05% Triton X-100 and 2 mM EDTA. The volumes of the lysis buffer added were 4 μl/mg for each liver lobe and 5 μl/mg for other tissues. After three cycles of freezing and thawing, the homogenates were centrifuged at 15,610 × *g* for 5 min. The supernatants were stored at –20°C until the luciferase assays were performed. Twenty microliters of supernatant was mixed with 100 μl luciferase assay buffer (Picagene®, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9506, EG & G Berthold, Bad Wildbad, Germany). The luciferase activity is indicated as the relative number of light units per gram tissue.

Statistical Analysis

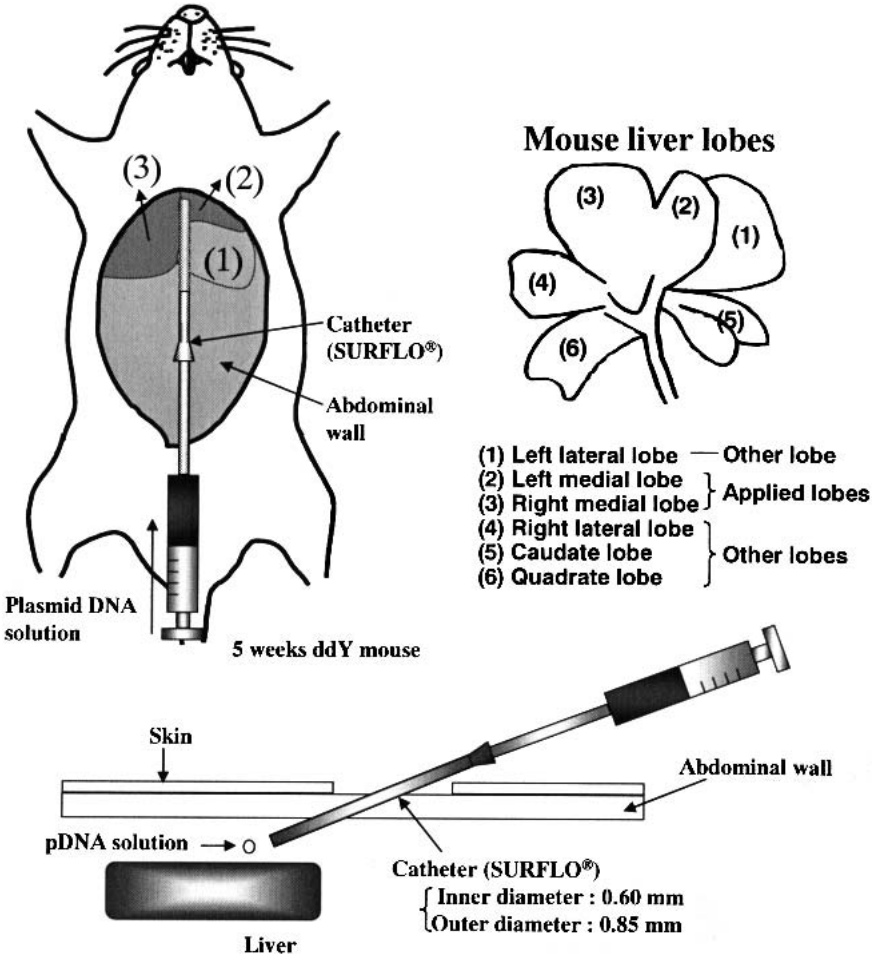
Statistical comparisons were performed by analysis of variance and by Dunnett's test. $p < 0.05$ was considered to be indicative of statistical significance.

RESULTS AND DISCUSSION

In this study, we evaluated the *in vivo* gene transfection using a catheter without cutting the abdomen in mice. The catheter was inserted on the liver right and left medial lobes, which are most outward, to administer the pDNA solution exactly (Scheme 1).

