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A novel gene delivery system using EGF receptor-mediated endocytosis

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Abstract

A monoclonal antibody to the human epidermal growth factor (EGF) receptor was conjugated with polylysine and the resulting conjugate was affinity-linked with DNA (gene). This novel gene delivery system utilizes receptor-mediated endocytosis and would be especially suitable for gene therapy for EGF receptor-overproducing squamous cell carcinomas.

Key words: EGF receptor; Endocytosis; Monoclonal antibody; Gene therapy

1. Introduction

In recent years, there has been a substantial interest in developing a delivery system of foreign genes into target cells for therapeutic purposes. These gene delivery systems mainly utilize retrovirus and adenovirus vectors through their infective abilities [1].

For the gene therapy of cancer, there are successful cases such as the cure of melanoma by introducing the tumor necrosis factor (TNF) gene into tumor infiltrating bone marrow cells [2]. In this case, bone marrow cells isolated from patients are treated with DNA in vitro by the calcium-phosphate precipitation method and electroporation and returned into patient blood. However, there is little way to direct gene transfer into specific target cells. If such gene delivery systems are developed, it would be extremely beneficial for cancer therapy.

Mouse monoclonal antibody (MoAb) B4G7 is an IgG class 2 and binds specifically to a protein portion of the EGF receptor [3]. This MoAb is uniquely internalized through endocytic mechanisms of EGF receptors and therefore it has been successfully used to construct an immunotoxin which targets EGF receptor-overproducing cancer cells [4–6]. Thus, this MoAb delivers toxic proteins into human cells. In this paper, we utilized B4G7 antibody as a vehicle to deliver a DNA segment (gene) into human cancer cells through EGF receptor.

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Abbreviations: EGF, epidermal growth factor; MoAb, monoclonal antibody; CAT, chloramphenicol acetyltransferase; SPDP, *N*-succinimidyl 3-(2-pyridyldithio) propionate; TNF, tumor necrosis factor, 3-AcCM; 3-acethyl chloramphenicol; CM, chloramphenicol; EBSS, Earle's balanced salt solution.

2. Materials and methods

2.1. Cell and cell culture

A squamous carcinoma cell line, NA (from Dr. K. Rikimura, Tokyo Medical Dental Univ., Tokyo, Japan) [7,8] and a human lung adenocarcinoma cell line, A549 were maintained in plastic dishes containing Dulbecco's modified Eagle's medium (DMEM, Gibco) plus 10% fetal calf serum at 37°C and 5% CO_2.

2.2. Monoclonal antibody(B4G7) against the human EGF receptor

Murine monoclonal antibodies (MoAbs) of IgG2 type that immunoreact with the human low-affinity type EGF receptors were produced by a hybridoma cell line B4G7 [3].

2.3. Plasmid preparation

The pSV2 CAT plasmid DNA [9] was grown in *E. coli*, isolated, and purified according to the method of Birnboim and Doly [10]. Purity was confirmed by 1% agarose gel electrophoresis demonstrating the absence of bacterial cellular DNA.

2.4. Modification of B4G7 with N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP)

B4G7 murine MoAb (1 mg/ml) in 100 mM potassium phosphate buffer, pH 8.0, EDTA (0.5 mM) was mixed with a 17-fold molar excess of SPDP added from a freshly made solution (10 mM) in ethanol. The mixture was incubated at 37°C for 30 min. Reactions were terminated by gel filtration at 4°C through columns of Sephadex G-25 equilibrated with 200 mM HEPES buffer, pH 7.9, containing EDTA (0.5 mM). About three dithiopyridyl groups were incorporated per molecule of antibody, measured as described previously [4,11], and then diluted with 10-fold volume of HBS buffer (20 mM HEPES pH 7.3, 150 mM NaCl) at 4°C, ultraconcentrated by filtration (Amicon), and filtered through 0.22-mm membranes (Millipore Corp.)

2.5. Modification of polylysine with 2-iminothiolane

Polylysine (1 mg/ml) in water was mixed with 0.5 M triethanolamine-HCl buffer, pH 8.0, and 0.1 M EDTA. The final concentration of triethanolamine and EDTA were 60 mM and 1 mM, respectively. The solution was then degassed and kept under nitrogen at 0°C, then treated with 2-iminothiolane (1 mM) at 0°C for 90 min under nitrogen. The excess reagent was removed by gel filtration at 4°C on a column of Sephadex G-25 equilibrated with 5 mM bis-tris/acetate buffer, pH 5.8, containing 50 mM NaCl and 1 mM EDTA. The pH of modified polylysine solution was raised to 7.0 by adding 1/10 volume of 0.5 M triethanolamine-HCl buffer, pH 8.0.

