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Targeted Gene Transfer for Adenocarcinoma Using a Combination of Tumor-specific Antibody and Tissue-specific Promoter

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We have developed a highly specific gene transfer method for adenocarcinoma using a monoclonal antibody against tumor-specific antigen coupled with a plasmid containing the carcinoembryonic antigen (CEA)-specific promoter. The chimeric CEA promoter (CC promoter), which contained an enhancer from the immediate early gene of cytomegalovirus and the CEA promoter, achieved 4- to 5-fold higher transgene expression in CEA-producing cells than the original CEA promoter while maintaining CEA specificity. Furthermore, a complex of a monoclonal antibody against Lewis Y antigen (LYA), the CC promoter-containing plasmid and cationic liposomes (DOTAP) achieved specific gene expression in CEA-producing and LYA-positive adenocarcinoma cell lines that was 200-fold more efficient than in CEA-non-producing and LYA-negative cell lines during a short *in vitro* incubation. This strategy may be applicable for clinical gene therapy.

Key words: Anti-Lewis Y antigen antibody — CEA promoter — CVM enhancer — Cationic liposome — Tumor-specific gene expression

Specific targeting of the transfer and expression of genes is essential for practical clinical gene therapy; however, no effective procedures for systemic administration of genes of interest followed by site (tissue)-specific expression have been established to date. Carcinoembryonic antigen (CEA) is one of the candidate molecules for a targeting strategy. CEA is widely expressed on the surface of neoplasms, and most colon[1)] and lung[2)] adenocarcinomas express particularly high levels of CEA on their surface. For this reason, use of the CEA-specific promoter for tumor-specific gene expression has been recently assessed.[3–5)] Even though the CEA promoter functions preferentially in CEA-producing tumor cells, it does not show as strong levels of activity as commonly used promoters such as the CMV or SV40 promoter.[3, 4)] We therefore constructed a strong CEA-specific promoter by cloning a CMV immediate early (IE) enhancer sequence[6)] into a site upstream of the CEA promoter sequence, and we found that this chimeric promoter (CC promoter) strongly expressed the transgene in a CEA-producing tumor cell-specific manner.

The use of a tissue-specific antibody as a DNA carrier may further improve the specificity of targeted gene therapy and has already been employed to target transgenes to specific cell types.[7–9)] In this study, we used a monoclonal antibody (H18A) directed against Lewis Y antigen (LYA)[10)] which is expressed on 60 to 90% of neoplasms,[11, 12)] and found that the complex of this antibody, the plasmid containing the CC promoter and cationic liposomes achieved rapid and highly specific gene transfection into CEA-producing and LYA-positive human colon (SW403) and lung (PC9) carcinoma cells. This strategy may provide effective *in vivo* gene transfer through systemic administration.

MATERIALS AND METHODS

Cell lines A human colorectal cancer cell line (SW403) was obtained from American Type Culture Collection (ATCC, Rockville, MD). A lung adenocarcinoma cell line (PC9) was kindly provided by Dr. N. Saijo (Tokyo). The melanoma cell line (316) was established in our laboratory. HeLa was established from cervical carcinoma. All cell lines were maintained in RPMI-1640 (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) complete medium (CM). They were cultured in an incubator containing 5% CO_2 at 37°C.

Analysis of soluble CEA production Media conditioned with 1×10^6 of cells for 72 h were analyzed for CEA concentration by radio immunoassay using CEA RIA BEAD (Dainabot, Chicago, IL).

Flow-cytometric analysis of cell surface antigens Cell surface CEA and LYA were analyzed by indirect immunofluorescence, using the supernatant from a hybridoma cell line (T84.66A3.1-H-11: ATCC) that produces a monoclonal antibody directed against CEA or a monoclonal

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antibody directed against LYA (H18A).[13] FITC-conjugated rabbit anti-mouse IgG (DAKO A/S: Glostrup, Denmark) was used as a second antibody. FACS analysis was performed by FACScan (Becton-Dickinson, San Jose, CA).

