Protective Effect of Montmorillonite on Plasmid DNA in Oral Gene Delivery into Small Intestine

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Montmorillonite, a bioinert clay mineral, was examined as a novel vector for an oral gene-delivery system. The complex of montmorillonite and plasmid DNA encoding the enhanced green fluorescent protein (EGFP) gene was prepared at various weight ratios, and then transfected into cultured intestinal epithelial cells (IEC-6) *in vitro*. The EGFP gene was clearly transcribed when the transfection was performed using the montmorillonite–plasmid complex at a weight ratio of 0.05 : 1. In contrast, no gene expression was detected by reverse transcription-polymerase chain reaction (RT-PCR) analysis when the transfection was performed with naked plasmid. Various plasmid preparations were given orally to mice, and the gene expression in the stomach and small intestine was examined by RT-PCR. Although no gene expression was detected in the mice receiving an oral administration of naked plasmid or polyethyleneimine–plasmid complex, the EGFP gene complexed with montmorillonite was expressed in the small intestine. These results indicate that montmorillonite protected the plasmid DNA from the acidic environment in the stomach and DNA-degrading enzymes in the intestine, and successfully delivered it into cells of the small intestine.

Key words montmorillonite; gene therapy; oral gene delivery; non-viral vector; clay

Oral gene therapy has the advantages of high patient compliance and ease of administration over traditional methods using invasive procedures, such as injections. Recombinant DNA reaching the small intestine can transform and be expressed by the epithelial cells. The gene product can then be secreted into the bloodstream to cure the genetic defect. Moreover, since oral DNA administration promotes both mucosal and systemic immune responses,[1] the delivery system can be applied to prophylactic treatments for allergy and viral infection. To realize the promise of oral gene therapy, safe and effective vectors must be developed. Such a vector should not be toxic to the gastrointestinal tract and, moreover, must protect the DNA from the acidic environment of the stomach and DNase released from the intestine. Clay minerals such as montmorillonite, illite, and kaolinite are well known to protect DNA from nucleases during natural bacterial genetic transformation.[2,3] The transformational ability of clay is believed to play a role in evolution and genetic exchange among unrelated organisms. Many efforts have been made to develop safe and efficient non-viral vectors.[4,5] We sought to exploit the transformational ability of clay for an oral gene delivery system, because clay minerals have already been used safely in human applications, such as anti-diarrheal medicine,[6] antacids, and cosmetics. Montmorillonite, a bioinert clay mineral, consists of a fine-grained hydrated aluminum silicate. The structure is an octahedral laminated sheet sandwiched between tetrahedral silicate layers. Since the interaction between clay and DNA has been well studied using montmorillonite,[7,8] we chose this mineral in this study to evaluate as a novel vector for an oral gene delivery system.

MATERIALS AND METHODS

Materials Sodium montmorillonite was obtained from Kunimine Industries Co., Ltd., Tokyo, Japan. Polyethyleneimine (PEI) with an average molecular weight of 70000 was purchased from Wako Pure Chemical Industries (Osaka, Japan) and used as a control as a well-known nonviral vector. Primers for detecting GFP gene expression were purchased from Invitrogen (Invitrogen Corp., Carlsbad, CA, U.S.A.). A 100-bp DNA ladder (TOYOBO Co., Ltd., Tokyo, Japan) was used as a molecular size marker for RT-PCR analysis.

Preparation of DNA-Vector Complex A plasmid, pCAGGS containing the enhanced green fluorescent protein (EGFP) gene[9] was used for the experiments. The complexes were prepared by mixing 500 μl of solution containing 50 μg plasmid DNA and 500 μl of suspension containing 50, 5, 2.5 or 0.5 μg montmorillonite, vortexing for 1 min, and incubating at room temperature for 1 h. The complex was vortexed for 1 min every 20 min during the incubation. Another complex was prepared by the same method mixing 50 μg plasmid DNA and 50 μg PEI.

