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The dominant negative H-ras mutant, N116Y, suppresses growth of metastatic human pancreatic cancer cells in the liver of nude mice

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(Summary)
In pancreatic cancer, the mutation of c-K-ras is a critical event of tumor growth and metastasis.1 We have previously demonstrated a dominant negative effect of N116Y on the growth of pancreatic cancer cells.2 To evaluate the potential of N116Y for suppressing the metastatic growth of pancreatic tumor cells, we made a replication-deficient recombinant N116Y adenovirus driven by the carcinoembryonic antigen (CEA) promoter (Ad CEA-N116Y). We demonstrated that the expression of N116Y, growth inhibition, and apoptotic death induction were all specific to pancreatic cancer cell lines (PCI-35 and PCI-43) that were promoter positive, whereas no growth retardation was observed in human embryonic pancreas-derived cell line 1C3D3 after Ad CEA-N116Y infection. We examined the effect of Ad CEA-N116Y on the metastatic growth of PCI-43 colonies in liver, which were generated by tumor injection into the spleen of nude mice. The results showed that Ad CEA-N116Y effectively reduced the number of metastatic colonies without any complication by injecting intrasplenically five days after tumor cell inoculation. Thus N116Y can selectively suppress the metastatic growth of pancreatic tumor cell by using the CEA promoter driven adenovirus vector indicating that N116Y gene therapy may be potentially useful for the treatment of pancreatic cancer patients with liver micrometastasis.

Short title: Suppression of metastatic cancer by N116Y
Key words: ras suppressor, pancreatic cancer, liver metastasis

Introduction

The poor prognosis of pancreatic cancer is due to several reasons such as difficulties in establishing diagnosis at an early stage, resistance to various therapies, and frequent metastasis to liver or other organs.1, 3-6 In particular, liver metastasis occurs frequently (about 60 %) after a surgical resection for pancreatic adenocarcinoma even if it is performed with a curative intent.5 No effective therapeutic modality has been established to prevent liver metastasis or to treat them successfully. In human

pancreatic cancer, point mutation of the c-K-ras protooncogene at codon 12/13 is frequently detected.7, 8 The somatic mutational activation of c-K-ras is considered a critical event in the emergence and the cell growth of most human pancreatic cancers.9-12 Although the molecular mechanisms of metastasis have not been well defined, activated ras promotes cell survival upon detachment from the extracellular matrix and may contribute to hematogenous metastasis.13 Indeed, K-ras detection in liver specimens is associated with future liver metastasis and poor prognosis in pancreatic cancer.1, 14 The therapeutic potential of suppressing endogenous oncogenic ras function therefore provides an attractive possibility. For example, farnesyltransferase inhibitors (FTI) prevent specific posttranslational modification of Ras oncoprotein resulting in dissociation of Ras from the plasma membrane and functional inactivation,15 and antisense K-ras RNA inhibits production of the K-ras (p21) protein.16 Although selective Ras inhibition is possible using these compounds, there is no way to specifically target cancer cells. The in vivo efficacy of FTI is also unclear since at a concentration where it is effective for tumor growth, it also appears toxic.17

We have pursued another strategy of suppressing intracellular Ras function using a dominant negative H-ras mutant, N116Y.2, 18-22 It was derived from the v-H-ras oncogene by substituting asparagine with tyrosine at codon 116, which is in the GTP-binding domain. N116Y is thought to prevent the activation of oncogenic Ras protein with which it competes for a guanine nucleotide exchange factor.18 N116Y expression significantly inhibited colony formation in selection medium when cotransfected into human pancreatic cancer cell lines with an oncogenic c-K-ras mutated at codon 12 (PCI-10, PCI-19, PCI-24, PCI-35, PCI-43, PCI-55, PCI-64, and PCI-66).2 Moreover, the N116Y transfectants (PCI-35-N116Y) have rendered nontumorigenic when injected subcutaneously into nude mice. Recently, we have reported that a recombinant N116Y expressing adenoviral construct driven by the cytomegalovirus promoter (Ad CMV-N116Y) suppressed the activation of extra-cellular signal-related kinase 2 (Erk2) after EGF stimulation in serum starved esophageal cancer cells (TE8, SGF3, and SGF7).22 In TE8 cells, Ad CMV-N116Y infection also induced cell cycle arrest and G1 phase accumulation.