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2.6. Conjugation of modified B4G7 with modified polylysine

Modified B4G7 in HBS buffer was mixed with an equal weight (about 5-fold molar excess) of modified polylysine. The mixture was kept under nitrogen at 4°C for 20 h. Finally, 2 mM iodoacetamide was added to block any remaining free sulfhydryl groups and incubation continued for 1 h at 25°C.

2.7. Purification of B4G7-polylysine conjugate

Conjugates were isolated by cation-exchange chromatography on a Mono S HR 5/5 column (Pharmacia) using a gradient from 80% A/20% B buffer to 100% B buffer (A, 50 mM HEPES pH 7.9; B, A + 3 M NaCl) [12]. The product fractions, eluting between 1.65 M and 2 M salt (NaCl), were dialyzed against HBS, and then filtered through 0.22-μm membranes to ensure that conjugates to be used did not contain precipitates.

2.8. Preparation of B4G7-polylysine/DNA complex and assay for DNA delivery

Complex formation of the DNA to the polylysine conjugated monoclonal antibody (B4G7) was accomplished as described by Curiel et al. [12]. After incubation at room temperature for 30 min, the complex was applied to 10-cm tissue culture dishes containing 5×10^5 NA cells grown in DMEM, 10% FCS (37°C, 5% CO_2). The cells were incubated at 37°C for 24 h, washed by 10% FCS/DMEM, and then maintained for 60 h. To evaluate reporter gene expression, cell lysates were prepared as described by Wu et al. [13], and assayed for chloramphenicol acetyltransferase activity as described by Gorman et al. [9]. In brief, cell extracts were incubated at 37°C with [^{14}C]chloramphenicol (DuPont New England Nuclear) in 0.25 M Tris-HCl, pH 7.5, to which was added acetyl coenzyme A (4 mM) for 60 min. A thin layer chromatography plate was autoradiographed using Imaging plate (Fuji Photo Film) at room temperature for 2 h, and analyzed by Fujix BAS2000 system (Fuji Photo Film).

2.9. Binding assay and competitive inhibition assay

The B4G7 MoAb was labeled with ^{125}I using Iodobeads [14]. [^{125}I]B4G7 binding was assayed as described previously [15]. Confluent cell cultures grown in 24-well plastic dishes were washed twice with 1 ml of ice-cold EBSS buffer on ice and treated with [^{125}I]B4G7 (approx. 30,000 cpm) with various concentrations of unlabeled B4G7, B4G7-polylysine conjugate and B4G7-polylysine/DNA complex in 1 ml of EBSS buffer for 2 h [15]. Then the cells were washed three times with 1 ml of EBSS buffer and solubilized in 1 N NaOH. The cell-associated radioactivity was determined in a Beckman 5500 gamma counter.

3. Results

B4G7 antibody and polylysine were activated separately with SPDP and 2-iminothiolane, and mixed together to form a conjugate via disulfide bonds. The resulting B4G7-polylysine conjugate was purified by Mono S column chromatography. This conjugate was then mixed with pSV2 CAT plasmid DNA to form an affinity complex. The resulting conjugate/DNA complex migrates differently from DNA itself under agarose gel electrophoresis (Fig. 1). The DNA/conjugate complex is formed in proportion to the DNA concentration. Since positive charges of polylysine interfere with the ethidium bromide staining of DNA, the complex unsaturated with DNA is not visible. The conjugate can be saturated with DNA and then becomes visible at the top of the gel. The DNA/conjugate complex apparently binds to the EGF receptor because they inhibited the binding of [^{125}I]B4G7 to the cells (Fig. 2). Then the DNA/conjugate complex was added into a culture of NA cells overexpressing EGF receptors and CAT activity was measured after 60 h.

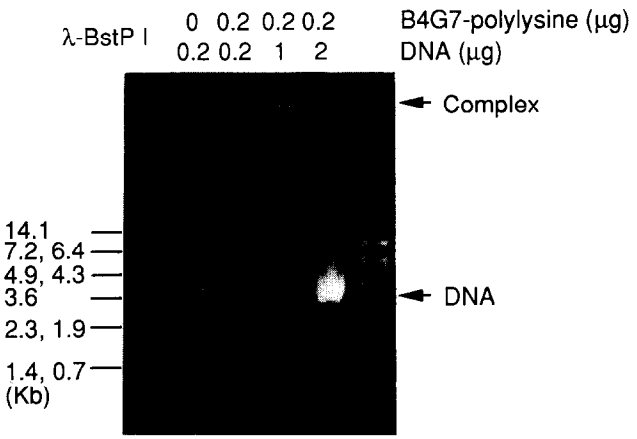

Fig. 1. Agarose gel electrophoresis of B4G7-polylysine/DNA complex. B4G7-polylysine conjugate (0.2 μg) was mixed with various amounts of pSV2 CAT plasmid DNA (0.2, 1, or 2 μg) and electrophoresed on agarose gel. The presence of DNA was visualized with ethidium bromide under UV illumination. DNA markers are I DNA digested with *Bst*PI

Significant CAT activity was expressed in these cells, whereas no CAT activity was detected when cells were treated with DNA alone or a mixture of antibody, polylysine and DNA (Fig. 3A). The CAT gene expression was detectable 12 h after transfection and increased until 72 h (data not shown). CAT activity was increased when the conjugate carrying 2-fold DNA was used for transfection (Fig. 3B). Thus, CAT gene DNA was delivered with B4G7 antibody through EGF receptors and expressed in the transfected cells.