***In Vitro* Transfection** Rat intestinal epithelial cells (IEC-6) were seeded onto tissue-culture plates (Nippon Becton Dickinson) at 4.7×10^4 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St Louis, U.S.A.) supplemented with 10% fetal bovine serum (ICN Biochemicals, Costa Mesa, CA, U.S.A.). The various DNA samples were added when cells were at a density of 1×10^6 cells/cm^2, and incubated for 3 h in serum-free DMEM. Each sample containd 6 μg of plasmid DNA. Serum was then added and the cells were cultivated for 48 h.

Oral Administration Six-week-old male BALB/c mice were fasted for 24 h, and then 300 μl of plasmid-vector complex or naked plasmid was orally administered. Each sample

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contained 50 μg of plasmid DNA. Taking into account the time required for the complex to reach the intestine, the mice were fasted for another 4—5 h. The stomach and small intestine were excised 24 h after the oral administration of plasmid. The small intestine was cut into three parts. The animal experiments were conducted according to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) IEC-6 cells and excised organs were homogenized, and the total RNAs were extracted by Isogen (Nippon Gene Co., Ltd., Tokyo Japan). The gene expression of EGFP was analyzed by RT-PCR using the following primers: forward 5′-ATCAAGGGCGAGGAGCTGTT-3′ and reverse 5′-GTAGGTCAGGGTGGTCACGA-3′. β-Actin gene expression was analyzed using the following primers: forward 5′-CATCCCCCAAAGTTCTAC-3′ and reverse 5′-CCAAAGCCTTCATACATC-3′.

RESULTS AND DISCUSSION

The effective weight ratio for the preparation of the montmorillonite and plasmid complex was examined *in vitro* using IEC-6 cells. PEI, a well-known nonviral vector,[10] was used as a positive control. A complex of PEI and plasmid at a weight ratio of 1 : 1 was added to the IEC-6 cells. After 48 h of cultivation, the gene expression of EGFP was analyzed by RT-PCR. As shown in Fig. 1, transfection using the PEI–plasmid complex was successful, in contrast to transfection by naked plasmid. Complexes of montmorillonite and plasmid at a weight ratio of 1 : 1, 0.1 : 1, 0.05 : 1, and 0.01 : 1 were also added to the IEC-6 cells. RT-PCR analysis showed that the EGFP gene was expressed in cells transfected with the montmorillonite–plasmid complex, and the weight ratio eliciting the strongest band intensity among those tested was 1 : 0.05 (Fig. 1). Since only a faint band was seen in the naked-plasmid-transfected cells, it was concluded that the montmorillonite acted as a vector for the transfection of mammalian cells. We used the weight ratio of 0.05 : 1 in the following experiments because the gene expression was markedly decreased above and below this proportion of montmorillonite.

Franchi *et al.* proposed a ‘cation bridge model’ for the adsorption of nucleic acids on clay minerals.[8] In this model, cations such as Na^+ and Ca^{2+} intercalate between the phosphate groups of the DNA and the surface of the clay, both of which are negatively charged, allowing interaction between the two polyanionic components. The bridge structure might contribute to the increase in the DNA’s stability, helping it to resist DNA-degrading enzymes. Since the monovalent Na^+ion can not form the bridge structure, the multivalent cations, Ca^{2+} and Mg^{2+}, existing in the medium might act for the bridge formation and assist to deliver the DNA into cultured IEC-6 cells. It is not clear whether the complex was incorporated into cells by phagocytosis or whether the plasmid entered the cells after being released from the montmorillonite. Further analysis is needed to clarify the mode of complex formation and the mechanism of transfection.

We next investigated whether we could achieve oral gene delivery into the small intestine using the montmorillonite–plasmid complex. The plasmid was orally administered

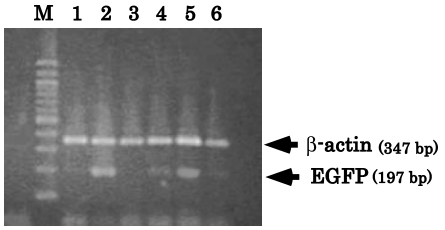

Fig. 1. RT-PCR Analysis of the EGFP Gene Expression in IEC-6 Cells Transfected with the Montmorillonite–Plasmid Complex

IEC-6 cells were transfected with naked DNA (lane 1), PEI–plasmid (lane 2), and montmorillonite–plasmid at weight ratio of 1 : 1 (lane 3), 0.1 : 1 (lane 4), 0.05 : 1 (lane 5), and 0.01 : 1 (lane 6). A 100-bp DNA ladder was used as a size marker (lane M).