In this study, we attempted to examine the suppressive effect of N116Y on progression of liver metastasis by the human pancreatic cancer cell line, PCI-43, in nude mice. We based on experimental model for liver metastasis that has been established by intrasplenic injection of tumor cells.23, 24 To accomplish selective expression of N116Y in tumor cells, we made an E1-deleted replication-deficient recombinant adenovirus, Ad CEA-N116Y, in which N116Y cDNA was driven by a human carcinoembryonic antigen (CEA) promoter.25,26 This construct is designed to express N116Y only in cells where the CEA promoter is active.

Results

In vitro analysis

Expression of carcinoembryonic antigen (CEA) was measured by enzyme immunoassay (EIA) in a normal human embryonic pancreas-derived cell line, 1C3D3, and in human pancreatic cancer cell lines, PCI-19, PCI-24, PCI-35, and PCI-43. The CEA secretion was not detected in 1C3D3, PCI-19, and PCI-24, while it was confirmed in PCI-35 and PCI-43 (Table 1). To analyze the CEA promoter activities in the normal human embryonic pancreas-derived cell line, 1C3D3, and in human pancreatic cancer cell lines, PCI-19, PCI-24, PCI-35, and PCI-43, quantitation by

luciferase assay was done as described in materials and methods (Fig. 1). Faint, control levels of CEA promoter activity were seen in 1C3D3, PCI-19, and PCI-24. The expression of luciferase expressed from the CEA promoter and luciferase cDNA constructs ranged from 1 to 3 % of that seen in cells transfected with positive control constructs driven by the SV 40 enhancer and promoter (pGL3-control). In PCI-35 and PCI-43, on the other hand, the CEA promoter activities were strong and the expression of luciferase was 40 and 100 % of that from the positive control construct, respectively. Based on this result, we concluded that the activity of the CEA promoter is negative in 1C3D3, PCI-19, and PCI-24 and positive in the PCI-35 and PCI-43 cell lines. To evaluate the transduction efficiency of the recombinant adenovirus into 1C3D3, PCI-19, PCI-24, PCI-35, and PCI-43 cells in vitro, beta-galactosidase activity was evaluated by using a recombinant adenovirus expressing beta-galactosidase under the control of CMV promoter (Ad CMV-Lac Z).22 In 1C3D3, 80 % of the infected cells exhibited positive staining at a multiplicity of infection (MOI) of 1000. In contrast, all of the human pancreatic cancer cell lines showed that 65-80 % of the cells were positively stained at a MOI of 200, and about 90 % of the cells were positively stained at a MOI of 400. To confirm that N116Y was expressed specifically in the CEA promoter activity positive cell lines, mRNA expression was evaluated by RT-PCR 48h after Ad CEA-N116Y infection at a MOI that gave 50% transduction efficiency (Fig. 2). Controls included infection with vector control or Ad CMV-N116Y.22 The 254 bp N116Y fragment was confirmed to be present in each cell line after Ad CMV-N116Y infection. In contrast, after Ad CEA-N116Y infection, the band was detected only in the cell lines, PCI-35 and PCI-43, whose activity of the CEA promoter was positive, confirming that expression of N116Y in these cell lines was specific. We next determined whether there was also a direct correlation between expression and suppression of cell growth after infection with Ad CEA-N116Y or Ad CMV-N116Y (Fig. 3). The number of cells was counted by trypan-blue exclusion staining on the indicated day. Cell growth was not affected when cells were exposed to vector control at a MOI of 400 or 600. Ad CMV-N116Y infection induced cell growth retardation in all cell lines by day 5. Growth after Ad CEA-N116Y infection was not affected at the same MOI in 1C3D3, PCI-19, and PCI-24. On the other hand, Ad CEA-N116Y remarkably suppressed the cell growth of PCI-35 and PCI-43 by day 5. In both cell lines, the difference in number of cells at this time was statistically significant as compared to vector infected controls ($p < 0.01$ and $p < 0.05$, respectively). To further compare the dominant negative effect of Ad CEA-N116Y to that of Ad CMV-N116Y and to determine whether Ad CEA-N116Y induces apoptosis selectively in CEA positive-pancreatic cancer cells, chromatin morphology was analyzed by nuclear staining with Hoechst 33342 (Fig. 4). In vector control- or Ad CEA-N116Y-infected 1C3D3 and CEA-negative PCI-24 cells, cell morphology was unchanged after 48h incubation (Fig. 4a, 4b). In CEA-positive PCI-43 cells, vector control-infection showed no change in nuclear shape and homogeneous nuclear staining (Fig. 4c), while Ad CEA-N116Y induced condensed and coalesced nuclei typical of apoptosis (Fig. 4d). Similarly, after incubation with Ad CEA-N116Y, most of CEA negative PCI-19 cells showed no change in nuclear shape and homogeneous nuclear staining. In CEA positive PCI-35 cells, on the other hand, condensed and coalesced nuclei typical of apoptosis were observed after Ad CEA-N116Y treatment. The percentage of apoptotic nuclei after Ad CEA-N116Y infection was unchanged in CEA negative cell lines (3.3% in 1C3D3, 2.8% in PCI-19, and 3.0% in PCI-24) as compared to that seen in uninfected control cells or vector control infected cells (1.3 to 3.0%), while it was