Plasmid construction The pCEA/GAL plasmid was constructed by subcloning a minimal CEA promoter sequence from the pCEA424/2CAT plasmid (kindly provided by Dr. J. Thompson, Germany)[3] into a site upstream of the luciferase (*luc*) gene of pGL2-basic (Promega, Madison, WI), then replacing the *luc* gene with a β-galactosidase (*GAL*) gene which had been excised from pSV-β-GAL (Promega). The cytomegalovirus (CMV) enhancer is derived from the *IE* gene of human CMV.[14] To make a chimeric promoter, the CMV enhancer was cloned into a site immediately upstream of the CEA promoter and named the CC promoter for forward insertion and CrC promoter for reverse insertion (pCC/GAL and pCrC/GAL). We also made a CEA2 promoter by cloning a tandem CEA promoter repeat into a site upstream of the *GAL* gene (pCEA2/GAL). The plasmid pSV-β-GAL (*GAL* gene driven by SV40 promoter: Promega) was used as a control. All plasmids were maintained and propagated in *Escherichia coli* strain DH5α cells. To isolate and purify the plasmid, a kit from Qiagen (EndoFree Plasmid Maxi Kit: Chatsworth, CA) was used according to the manufacturer's instructions. Plasmid concentrations and purity were determined by A_{260}/A_{280} measurements. All plasmids used in this study are shown in Fig. 1.

Evaluation of promoter activity Transfection experiments were performed using cationic liposomes (DOTAP: Boehringer-Mannheim, Indianapolis, IN). Twenty-four hours prior to transfection, 5×10^5 cells/well (SW403, SW1463 and PC9) or 2.5×10^5 cells/well (316 and HeLa) were transferred into a 12-well plate (CLUSTER 12: Costar, Cambridge, MA) and incubated in CM at 37°C, under 5% CO_2. Wells were then washed with serum-free media immediately prior to transfection. The total volume of 600 μl of serum-free media containing plasmid-DOTAP complex (1 μg of plasmid DNA coupled with 5 μg of DOTAP per 2.5×10^5 cells) was left for 8 h, and then the cells were placed into CM and subsequently incubated for 32 h at 37°C, under 5% CO_2. After incubation, cells were harvested and assayed for GAL activity.

Formation of anti-LYA antibody (H18A: H), DNA plasmid (D) and cationic liposome (L) complex (H-P-D-L) The monoclonal anti-Lewis Y antibody (H18A: H) was coupled to poly-L-lysine (P) (Sigma Chemical Co., St. Louis, MO) using 3-(2-pyridyldithio)propionate (SPDP: Pierce Chemical Co., Rockford, IL) as described.[15, 16] Briefly, 1 mg of H18A in 0.5 ml of 400 m*M* NaCl, 80 m*M* HEPES buffer, pH 7.9 was mixed with 6.2 μl of a 10 m*M* ethanolic solution of SPDP and incubated for 3 h at room temperature (molar ratio of 1:10) and then purified by Sephadex G25 gel filtration. The reaction mixture and a solution of 9.2 nmol of poly-L-lysine (average chain length of 300 lysine monomers), which had been modified with 3-mercaptopropionate (molar ratio of 1:4), were mixed and incubated under an argon atmosphere at room temperature for 24 h. The final complex (H-P) was isolated by cation-exchange chroma-

