Specifically Targeted Killing of Carcinoembryonic Antigen (CEA)-expressing Cells by a Retroviral Vector Displaying Single-Chain Variable Fragmented Antibody to CEA and Carrying the Gene for Inducible Nitric Oxide Synthase¹

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ABSTRACT

The generation of retroviral vectors that infect specific cell types through recognition of cell surface antigens is a promising and effective approach to targeted gene therapy of cancer. Carcinoembryonic antigen (CEA), a highly characterized, cell surface glycoprotein overexpressed by various tumor cells, provides a specific tool for tumor tissue-specific targeting by retroviral vectors. The conventional suicidal gene delivery systems need additional drugs other than their gene products. The inducible nitric oxide synthase (iNOS) gene product yields nitric oxide (NO), which directly induces autotoxicity and cytolytic bystander cells. In the present study, we have developed a novel bifunctional Moloney murine leukemia virus-based recombinant retroviral vector that displays a chimeric envelope protein containing a single-chain variable fragmented (scFv) antibody to CEA and carries the iNOS gene in the genome. The resultant bifunctional retroviral vector showed a specific delivery of the iNOS gene to human CEA-expressing carcinoma cells, resulting in the direct and efficient killing of CEA-expressing carcinoma cells by induction of apoptosis. This is the first report of successful killing of CEA-expressing cells by specific targeting of the iNOS gene. This approach may offer a one-step procedure for effective gene therapy of CEA-expressing tumors.

INTRODUCTION

Most experimental and clinical trials for gene therapy have adopted retroviral vectors originated from murine leukemia virus to transfer specific genes into target cells. To establish gene therapy as a feasible treatment of cancer, more emphasis will be required on the development of optimal gene delivery systems with greater tumor tissue specificity. One of the efforts of tissue-specific targeting is based on attempts to engineer the normal retroviral envelope protein. Recent advances in the genetic engineering field provide a concept for target cell specificity by changing the tropism of normal envelope retroviral receptor-binding domain with a ligand or a scFv that recognizes a specific cell surface receptor or antigen (1–6).

CEA is a highly glycosylated protein of about 180,000 and is widely expressed on the surface of tumor cells of various human tissues as well as on the epithelial cells of normal gastrointestinal tissues and fetal intestine (7). In normal adult human colon tissues, however, CEA is localized on the luminal surface of the single layer of columnar epithelial cells lining the upper parts of the crypts (8), so that normal CEA is not directly in contact with blood flow or tissue fluid. On the other hand, in tumor tissues that no longer conform to the single-layer organization by invading through the basement membrane in multicellular arrays, CEA is usually localized at all sides of the cell membranes and is directly facing blood flow or tissue fluid (8). Hence, tumor CEA can be a useful target molecule for gene therapy using anti-CEA antibody. We have previously prepared an monoclonal antibody clone F11–39, which shows a high affinity to CEA and discriminates CEA in tumor tissues from the other CEA gene family antigens in the normal tissues (9). A mouse-human chimeric antibody (Ch F11–39) generated from F11–39 has retained the same affinity with that of the parental monoclonal antibody and suppressed tumor growth both in vitro and in vivo when administered with lymphokine-activated killer cells (10, 11). Thus, the scFv form of this antibody seems to be applicable to the targeting of retroviral vectors to CEA-expressing tumor cells by expression in the envelope proteins of the virus particles that contain a certain killing or suicidal gene.

The second important issue in this approach is the selection of the gene used for cell killing. NO, one of the smallest biologically active molecules, is a potential toxin for immunological self-defense. The formation of NO from l-arginine in mammalian cells is catalyzed by NOSs including iNOS, endothelial NOS, and brain NOS (12, 13). Under physiological conditions, iNOS is absent from mammalian cells, but when cells are activated, it is induced and causes a long-lasting generation of NO, which is associated with a cytotoxic reaction against pathogens and tumor cells (13, 14). Previous studies have shown that iNOS transfected into mouse melanoma cells suppressed their tumorigenicity (15) and also lysed bystander murine cells under in vitro and in vivo conditions, which suggested that NO-mediated cell killing does not require transfection of every cell (16). However, there is no report thus far on the specific-targeted delivery of the iNOS gene against tumor cells.