4. Discussion

We have described a novel gene delivery system using the EGF receptor-specific monoclonal antibody. This

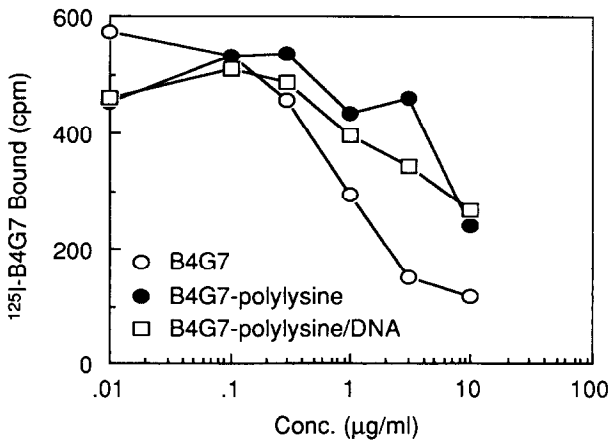

Fig. 2. Competitive inhibition of [^{125}I]B4G7 binding by B4G7-polylysine/DNA complex. Binding of [^{125}I]B4G7 antibody to A549 cells was measured in the presence of increasing amounts of B4G7-polylysine conjugate or B4G7-polylysine/DNA complex.

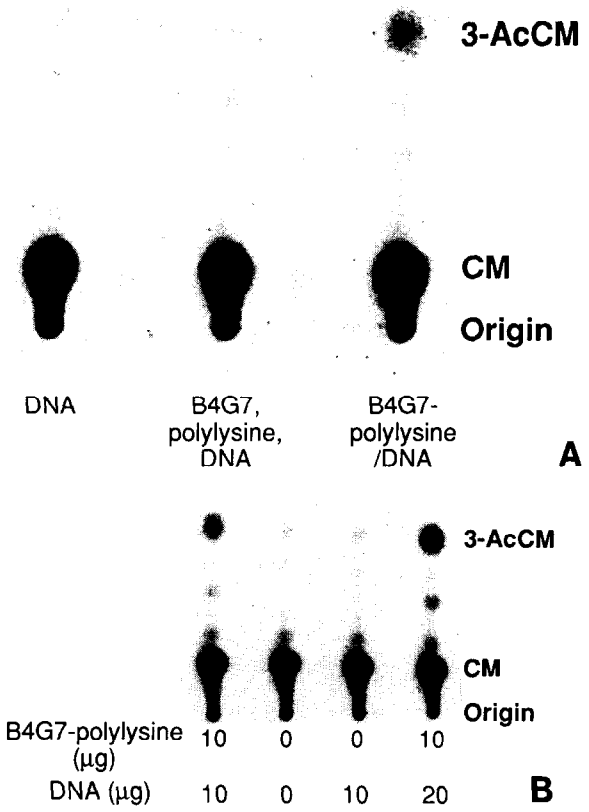

Fig. 3. CAT gene transfection by B4G7-polylysine/DNA complex. (A) Delivery of CAT gene through complex formation; NA cells were incubated with DNA only, a mixture of B4G7, polylysine and DNA, and B4G7-polylysine/DNA complex, respectively. CAT activity was detected as a production of 3-AcCM. (B) Dose dependent expression; NA cells were treated with complex of B4G7-polylysine (10 μg) with DNA (10 μg or 20 μg).

system utilizes a natural cellular mechanism which is receptor-mediated endocytosis and therefore little damage is given to the cells as compared to conventional gene transfer systems such as calcium-phosphate method and electroporation. Furthermore, the present system can avoid a potential problem of viral gene expression when a virus was used as a gene transfer vehicle. Previously, Wu et al. reported the gene delivery system using an asialoglycoprotein as a vehicle to target liver cells [13]. Our system is unique because a specific MoAb was used as a vehicle to target only the EGF receptor-expressing cells among various types of cells. In this regard, EGF receptor-overexpressing tumor cells like those derived from squamous cell carcinomas can be targeted as being successful in delivering toxic proteins [4–6]. In this paper, CAT gene was used as a reporter gene and its incorporation and expression was sufficient. Any genes can be delivered into target cells because attachment of DNA to the polylysine portion of conjugate is affinity binding. We are now using this system to develop gene therapy not only for EGF receptor-overproducing squamous cell carcinomas but also for certain genetic diseases.

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