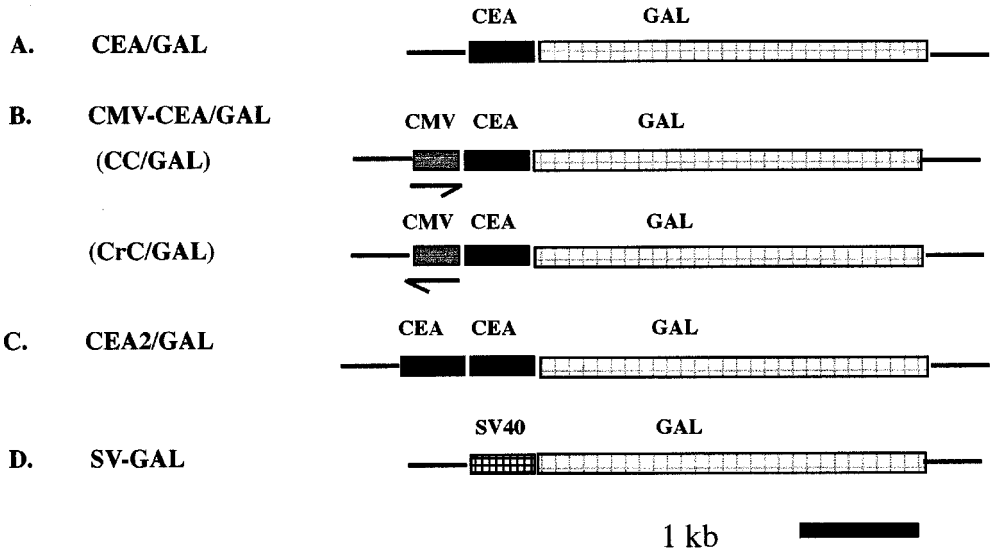

Fig. 1. Plasmids used in this study. *GAL* gene driven by CEA promoter (A), chimeric promoter containing CMV IE enhancer and CEA promoter (B), chimeric promoter containing a tandem CEA promoter repeat (C), and SV40 promoter (D). Procedures for plasmid construction (A, B and C) are mentioned in "Materials and Methods."

tography. Isotype-matched monoclonal antibody (Ig G3) of unknown specificity was used as a negative control (C). The aliquots were stored at −20°C until further procedures. To construct the H-P-plasmid DNA complex, 2 μg of pCC/GAL (D) in 75 μl of Hanks' balanced salt solution (HBSS) was added dropwise with gentle stirring to 75 μl of HBSS containing various amounts of H-P (1.0 μg, 0.5 μg or 0.25 μg of H18A coupled to poly-L-lysine) and incubated for 30 min at room temperature. The resulting three types of H-P-DNA plasmid (H-P-D) complex were examined for transfection efficiency. We also constructed the H-P-DNA-cationic liposome (H-P-D-L) complex by mixing H-P-D complex (containing 2 μg of pCC/GAL) in 150 μl of HBSS and 50 μl of HBSS containing 4 μg of DOTAP (L) and incubating the mexture at room temperature for 20 min. The transfection efficiency was then examined.

Kinetics of transfection with H18A-pCC/GAL-cationic liposome (H-P-D-L) complex Twenty-four hours prior to transfection, 5×10^5 cells/well (SW403 and PC9) or 2.5×10^5 cells/well (HeLa or 316) were transferred to 12-well plates and incubated in CM at 37°C, under 5% CO_2. H-P-D-L (using 1 μg of DNA per 2.5×10^5 cells) in a total volume of 200 μl of HBSS was transferred to 12-well plates in which the media were replaced with 400 μl of serum-free media immediately prior to the start of transfection and incubated for various times (0.5, 1, 2, 4 and 8 h) at 37°C, under 5% CO_2. We also used control antibody-pCC/GAL-cationic liposome (C-P-D-L) complex as a control. After transfection, wells were washed with CM to remove free plasmid complexes and subsequently incubated in CM for 24 to 32 h, and then the cells were harvested for GAL activity assay.

GAL activity assay Cells were harvested after incubation and assayed for GAL activity with a kit from Promega (β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer) according to the manufacturer's instructions. Briefly, cells were washed twice with phosphate-buffered saline and treated with reporter lysis buffer. Then the cells were scraped off, transferred into a microcentrifuge tube, vortexed and centrifuged. The cell extracts were transferred to 96-well microplates (Falcon 3072: Becton Dickinson, Lincoln Park, NJ) for GAL assay and protein concentration assay. The protein concentration of each extract was assessed with a kit from Pierce (BCA protein assay kit) according to the manufacturer's instructions. Data were expressed as milliunits of GAL activity per milligram protein of cell lysate (mU/mg) using a standard GAL sample and a standard albumin sample run in parallel.