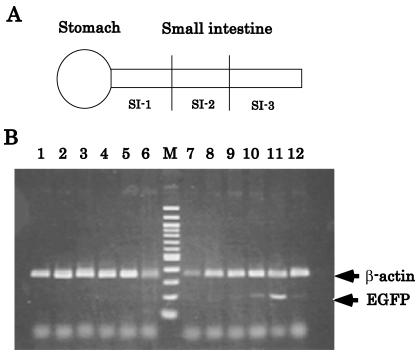

Fig. 2. RT-PCR Analysis of EGFP Gene Expression in the Stomach and Small Intestine

(A) Twenty-four hours after the administration of plasmid into mice, the stomach and small intestine were excised for analysis. The small intestines (SI) were cut into three pieces (SI-1, 2, and 3). Each SI piece was about 1.5-cm long. (B) Naked plasmid (lanes 1 to 4), PEI–plasmid (lanes 5 to 8), and montmorillonite–plasmid (lanes 9 to 12) were administered orally to mice. RT-PCR was conducted using RNA samples from the stomach (lanes 1, 5, 9), SI-1 (lanes 2, 6, 10), SI-2 (lanes 3, 7, 11), and SI-3 (lanes 4, 8, 12). A 100-bp DNA ladder was used as the size marker (lane M).

to mice in the presence or absence of the PEI or montmorillonite vector. Twenty-four hours after the administration, plasmid gene expression in the stomach and small intestine, which was cut into three parts (Fig. 2A), were analyzed by RT-PCR (Fig. 2B). No RT-PCR product was detected in the transfection by naked plasmid or the PEI-plasmid complex. In contrast, the transcription of EGFP gene was confirmed in the small intestine of mice transfected with the montmorillonite–plasmid complex. The highest expression was observed in the central part of the small intestine, and no band was detected in the stomach. These results indicate that the montmorillonite protected the plasmid DNA from the acidic environment of the stomach and from the DNA-degrading enzymes in the intestine, and successfully delivered the gene into cells in the small intestine. Since the surface charge of clay becomes positive at low pH, montmorillonite could strongly adsorb DNA in the stomach. Moreover, montmorillonite might provide favorable niche for escaping nuclease attack or adsorb nuclease at the different part to separate from DNA in the small intestine. Montmorillonite is thus considered to be useful for the protection of DNA in the oral gene delivery system.

We examined to detect expressed EGFP protein, but the fluorescence could not be observed both *in vitro* and *in vivo*. When the complex of montmorillonite and plasmid harboring LacZ gene was added to IEC-6 cells, the introduced gene was confirmed to be trasnslated to express β-galactosidase

activity with X-gal staining *in vitro* (data not shown). Even a small amount of expressed β-galactosidase could produce detectable amount of indigo dye by the enzymatic reaction. The effort to increase the transfection efficiency is necessary for the practical application of clay mineral to the oral gene therapy. It is possible that the complex could be charged slightly negative at neutral pH and difficult to contact with cell surface. In our preliminary experiments, the treatment of montmorillonite-LacZ-containing DNA complex with polycation synergistically enhanced the β-galactosidase activity in IEC-6 cells. The polycation might assist for the complex to contact with negatively charged cell surface. The study to increase the transfection efficiency is now underway.

This is the first report to show the protective effect of clay on mammalian oral gene delivery system. Roy *et al*. reported oral gene delivery using chitosan-DNA nanoparticles with the aim of treating food allergy.[11] They showed that oral allergen-gene immunization was effective in modulating anaphylactic responses in the mouse. Similarly, a clay mineral could be used as a vector for the prophylactic treatment of food allergy.

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