A Method for Oral DNA Delivery with N-Acetylated Chitosan

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Received December 9, 2003; accepted January 29, 2004

Purpose. The gastrointestinal tract poses a variety of morphological and physiological barriers to the expression of a target gene. In this work, N-acetylated chitosan is used as a gene delivery carrier for solving this problem.

Methods. Plasmid DNAs carrying the lacZ gene and interluekin-10 (IL-10) gene were mixed with N-acetylated chitosan. The N-acetylated chitosan/plasmid DNA complex was mixed into a food paste to feed mice. The transport and distribution characteristics of the plasmid along the intestinal mucosa were identified by β-galactosidase assay. In addition, the stomach and intestines were subjected to analysis for the production of IL-10.

Results. The efficiency of N-acetylated chitosan–mediated gene delivery to the intestines was observed to be higher than that of chitosan alone. In particular, this result was most significant in the case of the duodenum, where the LacZ gene was expressed most effectively through the use of N-acetylated chitosan. It was also demonstrated that the IL-10 gene was successfully transferred to intestines through this method.

Conclusions. A plasmid DNA was able to be orally delivered to the intestines using N-acetylated chitosan as a carrier. Thus, we have developed a dietary dose system for delivering a DNA vaccine for treating gastrointestinal diseases.

KEY WORDS: interleukin-10; intestine; N-acetylated chitosan; oral DNA delivery.

INTRODUCTION

Several methods that enhance the transfer of DNA into eukaryotic cells have been developed for gene therapy (1,2). A virus-based gene delivery is currently the most effective way to transfer a gene to cells. However, this method is highly prone to endogenous virus recombinations, oncogenic effects, and immunological reactions, which have limited the use of viral vectors for human gene therapy (3–7).

For drug-based chronic therapies, oral delivery has undeniably been the preferred route of administration, doubtlessly because of enhanced patient compliance. The gastrointestinal tract, however, sets a variety of morphological (e.g., epithelial cells, mucus) and physiological (e.g., enzymes, pH) barriers that can limit intestinal absorption of gene medicines. Therefore, mediators for gene delivery should be nontoxic and acid-resistant with better-controlled release mechanisms.

This is especially true for diseases occurring at the terminal part of the intestines, such as ulcerative colitis, in which oral-drug–based therapy is not very effective due to delivery problems and in turn makes surgery with the use of special techniques a current requirement. A more satisfactory prescription method is thus desirable, and this has spawned a considerable amount of research related to these challenges. An effective combination therapy was reported by Hirschfeld *et al.*, who used 5-ASA, an anti-inflammatory, and steroids (8). Some immunotherapy reports are also available (9,10), in which immune responses were changed by introducing an expression vector of interleukin-10 (IL-10) or tumor necrosis factor-α (TNF-α) through intravenous injection. In these studies, however, the controlled release of the vector into a target site remains the important issue to be addressed.

Chitosan α(1-4)2-amino 2-deoxy β-glican is a deacetylated form of chitin, an abundant polysaccharide present in crustacean shells. From a technological point of view, it is important that chitosan be nontoxic, biocompatible, biodegradable, acid-soluble, and positively charged (3). These properties increase transcellular and paracellular transport across mucosal epithelium, which is further indicative of their potential in oral gene delivery and protective mucosal immune responses (4).

Mumper *et al.* (5) were the first to propose chitosan as a gene delivery medium. A gentle mixture of chitosan and DNA solutions followed by incubation generated broad distributions of chitosan/DNA particulate complexes with mean sizes from 100 to 600 nm, depending on the molecular weight of the chitosan. Because chitosan was demonstrated to have the additional advantage of promoting adhesion and adsorption across a mucous surface (6), a series of oral vaccines employing this material has been developed. The initial report of a chitosan/DNA vaccine showed very encouraging results (7). Here, the possibility of a DNA vaccine for oral delivery without a viral vector was shown. Although the proper oral dose was not optimized, chitosan-mediated DNA vaccines for mixing into food were introduced as the first immunization system for an oral dose that had not been reported elsewhere.