Figure 1 shows the gene expression in the applied liver lobes, other liver lobes, kidney, spleen, heart, and lung at 6 h after liver surface and small intestine surface instillation of pDNA using catheter at a dose of 30 μg into mice. The gene expression levels represent more than 2×10^3 RLU/g tissues because each tissue mixed with substrates without the instillation of pDNA showed approximately 2×10^3 RLU/g tissues (5). Therefore, the results represent $>10^4$ RLU/g tissues in Fig. 1, which was considered as stable gene expression. The luciferase levels produced in the applied liver lobes after liver surface instillation of pDNA were significantly higher than those produced in other liver lobes and other tissues assayed, and ranged from approximately 5-fold higher in other liver lobes to 20–30 folds higher in other tissues (Fig. 1 A). After small intestine surface instillation of pDNA, on the other hand, the gene expression was detected in small amounts ($<10^4$ RLU/g tissue) in all tissues assayed (Fig. 1 B). The small intestine surface instillation is comparable to the intraperitoneal administration; therefore, the result of Fig. 1 B agrees with those of a previous study on the gene expressions after intraperitoneal administration of pDNA (6).

Figure 2 shows the time course of gene expression in the applied liver lobes and other liver lobes until 48 h after liver surface instillation of pDNA using a catheter at a dose of 30 μg into mice. The gene expressions of the applied liver lobes



Scheme 1. Experimental methods and mouse liver lobes.

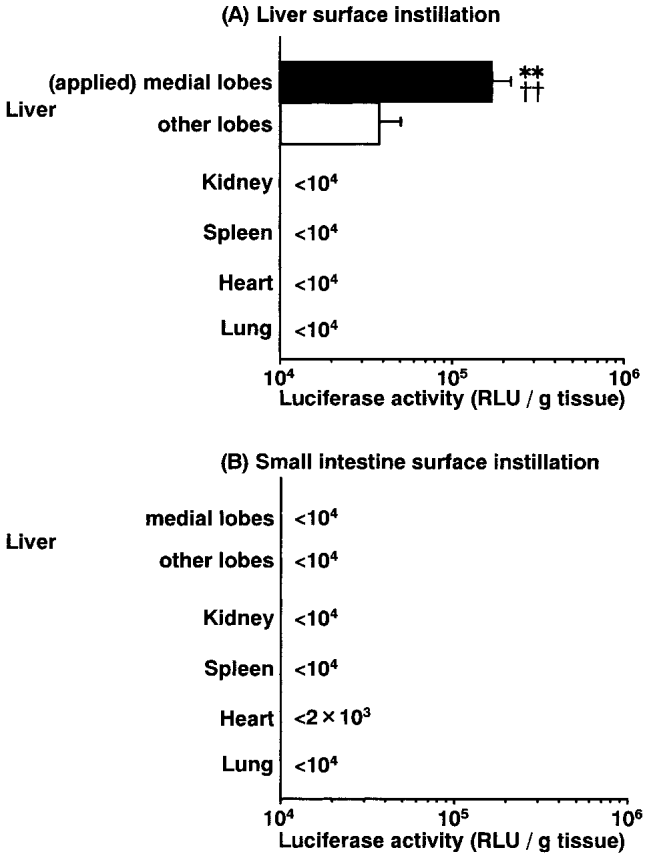


Fig. 1. The gene expression in the liver, kidney, spleen, heart, and lung at 6 h after liver surface instillation (A) and small intestine surface instillation (B) of pDNA at a dose of 30 μg (30 μl) into mice. Statistical comparisons were performed by Dunnett's test (** $p < 0.01$ vs. other liver lobes; †† $p < 0.01$ vs. other tissues). Each value represents the mean ± SE of at least twelve experiments.

from 2–24 h were always significantly higher than those of other liver lobes. The gene expression was detected in small amounts (<10^4 RLU/g tissue) in the kidney, spleen, heart, and lung. The highest gene expression in the applied liver lobes was observed at 6 h. The gene expression in the applied liver lobes was diminished thereafter and was detected in small amounts (<2×10^3 RLU/g tissue) at 48 h. Figure 3 shows the effect of the instillation doses of pDNA on gene expression in the applied liver lobes and other liver lobes at 6 h after liver surface instillation of pDNA using a catheter at doses of 5, 10, 15, 30, and 60 μg into mice. The gene expression was detected in small amounts (<10^4 RLU/g tissue) in kidney, spleen, heart, and lung. The gene expressions in the applied liver lobes at doses of 5, 10, 15, 30, and 60 μg were always significantly higher than those of the other liver lobes. The gene expressions in the applied liver lobes were enhanced by the increase of the pDNA doses and were constant at the pDNA doses from 15–60 μg.