In the present study, we have generated a bifunctional Moloney murine leukemia virus-based retroviral vector that displays a chimeric envelope protein including an anti-CEA scFv antibody derived from Ch F11–39 and carries the iNOS gene in the genome. The recombinant retrovirus showed a specific delivery of the iNOS gene to human CEA-expressing carcinoma cells and directly killed the infected cells by induction of apoptosis without any additional drugs.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The mouse fibroblast cell line NIH3T3, the NIH3T3-derived ecotropic retroviral packaging cells GP+E-86, and the amphotropic retroviral packaging cells PA317 were obtained from American Type Culture Collection (Rockville, MD). The human gastric carcinoma cell lines MKN-45, KATO-III, and MKN-74 were obtained from the Japanese Cancer Research Resources (Tokyo). All of the above cells were cultured in DMEM (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Bio-Whittaker, Walkersville, MD), 100 units/ml penicillin and 100 µg/ml streptomycin. A CEA-expressing CHO clone (CHO-CEA) and its parent CHO cell line were described previously (17).
and maintained in α-MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and penicillin and streptomycin. The cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂.

**Plasmid Construction.** The V₅₅ and Vₓ cDNA genes were obtained from the transfectoma Ch F11–39 (10) by RT-PCR using oligonucleotide primers corresponding to the 5' and 3' consensus amino acid sequences of immunoglobulin variable regions, and joined together using the flexible 14-amino-acid long 212 linker (18) by the overlap extension PCR method. The PCR products thus obtained were cloned into the pT7 blue vector (Novagen, Novagen, WI). The PCR products were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 10 μM calcium. The modified scFv cDNA was modified at its 5' and 3' ends with the following PCR primers introducing the unique XhoI and BstEII sites, respectively (underlined): 5'-TCTCGAGGACATCCAGATGACTCAGTCT-3' and 5'-TGGTGAGCTCGTCAGGAGACGAGATGACCAGAAG-3'. The modified scFv cDNA (728 bp, 242 amino acids) was inserted into the XhoI and BstEII restriction sites of the envelope expression vector pEnv 20.22 (kindly provided by Dr. N. V. Somia, Salk Institute, La Jolla, CA), which contains a strong cytomegalovirus immediate-early promoter (19), which yielded the plasmid pscFv-env (Fig. 1). This resulted in insertion of the scFv between the sixth and seventh amino acid of the ectropic envelope.

Mouse iNOS cDNA was inserted into the pLNCL retroviral vector with the cytomegalovirus promoter and a neomycin resistance gene (Clontech, Palo Alto, CA). Briefly, an adapter containing a HincII and an SphI site was inserted into the multiple cloning site of pLNCL. The pLNCL8 construct (Oxford Biomed. Res. Inc., Rochester Hills, MI) was digested with HincII and SphI, and the resulting 3.6-kb iNOS fragment was cloned into the HincII/SphI site of pLNCL, yielding the plasmid pLCN-iNOS.

The nucleotide sequences of all of the inserts were confirmed with the ABI Prism 377 DNA sequencing system (PE Applied Biosystems, Foster City, CA).