Because chitosan is soluble in an acidic pH range, it is not suitable as an oral delivery material. As described previously, the use of a chitosan-mediated DNA vaccine (11) led to the observation that hematopoietic cells were not stained for tracing the LacZ activity. In addition, the standard method in which chitosan is dissolved with acetic and/or hydrochloric acid is not very useful because it is difficult to force a mouse to ingest a chitosan solution with an acidic taste. On the other hand, it was reported (12) that chitosan, usually soluble in acid, becomes acid-resistant by N-acetylation of chitosan with acetic anhydride. Based on this fact, we present a novel method in which a chitosan/plasmid DNA complex is lyophilized and then N-acetylated. By combination of a 1% N-acetylated chitosan/plasmid DNA complex and 99% mouse food, a plasmid DNA for an oral dose administered to mice was able to be prepared.

By means of feeding mice with the acetylated chitosan/plasmid DNA complex mixed into a food paste, gene delivery and expression were observed in the mouse stomach and intestines. Our final objective was to develop a dietary dose system for delivering a DNA vaccine.

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0724-8741/04/0500-0838/0 © 2004 Plenum Publishing Corporation

METHODS

Materials

Chitosan with 100% deacetylation (MW 9.5×10^5) (13,14) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Acetic anhydride was supplied from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Food for mouse CL2 (gamma-radiation sterilization) was obtained from Clea Japan, Inc. (Tokyo, Japan).

Preparation of Chitosan Solution

Two grams of chitosan was dissolved in 50 ml of 5% (v/v) acetic acid and filled to 100 ml with sterilized water after adding 2 M sodium hydrate so that the final pH was 4.2. This chitosan solution (2% w/v) was filtrated with a cellulose filter (capture gain, 1 μm) under reduced pressure and stored at room temperature before use.

Preparation of a Reporter Gene Construct

pCMVLacZ (pBluescript II KS(+), Fermentas, Inc., Hanover, MD, USA) was introduced into an *Escherichia coli* strain (JM-109) and purified using Qiagen Plasmid Maxi Kits (Qiagen, Valencia, CA, USA). The purity of the plasmid DNA was certified by the OD_{260}/OD_{280} ratio and by distinctive bands of DNA fragments with the corresponding base pairs in gel electrophoresis after restrictive enzyme treatment of the DNA. The concentration of the plasmid DNA was determined using 1 (OD_{260}) = 50 μg of DNA. The plasmid was dissolved with a 0.1 M TE buffer (10 mM Tris HCl, pH 7.4, and 1 mM EDTA) and stored at –20°C until use.

Preparation of an IL-10 Gene Expression Plasmid

mIL-10 was purchased from the RIKEN Gene Bank and subcloned to pBluescript II KS(+). The fragment, pBluescript II KS-mIL-10, which was digested by BamHI, was then inserted into the pcDNA3.1(+) expression vector at the BamHI cloning site.

N-Acetylated Chitosan/Plasmid DNA Complex

The chitosan solution (2% w/v) and the plasmid DNA were gently mixed (100 mg DNA/ml). They were then flow-cast on Teflon dishes (60-mm diameters) and lyophilized overnight. The lyophilized chitosan/plasmid DNA complexes were N-acetylated with acetic 3% (v/v) anhydride in methanol under room temperature for 3 h. The resulting product of the N-acetylated chitosan/plasmid DNA complex was lyophilized overnight again.

Preparation of Mouse Food

As shown in Table I, N-acetylated chitosan/plasmid DNA complex and feed were milled for mixing. The composition of N-acetylated chitosan/plasmid DNA complex and feed was 99:1. Four kinds of samples were prepared, all of which were contained in the food: chitosan alone (group A), the chitosan/β-galactosidase plasmid DNA complex (group B), the N-acetylated chitosan/β-galactosidase plasmid DNA complex (group C), and the N-acetylated chitosan/IL-10 plasmid DNA complex (group D). These were stored at –20°C until use.

Table I. Composition of the Modified Foods for Mouse

Sample	Chitosan alone	Chitosan + plasmid DNA	Acetylated chitosan + plasmid DNA
Food	118.8 g	118.8 g	118.8 g
Chitosan	1.2 g	1.2 g	—
N-acetylated chitosan	—	—	1.2 g
Plasmid DNA	—	6 mg	6 mg
Sterilized H_2O	120 ml	120 ml	120 ml
Total	240 g	240 g	240 g

Administration of Chitosan/DNA Complex into Animals

C57BL mice (8 weeks, female; 6 in each group) were fed food containing the N-acetylated chitosan/plasmid DNA complex after 2 days of fasting. Five days later, the mice were sacrificed, and their stomachs and intestines were surgically removed. Animal studies were carried out according to the guidelines for the care and use of experimental animals, drawn up by the Committee for Animal Experiment of National Cancer Center, which meet the ethical standards required by the law and the guidelines about experimental animals in Japan.