RESULTS

CEA and LYA on the cell lines Adenocarcinoma cell lines (SW403 and PC9) showed a high frequency of CEA expression on the cell surface, whereas melanoma cells (316) and HeLa cells did not express any detectable CEA (Fig. 2A). These data correlated well with the levels of CEA in culture supernatants. CEA was produced from SW403 and PC9 cells at a rate of 43.0 ng/10^6 cell/72 h and 52.6 ng/10^6 cell/72 h, respectively. CEA was not detected in the culture supernatant of HeLa or 316 cells (<1.0 ng). LYA was also detected on SW403, PC9 cells, but not on the surface of HeLa and 316 cells (Fig. 2B).

CEA promoter activity in CEA-producing and -non-producing cells GAL activities in cell lines that were transfected for 8 h with the *GAL* gene driven by various promoters were analyzed 32 h after transfection. We used a plasmid-DOTAP complex weight ratio of 1:5. This ratio achieved maximal transfection efficiency for all cell lines used in this study (data not shown). While the CEA promoter showed higher activity in SW403 and PC9 cell lines than in HeLa and 316, this activity was only about 30% of the activity of the nonspecific SV40 promoter. The CC promoter showed 120–140% of the activity of SV40 promoter in SW403 and PC9 (about 450% of CEA promoter activity). Furthermore, the CC promoter maintained cell specificity, with less than 25% activity in CEA-non-producing cell lines (HeLa and 316) as compared to that achieved by the SV40 promoter. The reverse CMV enhancer (CrC) also augmented CEA promoter activity to almost the same degree in SW403 and PC9 cell lines as compared to the forward CMV enhancer and had equivalent specificity. On the other hand, the tandem repeat CEA promoter (CEA2) exerted a little less than twice the promoter activity achieved by the original CEA promoter (Fig. 3).

Transfection efficiency of H18A-directed plasmid complex The optimal antibody (H18A), plasmid DNA (pCC/GAL), cationic liposome (DOTAP) ratio and the kinetics of transfection for LYA-positive and CEA-producing adenocarcinoma cell lines were investigated. As indicated in Fig. 4, the highest transfection efficiency for 5×10^6 of SW403 or PC9 cells was achieved by complex no. 5, containing 2 μg of pCC/GAL coupled with 0.5 μg of H18A and 4 μg of DOTAP (complex no. 5: weight ratio, 4:1:8) under various conditions. However, the transfection efficiencies of antibody-plasmid complexes (no. 2, 4, 6) were not greater than those of DOTAP-containing complexes (no. 3, 5, 6). A plasmid-antibody weight ratio of 1:1 showed a lower transfection efficiency than a ratio of 2:1 (data not shown).

To examine the kinetics of transfection with complex no. 5 *in vitro*, we compared the transfection efficiency of H18A (H) and control antibody (C) (H-P-D-L and C-P-D-L). Although there were no notable differences observed over 4 to 8 h of transfection, H-P-D-L gave a significantly higher transfection efficiency than that achieved by C-P-D-L for LYA-positive and CEA-producing SW403 or PC9