**Reconstruction of Packaging Cells.** To establish a permanent clone of ectropic packaging cells for expression of the chimeric envelope displaying anti-CEA scFv antibody, 5 × 10⁵ GP+E-86 cells were cotransfected with 12 μg of the plasmid pscFv-env and 2 μg of the hygromycin-B phosphotransferase expression vector pcDNA3.1/Neo (Invitrogen, Carlsbad, CA) by calcium phosphate method. Clones were selected in the presence of hygromycin B (200 μg/ml; Wako Pure Chemical, Osaka, Japan) for 10–12 days. The positive clones were screened by RT-PCR using the primers for anti-CEA scFv (see above) and by an EPICS flow cytometer (Coulter, Miami, FL) using biotinylated CEA (20, 21) and FITC-conjugated avidin (Vector Laboratories, Burlingame, CA). The highest positive permanent packaging cell clone thus obtained was named GPEscFv-env. For control, the GP+E-86 cells were transfected only with the hygromycin-B phosphotransferase gene, selected and designated GPE-hgB.

**Production of Viruses.** Retrovirus carrying the iNOS gene was obtained by transfection of the PA317 packaging cells with pLNClNOS using the calcium phosphate method. After 48 h, the GPEscFv-env or GPE-hgB packaging cells were infected with the culture supernatant of the transfected PA317 cells in the presence of Polybrene (4 μg/ml; Sigma Chemical Co., St. Louis, MO) by spin infection at 2000 rpm for 2 h at 32°C. After washing, the successfully iNOS-infected cells were selected for 14 days in the medium containing 2 μM of the specific iNOS inhibitor NMA (Sigma; Ref. 15) and 600 μg/ml of G418 (Calbiochem-Novabiochem, San Diego, CA). For virus production, medium was harvested from confluent monolayers of the virus-producing cells 18–20 h after a medium change. The supernatant was filtered through a 0.45-μm pore filter (Millipore, MA) and used immediately for infection or stored at −80°C. The clones showing the highest virus titer for the NIH3T3 cells were chosen as virus-producing cells. The resultant recombinant retrovirus displaying anti-CEA scFv and carrying the iNOS gene was termed GPEscFv-env/iNOS and the retrovirus only carrying the iNOS gene as GPE/iNOS. To increase the virus titer, the viral supernatants were concentrated by centrifugation at 6000 rpm for 18 h at 4°C (5).

**Immunoblotting.** For detection of envelope proteins, 6 μl of viral supernatants were centrifuged as described above, and the pellet was dissolved in 100 μl of SDS-PAGE sample buffer. Proteins were then separated by SDS-PAGE on 8% gel and electroblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). After blocking, the membrane was reacted successively with anti-RVL gp70 antiserum (goat anti-Rouscher leukemia murine envelope gp69/71; Quality Biotech, Camden, NJ), biotinylated anti-goat IgG, and horseradish peroxidase-conjugated avidin (Vector). Proteins on the membranes were visualized by using the ECL system and Hyperfilm-ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

**RT-PCR.** Total RNA was isolated from virus-producing cells or infected cells using the High Pure RNA Isolation kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Isolated RNA (5 μg) was reverse transcribed by using the Superscript Preamplification System (Life Technologies, Rockville, MD). PCR amplification was performed using AmpliTaq Gold (Perkin-Elmer, NJ) and the primers were synthesized from published sequences with sense primer 5'-CTTCAGGAGACGAGAAGCCTG-3' and antisense primer 5'-GATCCTCACATCTGTTGACG-3' for iNOS; 5'-TACCGGCAAGGCGCGCGTTCTTCTT-3' and 5'-ATGCAGAAGACCG-GCTCCTCAAGC-3' for neomycin; 5'-GTTGGGGCGCTTGGACCAACAA-3' and 5'-CTCTTTGGATGTCACCGACATTTC-3' for mouse β-actin; 5'-TCTCTGCCAGGCGTGTCTACGAC-3' and 5'-TCTCTTAGGACGCGTGTCTACGAC-3' for human β-actin; and the above-mentioned oligonucleotides for anti-CEA scFv. PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

**NO Analysis.** The concentration of NO in the culture supernatants of the virus-producing cells was determined by a microplate assay as described by Ding et al. (22). Briefly, 100 μl of supernatant cultured in the absence of NMA was mixed with 100 μl of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid; Wako) at room temperature for 10 min in the dark. The absorbance at 540 nm was monitored by a microplate reader (Benchmark; Bio-Rad, Hercules, CA). NO concentration was determined using sodium nitrite as the standard.