Transgene Expression in Intestines

The intestinal tissues were frozen in an O.C.T. Embedding Medium (Sakura Finetek, Torrance, CA, USA) and cut into thin sections (three mice from each group). These sections were stained with 4-chloro-5-bromo-3-indolyl-b-galactosidase (X-gal) according to the standard protocols. After being stained at 37°C for 30 min, the tissues were photographed using a Nikon E1000 (Tokyo, Japan). The pictures were scanned into a computer and edited with imaging software that adjusts the brightness and contrast.

IL-10 Assay

The organs of the digestive system (stomach, duodenum, jejunum, ileum, colon, rectum) from mice administered with the N-acetylated chitosan/IL-10 plasmid DNA complex (n = 4) were added to 900 μl of a 1× lysis buffer (Promega, Madison, WI, USA), and the total IL-10 from each tissue was assayed with an IL-10 mouse ELISA kit (Pierce ENDOGEN, Iselin, NJ, USA).

RESULTS

Preparation of N-Acetylated Chitosan/Plasmid DNA Complex

Fig. 1 shows preparation of N-acetylated chitosan/plasmid DNA complexes. The mixture of chitosan solution and the plasmid DNA were flow-cast on Teflon dishes and lyophilized overnight. The lyophilized chitosan/plasmid DNA complexes were N-acetylated with acetic anhydride in methanol under room temperature for 3 h. The resulting product of the N-acetylated chitosan/plasmid DNA complex was lyoph-

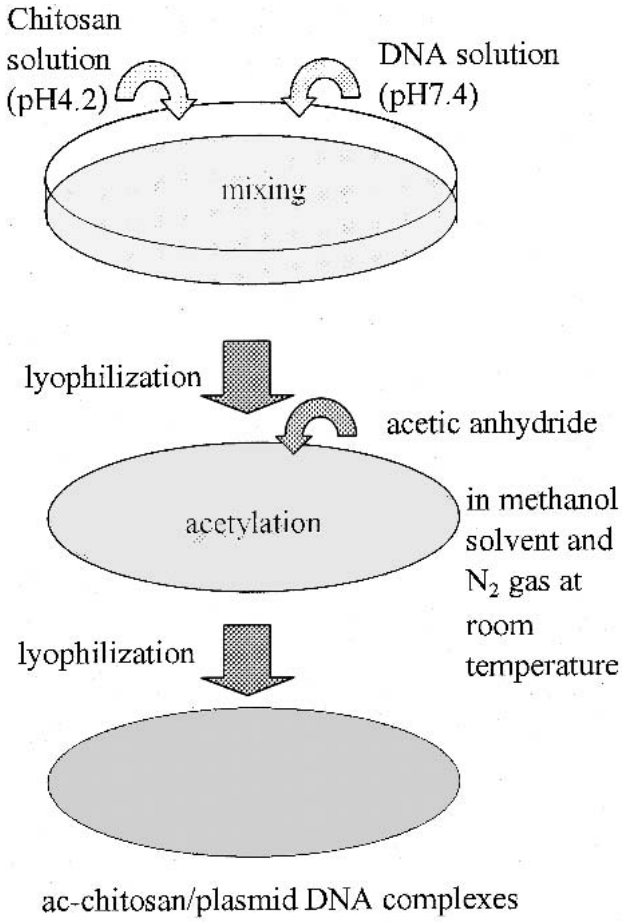

Fig. 1. Preparation of N-acetylated chitosan/plasmid DNA complexes. Plasmid DNAs were mixed with N-acetylated chitosan. The N-acetylated chitosan/plasmid DNA complex was lyophilized, ground, then mixed into a food paste to feed mice for 5 days after 2 days of fasting.

ilized overnight again. The N-acetylated chitosan/plasmid DNA complex and mice feed were milled for mixing.

Although the data are not shown, we performed an *in vitro* model experiment to assess the stability of N-acetylated chitosan. N-acetylated chitosan showed resistance for an artificial gastric cocktail (pH 1.0) and maintained ~50% degree of its form for 2 h, whereas non-acetylated chitosan dissolved quickly within 1 min.