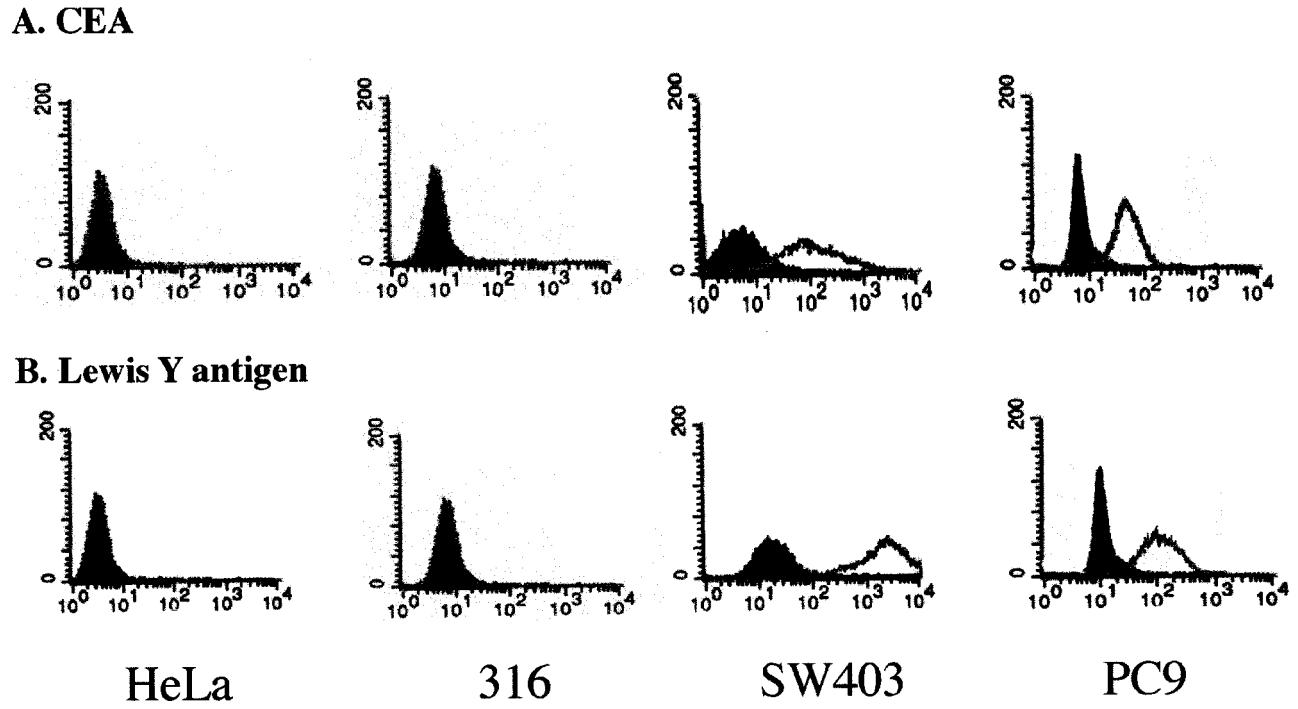

Fig. 2. Cell surface CEA expression (A) and LYA expression (B) on HeLa, melanoma (316), colorectal carcinoma (SW403) and lung adenocarcinoma (PC9) cells. Indirect immunofluorescence examination was performed with anti-CEA antibody or anti-LYA antibody (H18A) as detailed in "Materials and Methods."