dramatically increased in CEA positive cell lines (12.8% in PCI-35 and 12.3% in PCI-43) as compared to that seen in uninfected control cells or vector control infected cells (0.6 to 1.9%) ($p < 0.05$ and $p < 0.01$) (Fig. 4e). All Ad CMV-N116Y-infected cell lines showed changes in nuclear shape characteristic of apoptosis. The percentage of apoptotic nuclei seen after the recombinant adenovirus treatment showed no significant differences between Ad CEA-N116Y and Ad CMV-N116Y in CEA positive cell lines (12.8% vs 11.8% in PCI-35 and 12.3% vs 12.4% in PCI-43). Moreover, the percentage of apoptotic nuclei induced by Ad CEA-N116Y or Ad CMV-N116Y increased in a time-dependent manner in PCI-35 and PCI-43 and more than 70% of the cells appeared morphologically apoptotic by 72h (Fig. 4f). Therefore, Ad CEA-N116Y induces apoptosis selectively in CEA positive PCI-35 and PCI-43 cells and it has strong growth suppressive effects in these cell lines similar to effects seen with the Ad CMV-N116Y driven by the CMV promoter. Thus, in all cases, effects upon growth and apoptosis in vitro were directly correlated with expression of the Ad-N116Y constructs.

In vivo analysis

As a first step towards establishing a liver metastasis model, 1x106 of PCI-43 tumor cells were intrasplenically (i.s.) inoculated in nude mice.24 Five days later, 4x108 plaque forming units (pfu) of Ad CEA-Lac Z,27-30 a recombinant adenovirus expressing beta-galactosidase under the control of CEA promoter, was injected through the same route to confirm specific transgene expression in PCI-43 cells of the liver. Beta-galactosidase activity was evaluated by X-gal staining as a measure of infectivity and expression. After receiving intrasplenical Ad CMV-Lac Z, controls showed diffuse blue staining of the entire liver following X-gal staining. In contrast, the result of Ad CEA-Lac Z injected mice showed that only PCI-43 cells in liver were positively stained while the liver cells were negative (Fig. 5a). These results demonstrated specific transgene expression in PCI-43 cells of the liver. We then examined the effect of Ad CEA-N116Y expression on the metastatic growth of PCI-43 cells in liver. Six weeks after inoculation with 1 x 106 tumor cells per animal, mice were sacrificed and the number of metastatic colonies was counted as described in materials and methods. When 4 x 108 pfu of Ad CEA-Lac Z was injected one day after tumor cell inoculation, metastatic colonies were identified and the mean number of the colonies in replicate experiments was 28 (Fig. 5b). On the other hand, Ad CEA-N116Y treated mice had no viable PCI-43 colonies. To examine if Ad CEA-N116Y might have a suppressive effect on established liver metastases, 4 x 108 recombinant adenovirus were injected five days after i.s. injection of 1 x 106 PCI-43 cells by which time tumor cells had established metastatic colonies. The mean number of colonies of Ad CEA-Lac Z treated mice was 42, while Ad CEA-N116Y treated mice had only 10 (Fig. 5b). The difference between them was statistically significant at a level of $p< 0.01$. No obvious difference in the morphology of metastatic colonies between the groups was identified. Mice injected with either Ad CEA-N116Y or Ad CEA-Lac Z following injection of PCI-43 cells i.s. showed no notable complications and survived without any body weight loss during experiments. Thus, selective transgene expression in PCI-43 cells in the liver could be accomplished by i.s. injection of recombinant adenovirus under the control of the CEA promoter and Ad CEA-N116Y affected the established metastatic foci of the pancreatic cancer in the liver, effectively reducing the number of colonies.