Expression of the Plasmid DNA in Intestines

To determine whether a chitosan-mediated oral delivery method would be able to deliver plasmid DNA into intestinal organs, gene expression of bacterial β-galactosidase (LacZ) was assessed on whole organs (Fig. 2). For mice fed with chitosan alone (group A), nothing significant could be found in LacZ staining, but, for mice fed with chitosan/plasmid DNA (group B) and N-acetylated chitosan/plasmid DNA (group C), gene expression was confirmed at the duodenum, jejunum, ileum, and colon. Confirmation was thus obtained that N-acetylated chitosan (group C) was capable of much better delivery and showed strong expression of LacZ gene. These results suggest that N-acetylated chitosan may be more

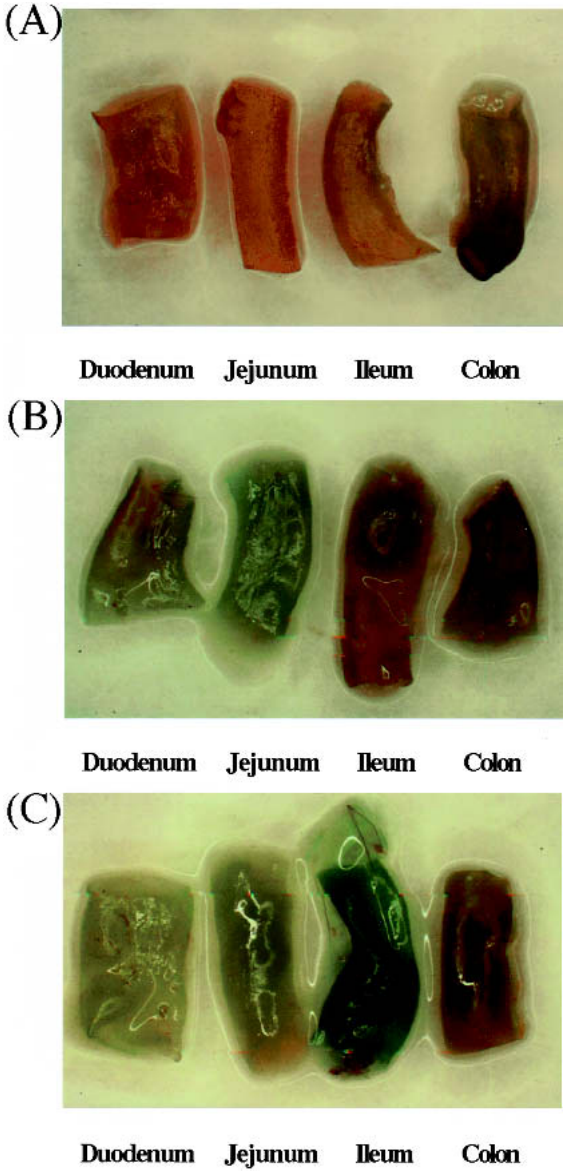

Fig. 2. Expression of the plasmid DNA in small intestines by oral delivery of (A) chitosan, (B) chitosan/plasmid DNA complexes, and (C) N-acetylated chitosan/plasmid DNA complexes. Five days after administration, the gastrointestinal organs including duodenum, jejunum, ilium, and colon were excised and fixed in 2% paraformaldehyde/0.2% glutalaldehyde at room temperature for 1 h. The β-galactosidase expression was detected by incubation in X-gal overnight at room temperature.

suitable as a delivery medium for oral gene therapy for intestinal diseases, such as ulcerative colitis.

Gene Expression on Histological Sections in Intestines

To confirm chitosan-mediated gene transfer and expression in cells, frozen sections of all of the tissues were prepared and their Lac Z gene expression was determined. The (B) and (D) photographs in Fig. 3 were obtained using hematoxylin-eosin staining for the ileum of groups A and C, respectively. The results indicated that the epithelial cells and the endothelial cells in the small intestines were stained intensely with