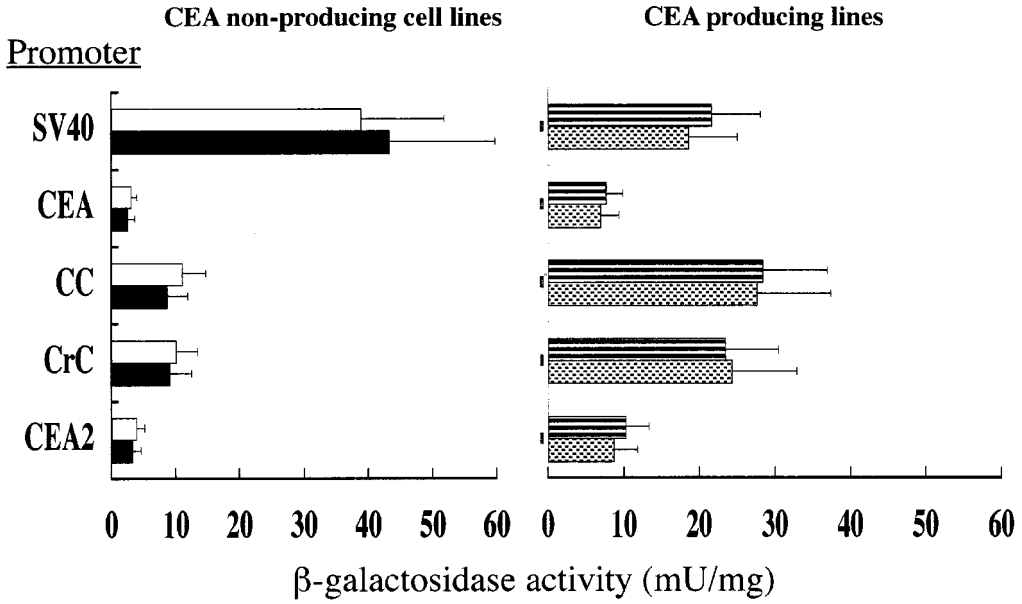

Fig. 3. Comparison of CEA promoter activities in CEA-negative (left) HeLa (open bar) and 316 (black bar), and CEA-positive (right) SW403 (line bar) and PC9 (dotted bar) cell lines. CEA, minimal CEA promoter; CC, minimal CEA promoter connected to the CMV enhancer; CrC, minimal CEA promoter connected to a reverse CMV enhancer; CEA2, tandem repeat minimal CEA promoter. The SV40 promoter was used as a standard control. Data represent the average of three experiments. Promoter activity was expressed as GAL activity which was measured as detailed in "Materials and Methods" and expressed as milliunit/mg (protein) of cell lysate. Data represent the average of three experiments.

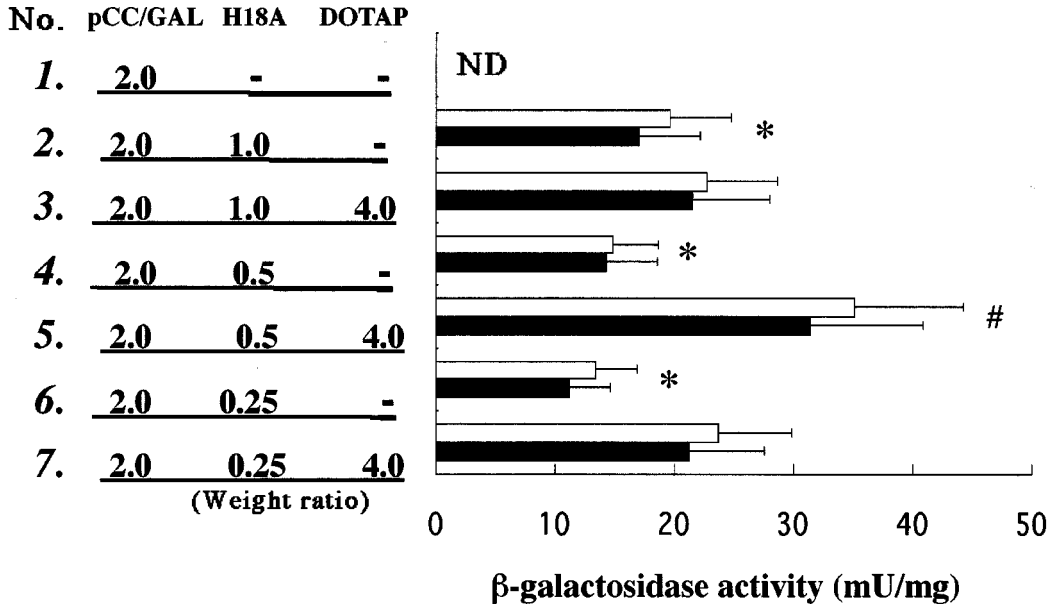

Fig. 4. Comparison of the transfection efficiencies of plasmid DNA (pCC/GAL) coupled with various amounts of antibody (H18A-polylysine complex) and cationic liposomes (DOTAP). The table on the left indicates the actual weight (μg) of each component used for the transfection of 5×10^6 SW403 (open bar) and PC9 cells (black bar). The procedure of plasmid complex formation or transfection was detailed in "Materials and Methods." Promoter activity was expressed as GAL activity, which was measured as detailed in "Materials and Methods" and expressed as milliunit/mg (protein) of cell lysate. Data represent the average of three experiments in a 2-h transfection period. ND: not detected. $*$ $P<0.05$ compared with complex no. 5 #.

cells in 0.5 h and 1 h transfections ($P<0.05$) (Fig. 5, SW403 and PC9). The H18A-specific advantages were almost completely abolished by the presence of 10 ng/ml of free H18A antibody in the culture media (data not shown). Furthermore, the average values of GAL activity after 1 h of transfection of CEA non-producing and LYA-negative HeLa and 316 cell lines were 0.12 and 0.13 (mU/mg), respectively (Fig. 5, HeLa and 316).