**Virus-binding Assay.** Target cells were detached from culture flasks by incubation in trypsin-EDTA solution and were resuspended in cold PBS supplemented with 2% BSA and 0.1% sodium azide. The cells (1 × 10⁶) were then incubated with the GPEscFv-env/iNOS or GPE/iNOS retrovirus for 30 min at 4°C for virus binding. The cells were washed with PBS and were incubated with goat anti-RVL gp70 antiserum diluted at 1:1000 in PBS. After washed three times, the cells were incubated with biotinylated goat IgG-FITC conjugate (DAKO, Glostrup, Denmark) for 30 min at 4°C and were analyzed by the EPICS.

**Viruses Infection and Virus Titer Assay.** Target cells were seeded at 1 × 10⁵ cells/well in 6-well plates (Becton Dickinson, Franklin Lakes, NJ). After 24 h, the target cells were infected with the GPEscFv-env/iNOS or GPE/iNOS retrovirus in the presence of Polybrene (4 μg/ml) by spin infection at 2000 rpm for 2 h at 32°C. After washing, the cells were cultured for 12 days in the presence of 2 μM NMA and 600 μg/ml G418, and the virus titer was determined after counting G418-resistant colonies and was calculated by the following formula: G418-R colony-forming units/ml = number of colonies/virus volume (ml) × replication factor × fraction of infected cells plated.

**In Vitro Cytotoxicity Assay.** The cytotoxicity of the GPEscFv-env/iNOS or GPE/iNOS retrovirus was detected by in vitro cytotoxicity assay. Target cells were infected as described above. The multiplicity of infection used was...
DNA was visualized by ethidium bromide staining. Electrophoresed together with molecular weight markers on 2% agarose gel. Samples were adjusted to 0.1. After washing, the cells were cultured for 7 days at 37°C in the absence of NMA. Noninfected control target cells were incubated for the same days in the plain medium. The remaining cells were collected in 96-well black plates (Labsystems, Helsinki, Finland), centrifuged, and incubated with the fluorescent dye H33342 (25 μg/ml; Calbiochem-Novabiochem) for 1 h at 37°C. Cells were washed and suspended in 100 μl of PBS, and their fluorescence intensity was measured at excitation wavelength of 355 nm and emission wavelength of 460 nm by using a Fluoroskan II fluorometer (Labsystems). The results were expressed as percentage survival, which was obtained by the formula: (mean test results - mean blank)/mean control × 100.

Analysis of DNA Fragmentation. Target cells were infected with the GPEscFv-env/iNOS or GPE/iNOS retrovirus as described above. After 7-day culture in the absence of NMA, the low-molecular-mass DNA was extracted from the infected cells using the Apop Ladder Ex Kit (Takara Biomedicals, Tokyo, Japan) according to the manufacturer’s instructions. Samples were electrophoresed together with molecular weight markers on 2% agarose gel. DNA was visualized by ethidium bromide staining.

RESULTS

Transcription of Anti-CEA scFv and iNOS Genes in Reconstructed Packaging Cells. The chimeric envelope expression vector pscFv-env was first transfected to the GP+E-86 cells to generate the reconstructed GPEscFv-env packaging cells, which can produce viral particles codepositing an anti-CEA scFv envelope chimeric protein and an unmodified envelope protein. The GPEscFv-env packaging cells were then infected with the culture supernatant of the PA317 cells transfected with pLNC-iNOS to produce the GPEscFv-env/iNOS retrovirus. To demonstrate that the resultant GPEscFv-env/iNOS retrovirus producing cells contained cDNAs for anti-CEA scFv and iNOS, the total RNA was isolated from different packaging cells and analyzed by RT-PCR. Anti-CEA scFv cDNA appeared as a 728-bp PCR product in two GPEscFv-env/iNOS clones (Cl-1 and Cl-2; Fig. 2A, Lanes 5 and 6). The GP+E-86 cells (Lane 2), the PA317 cells (Lane 3), and the GPE/iNOS retrovirus producing clone (Lane 4) showed no band for the scFv. The presence of the specific band of iNOS was also detected in two GPEscFv-env/iNOS clones (Cl-1 and Cl-2; Fig. 2B, Lanes 5 and 6) as well as in GPE/iNOS cells (Lane 4) but not in the GP+E-86 and PA317 cells. The presence of neomycin gene in these three packaging cells (Fig. 2C, Lanes 4–6) was also confirmed.