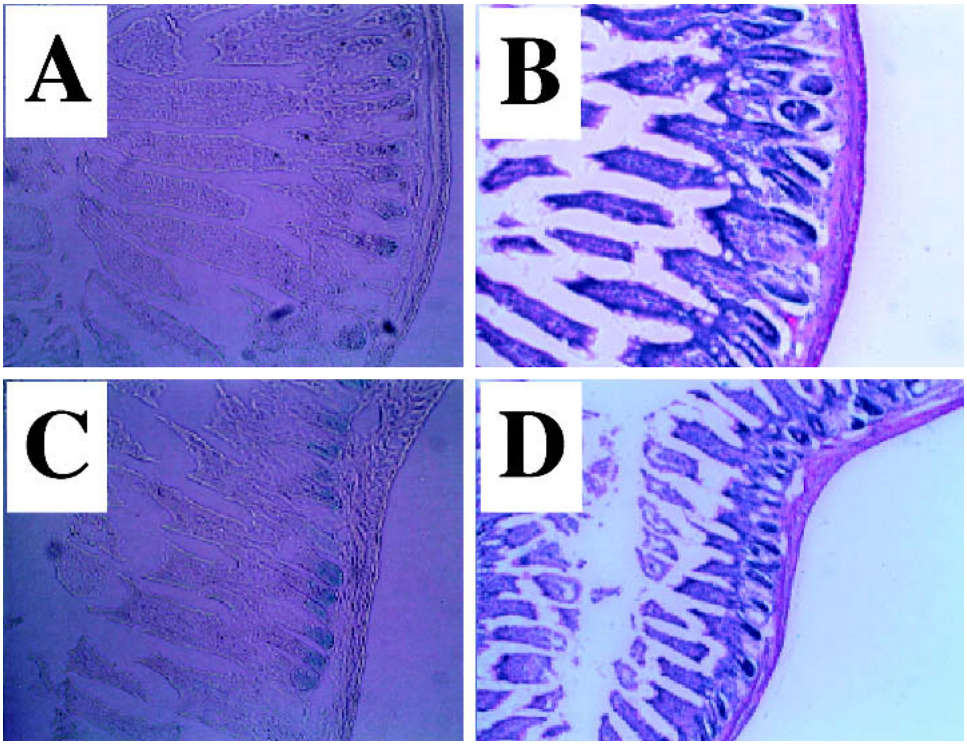

Fig. 3. β-galactosidase expression on histological sections in mouse small intestines 5 days after oral delivery of chitosan and N-acetylated chitosan/DNA complexes. Mice were fed (A) chitosan and (C) N-acetylated chitosan/DNA complexes at a dose of 160 μg plasmid DNA per day. Hematoxylin-eosin staining was performed in the same test sections (B and D).

X-gal 5 days after oral delivery of chitosan and N-acetylated chitosan/DNA complexes.

Oral IL-10 Gene Delivery with N-Acetylated Chitosan

Recombinant IL-10 suppresses inflammatory cytokine production (15). Evidence for disease protection was also observed in an *in vivo* IL-10 gene therapy against experimental autoimmune encephalomyelitis (16). To study whether our N-acetylated chitosan–mediated oral gene delivery method was effective for IL-10 gene delivery to the gastrointestinal tract, mice were fed a chitosan/plasmid DNA complex. As shown in Fig. 4, total IL-10 protein was clearly detected in the gastrointestinal tissues in mice that received the N-acetylated chitosan/plasmid DNA complex (group D). In contrast, mice with control N-acetylated chitosan alone, IL-10 plasmid DNA alone, or saline showed no IL-10 gene expression (data not shown). These results suggest that our N-acetylated chitosan-mediated oral gene delivery method allows efficient gene delivery into the gastrointestinal tissues.

DISCUSSION

It is generally considered that the delivery system is a key factor for effective and successful gene therapy. However, there have been few reports on successful oral gene delivery systems, because it is very difficult to introduce target genes into the desired therapeutic oral cells and tissues. Oral delivery of the DNA, though possessing certain obvious advantages, remains problematic due to the acidic environment of the stomach. Therefore, proper methods of protecting the DNA during its delivery to the target organ must be found.

Chitosan has been an attractive gene carrier because of its highly positive charges and low toxicity to cells (17). However, gene expression via oral delivery of a chitosan/DNA complex has not yet been intensely studied. The primary reason is presumed to be that the chitosan/DNA complex would be instantly destroyed under the acidic conditions of the stomach prior to its delivery to a final destination, such as the intestines. Even if delivery of a chitosan/DNA complex to the intestines were possible, it would still be difficult to transport the genes across mucosal epithelium.