DISCUSSION

A number of cancer gene therapy strategies have been developed in recent years, but several obstacles to clinical application still remain. One of the most important problems is effective targeting of the gene of interest to cancer cells *in vivo*. We have been focusing on a targeting method that uses the combination of a plasmid containing a CEA-specific promoter and anti-LYA monoclonal antibody for CEA-producing and LYA-positive colorectal cancer or lung adenocarcinoma cell lines. As shown in Fig. 3, the plasmid containing the minimal CEA promoter sequence specifically expressed GAL in CEA-producing cell lines, but not in CEA-non-producing cell lines. However, the activity of the CEA promoter was only about one-fourth of that of the SV40 promoter. In order to enhance CEA promoter activity while maintaining specificity, a chimeric (CC) promoter was constructed by cloning a human CMV immediate early enhancer sequence upstream of the CEA promoter sequence, and this was tested for activity. Boshart and colleagues reported that the human CMV enhancer sequence has little cell-type or species specificity and that its activity is several fold stronger than that of the SV40 enhancer.[6] This CMV enhancer sequence successfully augmented CEA promoter activity by up to 5-fold in CEA-producing cell lines, with retention of the specificity. Richards and colleagues have constructed the active CEA-specific promoter by cloning small sequences from the 5′ sequence of the CEA gene.[17] We did not compare the activity and specificity of their promoter and ours, but our strategy is simple and can be utilized for other tissue-specific promoters such as the tyrosinase promoter for melanoma[18, 19] and the α-fetoprotein promoter for hepatocellular carcinoma.[20]

A number of targeting molecules have been used as DNA carriers, including transferrin (Tf) for Tf receptor-positive cells,[15] asialoglycoprotein for asialoglycoprotein receptor-positive hepatocytes,[21] and monoclonal antibodies for antigen-positive cells.[7–9, 22] In this study, we used a monoclonal antibody (H18A) against LYA, which is a stage-specific embryonic antigen-1 (SSEA-1)[23] specifi-