Figure 4 shows the effect of the instillation volumes of pDNA using the catheter on gene expression in the applied liver lobes, other liver lobes, and spleen at 6 h after liver surface instillation of pDNA at a dose of 30 μg into mice. The gene expression was detected in small amounts (<10^4 RLU/g tissue) in kidney, heart, and lung. The gene expressions in the applied liver lobes at volume of 15, 30, and 60 μl were significantly higher than those of the other liver lobes. However, the gene expression in the applied liver lobes at a volume of 90 μl was not significantly higher than those of the other liver lobes. Thus, the gene expressions in the applied liver lobes were diminished with increase in instillation volume. This may be due to the spreading of the pDNA solution from applied liver lobes.

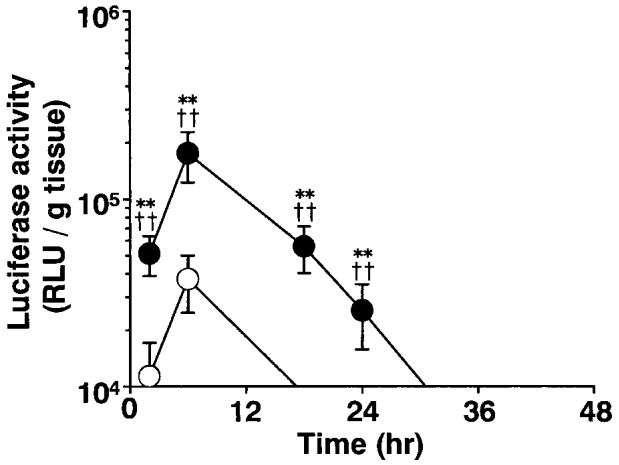


Fig. 2. The time course of gene expression in the applied liver lobes (●) and other liver lobes (○) until 48 h after liver surface instillation of pDNA at a dose of 30 μg (30 μl) into mice. Statistical comparisons were performed by Dunnett's test (** $p < 0.01$ vs. other liver lobes; †† $p < 0.01$ vs. other tissues). Each value represents the mean ± SE of at least ten experiments.

Gene transfer to hepatocytes should be of great therapeutic potential since hepatocytes are responsible for the synthesis of a variety of proteins, which play important physiologic roles. In addition, liver cancer, viral hepatitis, and cirrhosis, which are non-congenital refractory diseases are also subject for gene therapy treatment (2). There has been much interest in *in vivo* gene transfer to the liver, as an alternate to

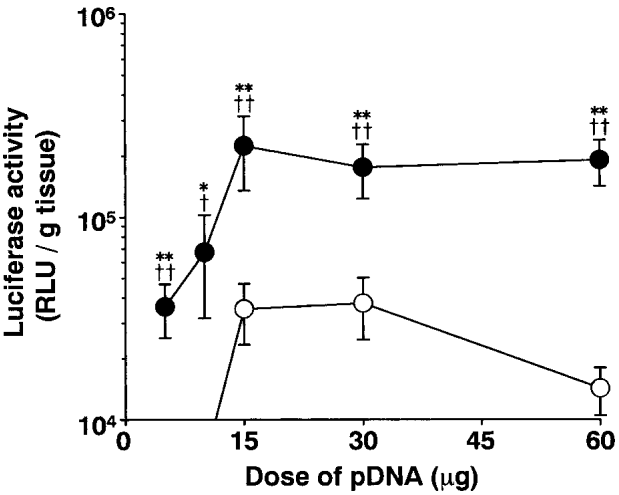


Fig. 3. The effect of instillation doses on gene expression in the applied liver lobes (●) and other liver lobes (○) at 6 h after liver surface instillation of pDNA at doses of 5, 10, 15, 30, and 60 μg (30 μl) into mice. Statistical comparisons were performed by Dunnett's test (* $p < 0.05$, ** $p < 0.01$ vs. other liver lobes; † $p < 0.05$, †† $p < 0.01$ vs. other tissues). Each value represents the mean ± SE of at least ten experiments.