Discussion

Specificity is a major aim in using gene therapy for cancer treatment. In this study, tumor-specific gene expression has been achieved by using promoter sequences that are only activated in proliferating or in oncogenic cells. The CEA promoter provides this specificity since the protein that induces this promoter is expressed at high levels in gastro-intestinal tumors including pancreatic cancer (nearly 45 % of the case) but not in normal tissues.1, 31 Several studies have demonstrated the usefulness of this promoter.27-30, 32 DiMaio et al. have demonstrated specific gene expression for pancreatic cancer cells (BXPC3) by using a retroviral vector in which the promoter for CEA linked to a herpes simplex virus thymidine kinase (HSV-tk) gene was cloned.32 Tanaka et al. have also shown selective HSV-tk gene expression for gastric cancer cells (MNK45) using an adenovirus vector driven by the CEA promoter.27, 29 In our present study, using the N116Y cDNA as a therapeutic gene, we tried to express N116Y in a tumor-specific fashion for pancreatic cancer cells (PCI-35 and PCI-43) by a CEA promoter driven adenovirus vector. After Ad CEA-N116Y infection, N116Y was expressed in the promoter specific manner in vitro. We asked whether similar selectivity could be obtained in our in vivo liver metastasis model and found that it could be, with little or no adverse effect to normal tissues. For selective adenoviral delivery through the portal vein, we injected recombinant adenovirus into the spleen. A previous study showed positive X-gal staining in CEA-producing gastric cancer cell lines (MKN28 and MKN45) after Ad CEA-Lac Z infection,27 while another study showed that the CEA promoter provides scarcely any transgene expression in CEA-negative hepatic cell lines (Hep3B and HuH7).33 In our current studies, when Ad CEA-Lac Z was injected into the spleen, beta-galactosidase expression was detected only in metastatic human pancreas cancer cells in mouse liver, but not in the normal tissue itself. These results formed a striking contrast to that of the diffuse liver staining seen after infection with a recombinant adenovirus carrying the beta-galactosidase gene under control of the potent CMV promoter.34 In addition, none of the replication-deficient Ad CEA-N116Y treated mice showed body weight loss, diarrhea, or other side effects. These results suggested that selective gene expression could be accomplished through intrasplenic injection and the use of the CEA promoter-driven adenovirus vector, and that the potential adverse effects of N116Y to normal tissues were avoided in vivo. Our results, therefore, confirmed the effectiveness of the CEA promoter in tumor targeting.

Among the characteristics of cancer, metastasis accounts for the majority of cancer deaths.35 It has been suggested that activated K-ras played an important role in tumor invasion and metastasis through inducing expression of matrilysin, a member of the matrix metalloproteinase family, in a colon cancer cell line (SW1417).36 Activation of K-ras may also contribute to liver metastasis and further expansion in pancreatic cancers.17 We, therefore, examined whether or not N116Y could effectively suppress the growth of established micrometastasis in liver through its potent dominant negative effect on Ras activation. The model that we used in this study simulated several steps of liver metastasis. The process included tumor cell transport through the portal vein, adherence to vascular endothelial cells, extravasation, invasion within the liver, and further growth and expansion.35 Consistent with this model, the number of metastatic liver colonies was reduced by Ad CEA-N116Y treatment compared to that of Ad CEA-Lac Z injection into the spleen. The reduced colony numbers of pancreatic cancer cell colonies in the liver after control Ad CEA-Lac Z treatment for one day (28 colonies) compared with those 5

days after tumor cell inoculation (42 colonies) in our present experiments may be related to a possible weak tumor suppressive capacity of Lac Z gene expression30 or to transient growth inhibition by viral infection. When we injected Ad CEA-N116Y one day after tumor cell inoculation, no metastatic colonies were detected in liver sections. This result suggested the possibility that N116Y could prohibit tumor cells from attaching to the endothelial cells or could inhibit their extravasation. As the cells expressed N116Y were forced into apoptosis, they may also be incapable of not only adhering to the endothelial cells but also progressing with further process. When we injected Ad CEA-N116Y five days after tumor cell inoculation, the number of liver metastatic colonies was reduced compared to that of Ad CEA-Lac Z treated mice. The result suggested that N116Y could prohibit tumor cells from invading the liver and induced cell growth arrest or apoptosis in Ad CEA-N116Y treated mice. The same phenomena that were observed in vitro occurred in tumor cells in Ad CEA-N116Y treated mice and the number of colonies was reduced.