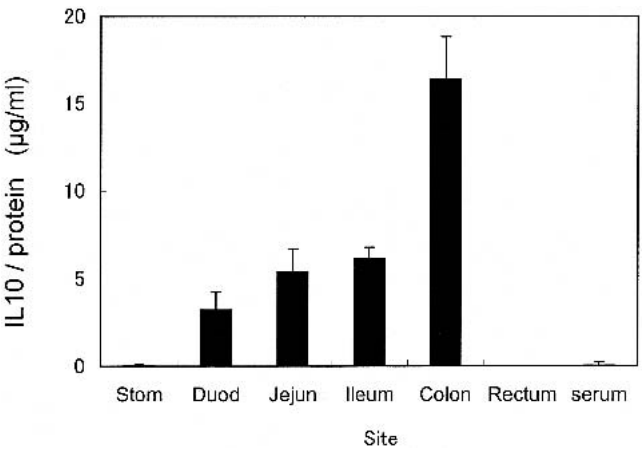

Fig. 4. Distribution profiles of a total IL-10 with N-acetylated chitosan/plasmid DNA complexes along the gastrointestinal tract and serum (n = 4). Experiments with food alone, N-acetylated chitosan alone, and IL-10-expressed vector alone (each group: n = 4) were also performed.

To overcome these problems, we investigated whether N-acetylated chitosan would allow efficient expression of genes in the intestines via an oral gene delivery system. Our results clearly show that an N-acetylated chitosan/DNA complex in food is successfully delivered to the target intestines and expressed LacZ reporter gene in epithelial cells. The expression of the transferred gene was apparent up to 5 days after the administration. The unique properties of each N-acetylated chitosan/DNA complex are thought to contribute to the transfection efficiency of the system as a whole. When the chitosan/DNA complex in digestive food passes from the stomach to the duodenum, the positively charged amino acid groups of the chitosan immediately become neutrally charged by the change of its environmental medium (from acidic to alkaline). This results in a dramatic removal of the electrostatic interaction between the positive amino groups of chitosan and the negatively charged phosphate moieties along the DNA backbone and thus leads to the swift release of DNA molecules from the complex. Pharmacokinetic and biodistribution studies (7), however, showed that the use of N-acetylated chitosan enabled a more prolonged retention of DNA in the human body than did the chitosan/DNA complex. This is because N-acetylated chitosan is only partially neutralized by the medium change. As a result, the interaction can be vigorously maintained, and the DNA can be carried longer.

The overproduction of immune-inflammatory cells and their mediators has consistently been implicated in the pathogenesis of human inflammatory bowel disease (IBD). Moreover, patients often experience intermittent remission and reactivation of their disease, suggesting a fluctuating balance between pro- and anti-inflammatory cell types and mediators. The expectations that exogenously administered IL-10 may be an effective therapeutic treatment of patients with IBD are based largely on the outcome of murine studies.

Each chitosan, N-acetylated chitosan (chitin), and their derivatives can induce an immunological adjuvant effect (9,10,18–20). Ito *et al.* (21) showed that the low-molecular-weight (LMW) chitosan (MW 25,000–50,000) had potent gastric cytoprotective and ulcer healing promoting actions. Furthermore, in this article we considered that chitosan and its derivatives might be primarily absorbed after being transformed into their oligosaccharides by chitosanase secreted from intestinal bacterial flora and by lysozyme in intestinal juice. Then, their monosaccaride, N-acetyl D-glucosamine might be used for formation of granulation tissue and angiogenesis.

In summary, our N-acetylated chitosan/DNA complex can be considered as a DNA carrier to deliver DNA to the terminal part of the intestines, where ulcerative colitis occurs and is difficult to treat. We believe that our results demonstrate the possible use of this method as a medical treatment of IBD without resorting to surgery requiring mechanical ventilation.

ACKNOWLEDGMENTS

We gratefully thank Ms. Maki Abe, Ms. Masako Hosoda, Ms. Kazumi Kimura, Dr. Hanako Yamamoto, Dr. Hideo Sasaki, Dr. Kotaro Hirai, and Dr. Takeji Takamura-Enya for their technical assistance. We are also grateful for the contributions of Associate Professor Naoji Kubota, Oita Medical University, and Dr. Kyong-Hoon Lee, Northwestern University, Chicago. We give special thanks to Ms. Naoka Shimada, Ms. Emi Tsuchitani, Ms. Midori Kono, Associate Professor Sadayori Hoshina, and Professor Katsuhiko Machida, Jikei University, School of Medicine, for their assistance with the preparation of the manuscript. This work was supported in part by a Grant-in-Aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control, a Grant-in-Aid for Scientific Research on Priority Areas Cancer from Ministry of Education, Culture, Sports, Science and Technology, and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan.

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