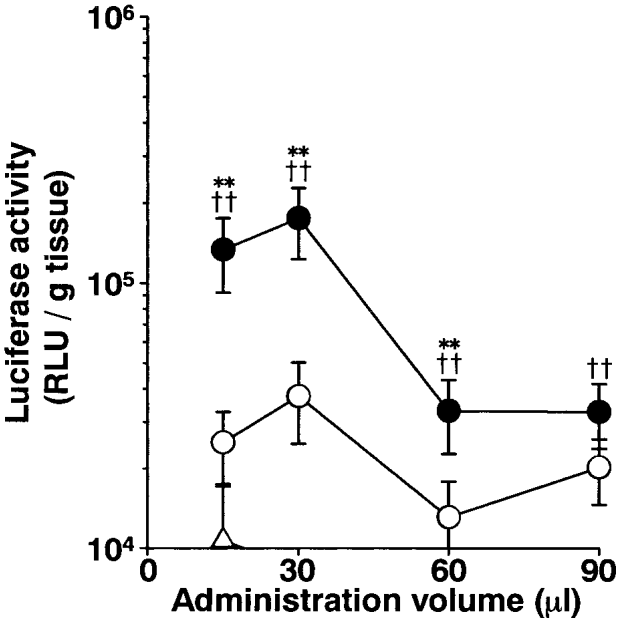


Fig. 4. The effect of instillation volumes on gene expression in the applied liver lobes (●), other liver lobes (○), and spleen (△) at 6 h after liver surface instillation of pDNA at a dose of 30 μg into mice. Statistical comparisons were performed by Dunnett's test (** $p < 0.01$ vs. other liver lobes; †† $p < 0.01$ vs. other tissues). Each value represents the mean ± SE of at least twelve experiments.

ex vivo methods, which require invasive surgery (7). Consequently, various viral and nonviral gene transfection vectors were developed to enhance the transfection efficiency in the liver. Despite the high transfection efficiency of viral vectors, their usefulness in gene therapy has been limited. Alternatively, the use of nonviral vectors has attracted great interest for *in vivo* hepatic gene transfection because they lack some of the risks inherent in viral vector systems. Naked pDNA-mediated gene transfection is an ideal method for clinical application. However, the transfection efficiency in the liver is very low despite their high uptake by the liver after intravenous injection of naked pDNA (8). Although it was reported that the direct injection of genes to the organ should yield the organ-selective gene transfection, there is great concern about safety because of injury to organs by needles and the administration volume limits etc.

The gene expression levels in tissues following the instillation of pDNA to the liver surface in mice by cutting the abdomen, in the previous report (5), were approximately 10-fold higher than those following the instillation of pDNA using catheter to the liver surface without cutting the abdominal wall (Fig. 1). This may be due to the change of the spread of pDNA solution or the change of the physiologic conditions in the intraperitoneal cavity by cutting the abdominal wall.

When the drugs were administered by the vasculature route, they were distributed to the whole body via the blood stream, leading to inadequate organ-selective and diseased site-selective drug delivery. Thus, we have originally worked out that the drug was found to be adequately absorbed by the liver (9), kidney (10), and gastric serosal (11) surface and to be accumulated site-selectively in the liver, kidney and stomach in rats, respectively. As for the gene delivery system, the liver- and site-selective gene expression was observed after liver surface instillation using catheter in mice (Figs, 1–4), suggesting that liver surface instillation of pDNA could be a novel administration method of pDNA for the liver- and site-selective gene transfection. In addition, transgene expression periods using nonviral gene transfection systems are transient (12), and accordingly pDNA should be used particularly for long-term and/or repeated administration for the efficient gene therapy in the liver. The liver surface instillation of pDNA using a catheter is not stressful against the liver, indicating that the continuous administration of pDNA could also be possible by using a catheter to enable long-term hepatic gene transfection.