Pancreatic cancers often metastasize to the liver following surgery. In pancreatic cancer patients, the preoperative or intraoperative portal injection of recombinant adenovirus carrying the N116Y gene may have an effect on liver metastasis based upon our results in this study. It may also be possible to treat massive metastatic liver foci using a high local concentration of Ad CEA-N116Y by delivery with selective intra-arterial catheter infusion, or direct intra-tumoral injection.37, 38 A phase I study of gene therapy using a recombinant adenovirus showed that the mRNA specific to the therapeutic gene (p53) was detected after direct injection to the head and neck tumor in spite of a high level of anti-adenovirus antibody in serum.39 This result is encouraging because it shows that transgene expression can be accomplished using adenovirus vectors despite the existence of humoral immunity. It also suggested that adenovirus vectors with both E1 and E4 deletions or additional ablations of E2A have advantages in terms of safety and efficacy over first-generation constructs for liver-directed gene therapy.40, 41 As we demonstrated here, N116Y gene is a potent tumor suppressor gene and is a potential candidate for cancer gene therapy.

Material and methods

Cell lines

The human embryonic pancreas-derived cell line, 1C3D3, was established by Dr. Ishikawa (Tokyo Jikeikai Medical University, Tokyo, Japan) and provided from the RIKEN cell bank (Tsukuba, Japan). This cell line was maintained in Ham's F 10-cell culture medium, supplemented with 5 % fetal bovine serum, 10 % newborn bovine serum, and 2.5 % horse serum. Human pancreatic cancer cell lines, PCI-19, PCI-24, PCI-35, and PCI-43, were established from surgically resected, primary carcinoma tissues.2 Among these cell lines, PCI-35 and PCI-43 are CEA-producers.24 These pancreatic cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal calf serum and 2 mM L-glutamine. All cells were maintained in 5 % carbon dioxide at 37 _C. Cell culture reagents were obtained from GIBCO BRL (Life Technologies Inc. (Frederick, MD).

Plasmids and recombinant adenoviruses

Plasmid pSV2 neo-N116Y and Plasmid pCEA CAT were generously provided by Dr. T. Y. Shih (NCI, Frederic, MD) and Dr. Osaki (Osaka University, Osaka, Japan), respectively.18, 25 The 2.2 kb BamHI-BamHI fragment containing the N116Y cDNA was inserted into the Sal I site of pCEA CAT using Xho I linker to construct pCEA-N116Y CAT. The 2.7 kb Bgl II-Xba I fragment containing the CEA promoter and N116Y cDNA was cloned into the Bgl II/Xba I digested pGL3-basic vector (Promega; Madison, WI)) to construct pGL3-CEA-N116Y.26 The Bgl II-Bgl II fragment containing CEA promoter, N116Y cDNA, and SV 40 polyadenylation signal was ligated with the Bgl II-Bgl II fragment containing the shuttle region of plasmid pCA 14 (Microbix Biosystems Inc., Toronto, Canada) to construct pCA 14-CEA-N116Y. The shuttle plasmid, pCA 14-CEA-N116Y, and pJM 17 plasmid were cotransfected into the 293 transformed human embryonic kidney cells by calcium phosphate precipitation to generate replication-deficient adenovirus, Ad CEA-N116Y.42 The CMV promoter driven, replication-deficient recombinant adenovirus Ad CMV-N116Y, Ad CMV-Lac Z containing a beta-galactosidase cDNA and viral vector control were prepared as described previously.22 The replication-deficient adenovirus, Ad CEA-Lac Z, expressing beta-galactosidase under the control of the CEA promoter was provided from the RIKEN gene bank (Tsukuba, Japan).27 Each recombinant adenovirus was expanded in the 293 cells and was purified by banding in cesium chloride and dialysis for 8 h at 4 _C against two changes of phosphate-buffered saline (PBS) containing 10 % glycerol. The viral titers were determined by plaque forming activity in 293 cells.42