Production of NO by Reconstructed Packaging Cells. High levels of NO (range, 32–38 μM) were observed in two GPEscFv-env/iNOS retrovirus producing clones, Cl-1 and Cl-2, and the GP+E-86, GPEscFv-env and GPEX cells produced much lower levels of NO in the absence of NMA (Fig. 3).

Expression of Anti-CEA scFv Chimeric Envelope Protein in Reconstructed Packaging Cells and Recombinant Retrovirus. To demonstrate that the chimeric envelope protein was transported to and correctly oriented in the cell membrane, we analyzed the reconstructed packaging cells by flow cytometry using biotinylated CEA. The peak was shifted on the chimeric envelope expressing clone GPEscFv-env, whereas no shift was found on the clone GPE-hgB (Fig. 4A).

Incorporation of the chimeric envelope protein into the recombinant GPEscFv-env/iNOS retrovirus was confirmed by immunoblotting of the virus particles with anti-envelope antiserum. The GPE/iNOS retrovirus contained only the normal envelope protein of about Mr 70,000 (Fig. 4B, Lane 1), whereas the GPEscFv-env/iNOS retrovirus contained the chimeric envelope protein of about Mr 97,000 as well as the normal envelope protein (Lane 2), which confirmed the expected size of scFv to be about Mr 27,000. These findings demonstrated that the chimeric envelope protein, as well as the normal envelope protein, was incorporated into the GPEscFv-env/iNOS retrovirus.

Specific Binding of Recombinant Retrovirus to CEA-expressing Cells. The recombinant GPEscFv-env/iNOS retrovirus, which expresses the anti-CEA scFv chimeric envelope protein, was examined for the binding activity to CEA-expressing cells by flow cytometry (Fig. 5). The GPEscFv-env/iNOS retrovirus showed significant binding activities to MKN-45, KATO-III, and CHO-CEA cells (Fig. 5, A–C), all expressing CEA on their cell surface, but did not do so to CEA-nonexpressing MKN-74 cells (Fig. 5D) and parental CHO cells (Fig. 5E). The GPE/iNOS retrovirus, displaying only normal envelope protein, was unable to bind to CEA-expressing nor to CEA-nonexpressing cells (Fig. 5, A–E). These results indicated that the GPEscFv-env/iNOS retrovirus specifically bound to CEA-expressing cells.

Blanks ± SD. Bars
Specific Infection of Recombinant Retrovirus to CEA-expressing Cells. Normally, wild-type ecotropic viruses do not infect human cells. We tested whether the GPEscFv-env/iNOS retrovirus infects human CEA-expressing cells. As expected, both the GPE/iNOS and GPEscFv-env/iNOS retroviruses showed high titers against the mouse NIH3T3 cells (Table 1). However, only the GPEscFv-env/iNOS retrovirus, which displayed the anti-CEA scFv chimeric envelope protein, infected CEA-expressing cells of human and hamster origins (Table 1).

Transcription of iNOS Gene in Infected CEA-expressing Cells. To demonstrate the expression of the iNOS gene in infected CEA-expressing cells, the total RNA was extracted from CEA-expressing and nonexpressing cells infected with the GPEscFv-env/iNOS or GPE/iNOS retrovirus, which displayed the anti-CEA scFv chimeric envelope protein, infected CEA-expressing cells of human and hamster origins (Table 1).