With respect to the administration method of the liver surface instillation, we have already demonstrated that the liver site-selective drug accumulation was enhanced by gradually and continuously instilling a small amount of drug solution on the liver surface (9). Recently, the implantable infusion pumps have been developed for treatment of several diseases (13) and endoscopic and laparoscopic operation techniques have made remarkable progress (14). Furthermore, continuous ambulatory peritoneal dialysis is an extremely popular treatment modality for end stage renal failure (15), indicating that the skill for inserting the catheter on the intraperitoneal organs would be advanced. Taken these into consideration, the application of the suitable medical skills should make possible the clinical application of pDNA on the liver surface.

In summary, we have demonstrated the liver- and lobe-selective gene transfection following the instillation of naked pDNA using the catheter on the liver surface in mice. The transfection levels of the applied liver lobes were significantly higher than that of other liver lobes and other tissues (Figs, 1–4). The liver- and lobe-selective gene transfection method is expected to be a safe and effective treatment against localized hepatic cancer. Also, the information on present study could provide a novel site-selective gene transfection system to the other organs.

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REFERENCES

1. C. R. Dass and M. A. Burton. Lipoplexes and tumours. *J. Pharm. Pharmacol.* **51**:755–770 (1999).
2. M. Nishikawa and M. Hashida. Nonviral approaches satisfying various requirements for effective *in vivo* gene therapy. *Biol. Pharm. Bull.* **25**:275–283 (2002).
3. R. Heller, M. Jaroszeski, A. Atkin, D. Moradpour, R. Gilbert, J. Wands, and C. Nicolau. In vivo gene electroinjection and expression in rat liver. *FEBS Lett.* **389**:225–228 (1996).
4. S. Kuriyama, A. Mitoro, H. Tsujinoue, T. Nakatani, H. Yoshiji, T. Tsujimoto, M. Yamazaki, and H. Fukui. Particle-mediated gene transfer into murine livers using a newly developed gene gun. *Gene Ther.* **7**:1132–1136 (2000).
5. S. Kawakami, R. Hirayama, K. Shoji, R. Kawanami, K. Nishida, M. Nakashima, H. Sasaki, T. Sakaeda, and J. Nakamura. Liver- and lobe-selective gene transfection following the instillation of plasmid DNA to the liver surface in mice. *Biochem. Biophys. Res. Commun.* **294**:46–50 (2002).
6. A. R. Thierry, Y. Lunardi-Iskandar, J. L. Bryant, P. Rabinovich,

R. C. Gallo, and L. C. Mahan. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc. Natl. Acad. Sci. USA* **92**:9742–9746 (1995).
7. M. Grossman, S. E. Raper, K. Kozarsky, E. A. Stein, J. F. Engelhardt, D. Muller, P. J. Lupien, and J. M. Wilson. Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nat. Genet.* **6**:335–341 (1994).
8. K. Kawabata, Y. Takakura, and M. Hashida. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* **12**:825–830 (1995).
9. J. Nakamura, Y. Yoshida, K. Mera, T. Mukai, K. Nishida, and H. Sasaki. Continuous microinstillation of phenol red on liver surface for liver site-selective delivery. *Biol. Pharm. Bull.* **22**:713–715 (1999).
10. S. Kawakami, T. Horimoto, K. Nishida, R. Hirayama, T. Mukai, M. Nakashima, H. Sasaki, T. Sakaeda, and J. Nakamura. Kidney- and site-selective delivery of 5-fluorouracil utilizing the absorption on the kidney surface in rats. *Biol. Pharm. Bull.* **25**:928–930 (2002).
11. J. Nakamura, A. Tsurumaru, K. Mera, T. Mukai, K. Nishida, and H. Sasaki. Absorption of drugs applied to the gastric serosal surface in rats. *Pharm. Pharmacol. Commun.* **5**:519–522 (1999).
12. F. Liu, Y. K. Song, and D. Liu. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**:1258–1266 (1999).
13. K. D. Hepp. Implantable insulin pumps and metabolic control. *Diabetologia* **37**:S108–S111 (1994).
14. T. A. Stellato. History of laparoscopic surgery. *Surg. Clin. North Am.* **72**:997–1002 (1992).
15. C. Y. Kim, A. Kumar, L. Sampath, K. Sokol, and S. Modak. Evaluation of an antimicrobial-impregnated continuous ambulatory peritoneal dialysis catheter for infection control in rats. *Am. J. Kidney Dis.* **39**:165–173 (2002).