Enzyme immunoassay for CEA production

CEA produced by the cells was measured by EIA. 5x105 cells were seeded in six-well tissue culture plates (Corning, New York, NY) and incubated for 48h at 37 _C. The medium was then replaced with new medium. After another 48h incubation, the cells were counted, and the supernatant was collected. The CEA content in the supernatant was measured by Bayer Immuno 1 CEA system (Bayer Medical Co., Ltd., Tokyo, Japan). In this assay, the minimal detectable levels of CEA were 0.2 ng/ml. The results are presented as the mean determined from three separate experiments as described previously.27

CEA promoter activities of the cell lines

The CEA promoter activities were evaluated by luciferase assay. The CEA promoter and luciferase cDNA constructs were made using standard techniques from pGL3-basic vector, which was used as internal control. The Bgl II-Bam HI fragment containing the CEA promoter sequence of pCEA-N116Y CAT was inserted into the Bgl II site of pGL3-basic vector to construct pGL3-CEA. The luciferase cDNA constructs driven by the SV 40 enhancer and promoter (pGL3-control vector)) were purchased from Promega. For transient luciferase assays, cells were transfected using a lipid-DNA complex containing 12 _l of LIPOFECTAMINE Reagent (GIBCO) and 3 _g of pGL3-basic, pGL3-CEA, or pGL3-control. Eighteen hours after the addition of the DNA/LIPOFECTAMINE Reagent complexes, the media was changed. The cells were incubated for 48 h, and then cell extracts were prepared using Reporter Lysis Buffer (Promega). Luciferase assays were performed using the Luciferase Assay System (Promega) and a luminometer (EG & G Berthold, Bad Wiledbad, Germany). All values were standardized to the expression of luciferase expressed from the pGL3-control as described previously.31

Adenoviral infection in vitro

The transduction efficiency of the recombinant adenovirus in 1C3D3, PCI-19, PCI-24, PCI-35, and PCI-43 cells in vitro was evaluated by using Ad CMV-Lac Z. 5x104 cells were seeded in six-well tissue culture plates and incubated for 24h at 37 _C. After washing with PBS, the cells were infected with Ad CMV-Lac Z at different multiplicity of infection (MOI) ranging from 0 to 1,000 for 1 h. The infected cells were incubated in complete medium for 48 h. Then cells were washed twice with ice-cold PBS and fixed with PBS containing 1.2 % glutaraldehyde for 5 min. beta-galactosidase activity was evaluated by incubation in staining solution containing 5 mM K3Fe(CN)5, 5 mM K4Fe(CN)5, 2 mM MgCl2, and 0.6 mg/ml of 5-bromo-4-chloro-3-beta-D-galactopyranoside (X-gal) at 37 _C overnight. Transduction efficiency was indicated by counting the percentage of positive cells identified by X-gal staining.22

Detection of N116Y mRNA expression by RT-PCR

To examine N116Y mRNA expression in the infected cells, RT-PCR was performed. Total RNA was isolated with TRIZOL Reagent (GIBCO) from adenoviral infected cells. The RNA was treated by Deoxyribonuclease I (GIBCO) for 15 min at room temperature before hand. Each 20 _l cDNA synthesis reaction contained 1 _g of purified RNA prepared as described above, 1x First Strand Buffer (Boehringer Mannheim, Germany), 0.5 _g of Oligo(dT)12-18 (Boehringer), 10 mM DTT (Boehringer), 0.5 mM dNTP Mix (0.5 mM each dATP, dGTP, dCTP, and dTTP), and 200 units of SUPERSCRIPT(tm) II (GIBCO). The reaction mixture was incubated for 50 min at 42 _C and then heated for 15 min at 70 _C to inactivate the reaction. Each PCR reaction contained 2 _l of RT reaction product as template DNA, 1x PCR buffer (Boehringer), 160 _M of each deoxynucleotide, 1 unit of Taq DNA polymerase (Boehringer), and 10 pmol of each 5' and 3' primer pair specific to N116Y cDNA.2 Amplification conditions were 94 _C for 1 min, 55 _C for 30 sec and 72 _C for 1 min for 35 cycles. Equal aliquots (10 pmol) of secondary glyceraldehydephosphate dehydrogenase (GAPDH) primer sets were added at the 6th cycle (30 cycles remaining) by the primer-dropping method.43 PCR were performed in 25 _l reaction volumes using the GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems,

Norwalk, CT). Aliquots of PCR reaction products were electrophoresed through 2 % agarose gels containing 0.2 _g/ml of ethidium bromide.