Targeted Killing of CEA-expressing Cells by Infection with Recombinant Retrovirus. To assess the cytotoxic activity of the recombinant retrovirus, we infected CEA-expressing and nonexpressing cells for 2 h with the GPEscFv-env/iNOS or GPE/iNOS retrovirus, and incubated them without the iNOS inhibitor NMA. All of the cells tested did not produce endogenous iNOS (data not shown). When the target cells were infected with the GPEscFv-env/iNOS retrovirus, only 18–25% of CEA-expressing carcinoma cells survived after 7 days, whereas CEA-nonexpressing cells grew normally (96% of the control; Fig. 7) as did all of the target cells infected with the GPE/iNOS retrovirus (data not shown). These findings indicated the specific killing of the target cells by the GPEscFv-env/iNOS retrovirus carrying the iNOS gene through the recognition of the cell surface antigen with the chimeric envelope protein.

Apoptosis of CEA-expressing Cells Infected with Recombinant Retrovirus. We next examined possible induction of apoptosis in CEA-expressing cells after infection with the recombinant retrovirus. The data shown in Fig. 8 reveal that significant DNA fragmentation...
particles specifically transfected the gene to tumor cells. Our recombinant GPEscFv-env/iNOS retroviral iNOS to be no publications describing the specific targeting of the expression of endogenous iNOS in the human carcinoma and hamster a human colon cancer cell line WiDr does not express iNOS (27). The primers of iNOS in the presence of NMA. RT-PCR analysis was performed with specific primers of iNOS and β-actin as an internal control.

**DISCUSSION**

For specific targeting to tumor cells, we have altered the tropism of retroviral vector to infect through the recognition of the CEA molecule on the tumor cell surface. Previous reports have stated that the generation of retroviral vectors incorporating a chimeric envelope protein with a scFv antibody should be fused close to the NH₂ terminus of ecotropic Moloney envelope protein and that the ratio of normal envelope:chimeric envelope protein in the viral membrane appeared to determine the efficiency of infection (1, 23, 24). Our anti-CEA scFv antibody was inserted between the sixth and seventh amino acid residues from the NH₂ terminus of the ecotropic envelope protein. The resultant GPEscFv-env/iNOS retroviral particles codisplayed the chimeric envelope and normal envelope proteins, and their ratio was almost the same (Fig. 4B). Furthermore, when tested on murine NIH3T3 cells, the infectivity of the GPEscFv-env/iNOS retroviruses did not decrease as compared with that of the wild-type GPE/iNOS (Table 1). Consequently, the infection titer of the GPEscFv-env/iNOS retroviruses on CEA-expressing cells was about 10 times higher than that reported by using another anti-CEA scFv gene (5). The main reasons for the high virus titer might be that our anti-CEA scFv gene was cloned from the Ch F11–39 transfectoma, which produces a chimeric antibody with a very high affinity to CEA (9, 10), and that the virus was generated from a helper virus-free ecotropic retroviral packaging cell line (GP+E-86; 25), although the titer obtained seems to be still insufficient for future human gene therapy applications.

Although several gene therapy strategies using the iNOS gene have been reported for various diseases, only a few reports described the use of the iNOS gene in cancer gene therapy (15, 26), and there seem to be no publications describing the specific targeting of the iNOS gene to tumor cells. Our recombinant GPEscFv-env/iNOS retroviral particles specifically transfected the iNOS gene into CEA-expressing tumor cells (Fig. 6). However, endogenous iNOS expression in human carcinoma cells is very diverse (27). Some human colon cancer cell lines (SW480, SW620, and DLD-1) express mRNA for Ca²⁺-dependent endothelial iNOS as well as for Ca²⁺-independent iNOS, whereas a human colon cancer cell line WiDr does not express iNOS (27). The expression of endogenous iNOS in the human carcinoma and hamster cell lines used in the present study was undetectable even by RT-PCR (data not shown), which indicated that the iNOS gene transferred to these cells could induce and maintain the high production of NO.