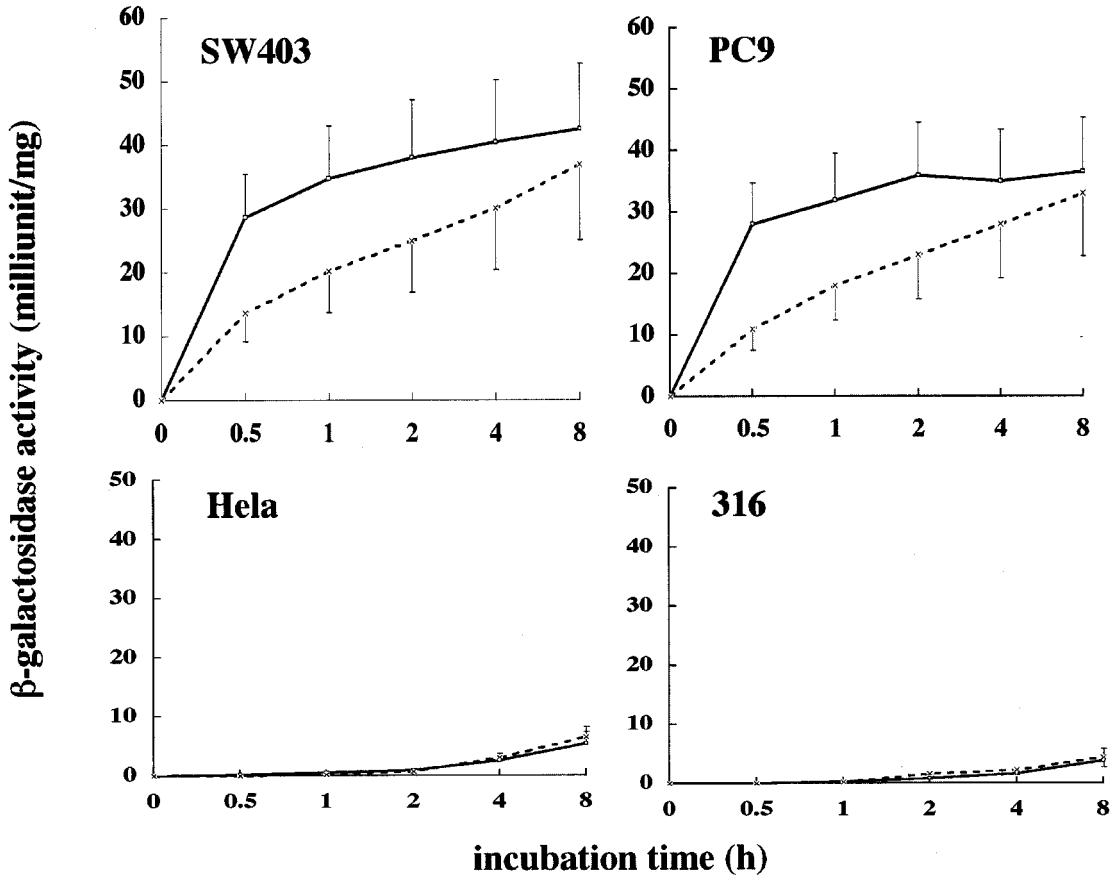

Fig. 5. Kinetics of transfection of LYA-positive and CEA-producing cell lines (SW403 and PC9) or LYA-negative and CEA-non-producing cell lines (HeLa and 316) using plasmid (pCC/GAL: D) coupled to antibody (H-P: H18A coupled with polylysine, C-P: control nonspecific antibody coupled with polylysine) and cationic liposomes: L (weight ratio of 4:1:8). H-P-D-L (solid line), C-P-D-L (dotted line). Formation of the antibody-pCC/GAL-cationic liposome ternary complex was described in "Materials and Methods." Cells were placed in 12-well plates and incubated overnight. After washing of the wells, plasmid complexes were placed in each well for each indicated period (from 0.5 to 8 h), then the wells were washed and incubation was continued for 24 to 32 h. GAL activities were measured and expressed as milliunit/mg of cell lysate (protein). The procedures are described in "Materials and Methods." Data represent the average of three experiments.

cally expressed on the surface of cancer cells as well as embryonic cells. Antibodies directed against LYA have been widely used for experimental and clinical tumor targeting.[24–27] Although LYA is not expressed in all adenocarcinoma cells, the advantage of employing the anti-LYA antibody (H18A) for both LYA- and CEA-positive tissue is that LYA, unlike CEA, is rarely shed from the cell. Furthermore, use of an anti-CEA antibody coupled with a CEA promoter may potentially also target CEA-expressing normal tissue, causing unwanted adverse effects when applied to clinical gene therapy.

Since many previous studies used poly-L-lysine (P),[7–9] we also used P to construct an antibody (Ab)-plasmid (D) complex. We prepared two types of complex: Ab-P-D and Ab-P-D-cationic liposomes (L). As shown in Fig. 4, Ab (H18A)-P-D-L complexes achieved a higher transfection efficiency than Ab-P-D complexes. This result may be because the anti-LYA antibody can target LYA-positive cells, but might not be naturally endocytosed, and the antibody may not efficiently lead to plasmid DNA internalization by the target cells. Another possible explanation would be that an antibody-plasmid weight ratio of 1:2 or less may be sufficient to obtain target cell binding, and an excess of antibody and poly-L-lysine may interfere with the plasmid by forming a DNA-cationic liposome complex, which may have more potent nonspecific transfection activity.

As shown in Fig. 5, 8-h *in vitro* transfection showed no significant advantage for H18A conjugation over the control antibody. However, short transfection periods, espe-

cially 0.5 to 1 h, resulted in a significant increase in GAL activity in H18A conjugate-treated wells, with the average GAL activity at 0.5-h transfection reaching 80% of that achieved by an 8-h transfection. This rapid antibody-directed attachment to the target cell may provide a great advantage when using these complexes for systemic administration. Further examination will be necessary to determine efficacy *in vivo*.

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