Cell growth assay and detection of apoptotic cells in vitro

5x104 cells (1C3D3, PCI-19, PCI-35, and PCI-43) or 2x104 cells (PCI-24) were plated in 6-well plates in triplicate. The cells were infected with vector control or either of the Ad-N116Y constructs (Ad CEA-N116Y or Ad CMV-N116Y) at a MOI of 400 (PCI-19, PCI-24, PCI-35, and PCI-43) or 600 (1C3D3) on day 0. The infected cells were harvested and counted after trypan blue exclusion staining at day 1, 3, and 5. Microscale analysis of cell death by apoptosis was performed by using fluorescent DNA-binding dye, Hoechst 33342. 1x104 cells per well were seeded in 24-well tissue culture plates and incubated for 24 h at 37 _C. Then the cells were incubated with Ad CEA-N116Y or vector control MOIs of 400 or 600. After 1 h incubation with recombinant adenovirus, 1 ml of complete media was added into each well and cells were incubated for 24-72 h at 37 _C. The cells were stained with 1 _g /ml of Hoechst 33342 and analyzed for apoptosis under fluorescence microscopy (Nikon, Tokyo, Japan). The number of apoptotic cells in each microscopic field was summed and expressed as a percentage of the total number of cells as described.44, 45 Ad CMV-N116Y was used as positive control.

Liver metastasis model

Male athymic BALB/c nude mice of six weeks old were purchased from Japan SLC Co., Shizuoka, Japan. All the mice were maintained under specific pathogen-free conditions according to the institute's guidelines for care and use of experimental animals. PCI-43 cells were used to establish liver metastasis model. The metastatic potential of the cell line to nude mouse liver was demonstrated previously.24 Mice were anesthetized with diethylether and the abdomen was incised to expose the spleen. 1x106 cells in 100 _l of PBS were injected into the spleen through a 30-gauge needle. The wound was closed with 3-0 nylon. Five days after tumor cell inoculation, 4x108 plaque forming units (pfu) of Ad CEA-Lac Z was injected into the spleen following anesthesia and a second laparotomy. After three days, animals were sacrificed and the livers were fixed with 2 % formaldehyde in PBS (4_C). Tissues were cut evenly into three slices and incubated in a PBS solution containing 5 mM K3Fe(CN)5, 5 mM K4Fe(CN)5, 2mM MgCl2, and 1 mg/ml of X-gal for 18 h at 37 _C.29 After staining, tissues were embedded in paraffin, sectioned, adhered to glass slides, and counter stained with hematoxylin and eosin.46 Ad CMV-Lac Z was used as control for adenoviral transduction of murine cells. One day or five days after tumor cell inoculation, 4x108 pfu of Ad CEA-Lac Z or Ad CEA-N116Y was injected into the spleen. Mice were killed six weeks later and livers were fixed in PBS containing 10 % formaldehyde. Each tissue was cut evenly into three slices and processed for hematoxylin and eosin staining. Total number of metastatic colonies from three slides was counted microscopically.24 Statistical analysis of the differences between the mean was calculated using Student's t test.

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Titles and legends to figures

Figure 1: CEA promoter activities evaluated by luciferase assay. All values of the CEA promoter and luciferase cDNA constructs (pGL3-CEA) were standardized to the expression of luciferase expressed from pGL3-control. pGL3-basic was used as an internal control. Data are presented as the mean determined from three separate experiments. Bars show SD.

Figure 2: Detection of N116Y mRNA expression by RT-PCR in human embryonic pancreas-derived cells (1C3D3) and pancreatic cancer cell lines (PCI-19, PCI-24, PCI-35, PCI-43). For each cell line, Lane C; vector control infected cells, Lane E; Ad CEA-N116Y infected cells, Lane M; Ad CMV-N116Y infected cells. GAPDH, glyceraldehydephosphate dehydrogenase as an internal control.

Figure 3: Effect of Ad CEA-N116Y or Ad CMV-N116Y on cell growth in vitro. In PCI-35 and PCI-43, the difference in number of cells after Ad CEA-N116Y infection and vector control infection was statistically significant at day 5 ($p < 0.01$ and $p < 0.05$, respectively). Data are expressed as the mean determined from three separate experiments. Bars show SD.