Our recombinant GPEscFv-env/iNOS retrovirus also revealed significant cytotoxicity against CEA-expressing cells. To our knowledge, this is the first report of successful killing of CEA-expressing tumor cells by specific targeting of the iNOS gene. The cell death was directly correlated with the amount of NO produced. We have also found that the NO production was augmented with the extension of culture time (data not shown). These findings suggest that NO-mediated cytotoxicity is directly related to the production of NO in culture supernatants. However, the cell survival was not completely inhibited in the present study. The exact reasons for this are unknown, but it may reflect the relatively low efficiency of infection. Higher multiplicities of infection of recombinant retrovirus may lead to a higher number of cells infected and killed. Another possibility is that expression of CEA on the tumor cells is very heterogeneous. It is conceivable that a small number of tumor cells, poorly expressing CEA, could escape from the infection and cytotoxic effect.

It has been reported that the NO released by activated cells can induce apoptosis through several mechanisms, including break of

![Fig. 6. Detection of iNOS transcripts in CEA-expressing cells infected with the GPEscFv-env/iNOS retrovirus. Total RNA was isolated from MKN-74 (Lanes 1 and 3), MKN-45 (Lanes 2 and 6), CH-CEA (Lanes 3 and 7), and KATO-III (Lanes 4 and 8) cells infected with the GPEscFv-env/iNOS retrovirus (Lanes 5–8) and the GPE/iNOS retrovirus (Lanes 1–4) in the presence of NMA. RT-PCR analysis was performed with specific primers of iNOS and β-actin as an internal control.

![Fig. 7. In vitro cytotoxicity assay of the GPEscFv-env/iNOS retrovirus. Cells were infected with the GPEscFv-env/iNOS retrovirus for 2 h and cultured for 7 days without NMA. The viability of cells was calculated as described in “Materials and Methods.” This is one representative experiment of three. Bars, SD.

![Fig. 8. Induction of DNA fragmentation to CEA-expressing by infection with the GPEscFv-env/iNOS retrovirus. The cells infected with the GPEscFv-env/iNOS retrovirus were the CEA-nonexpressing MKN-74 cells (Lane 1) and three CEA-expressing cells: MKN-45 (Lane 2), KATO-III (Lane 3), and CHO-CEA cells (Lane 4). MKN-45 was also infected with the GPE/iNOS retrovirus (Lane 5). Lane 6, molecular weight markers. This is one representative experiment of three.

374
DNA strands by NO$_2$ (28) or by formation of peroxynitrite (29). Induction of DNA fragmentation to CEA-expressing cells by high levels of NO productions was also confirmed in the present study (Fig. 8).

Our specifically targeted killing approach has several features that make it attractive for clinical gene delivery. First, the recombinant retrovirus obtained specifically bound to CEA-expressing cells. This prevents the uptake of virus by nontarget cells or CEA-nonexpressing cells, which would result in lesser side effects. Second, the targeted cells were directly killed by the biological product of the therapeutic gene, which is very convenient for practical retroviral targeted delivery, because the established suicidal gene therapy systems using, e.g., herpes simplex virus-thymidine kinase (HSV-TK) and cytosine deaminase (CD) need the additional drug treatments (30, 31). Furthermore, the “bystander effect” of NO may overcome the limitation of retroviral vector gene transfer efficiency (16).

Although serum CEA levels vary among the cancer stages, immunohistochemical studies have shown high positivity of CEA in almost all gastrointestinal cancers (32). Therefore, our strategy may be more useful and effective than other conventional suicidal gene therapy approaches in clinical application. We are in the process of performing additional studies for an in vivo approach of targeted cell killing using a relevant animal model system.

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