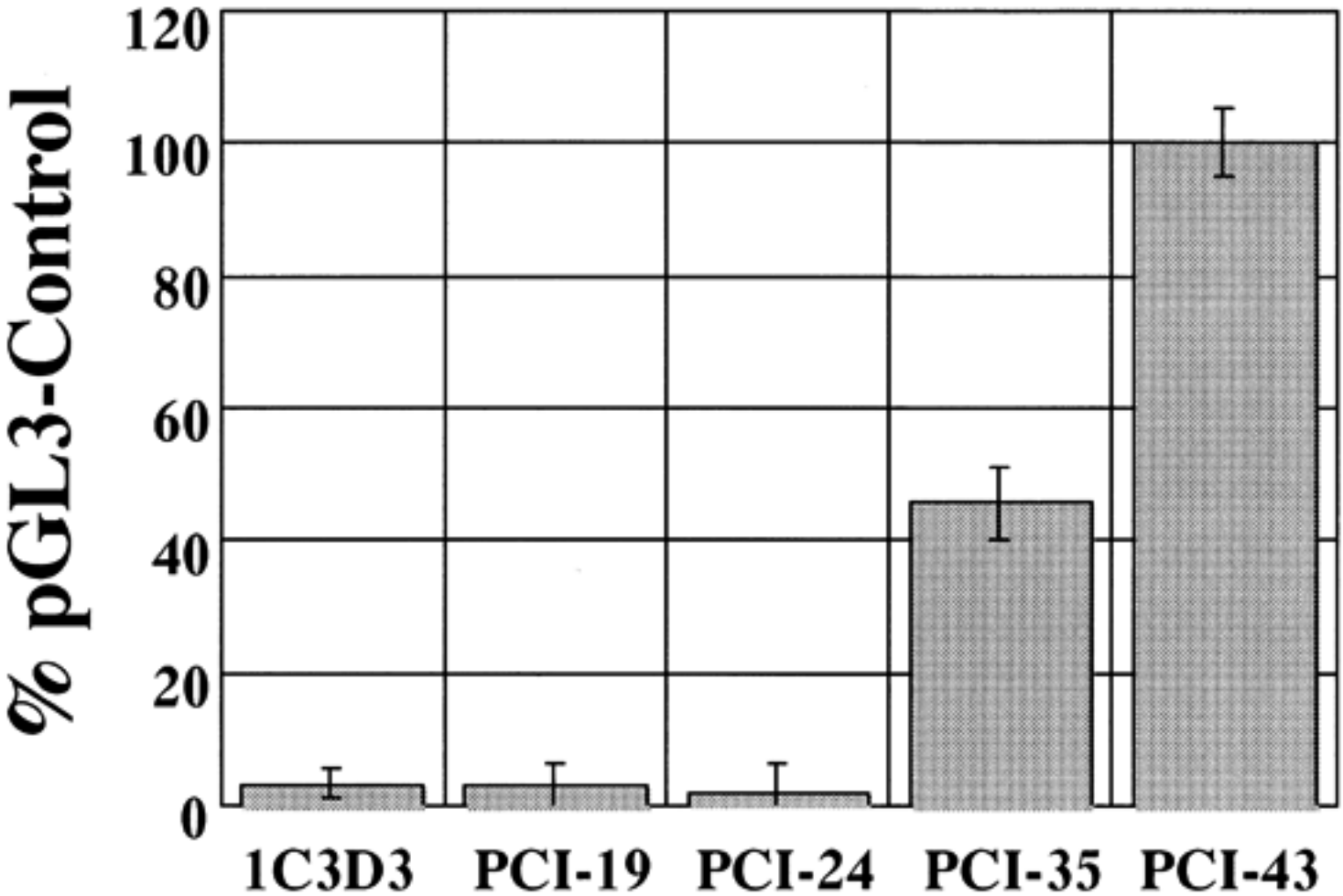
Figure 4: Detection of apoptotic cell death in vitro by fluorescence microscopy. (a) vector control-infected PCI-24 cells, (b) Ad CEA-N116Y-infected PCI-24 cells, (c) vector control-infected PCI-43 cells, (d) Ad CEA-N116Y-infected PCI-43 cells. (e) Quantification of apoptotic nuclei as measured by chromatin morphology after 48h incubation with vector control, Ad CEA-N116Y or Ad CMV-N116Y. (f) A time course for induction of apoptosis by Ad CEA-N116Y or Ad CMV-N116Y in PCI-35 and PCI-43 cells. Data are presented as the mean determined from two separate experiments. Bars show SD.

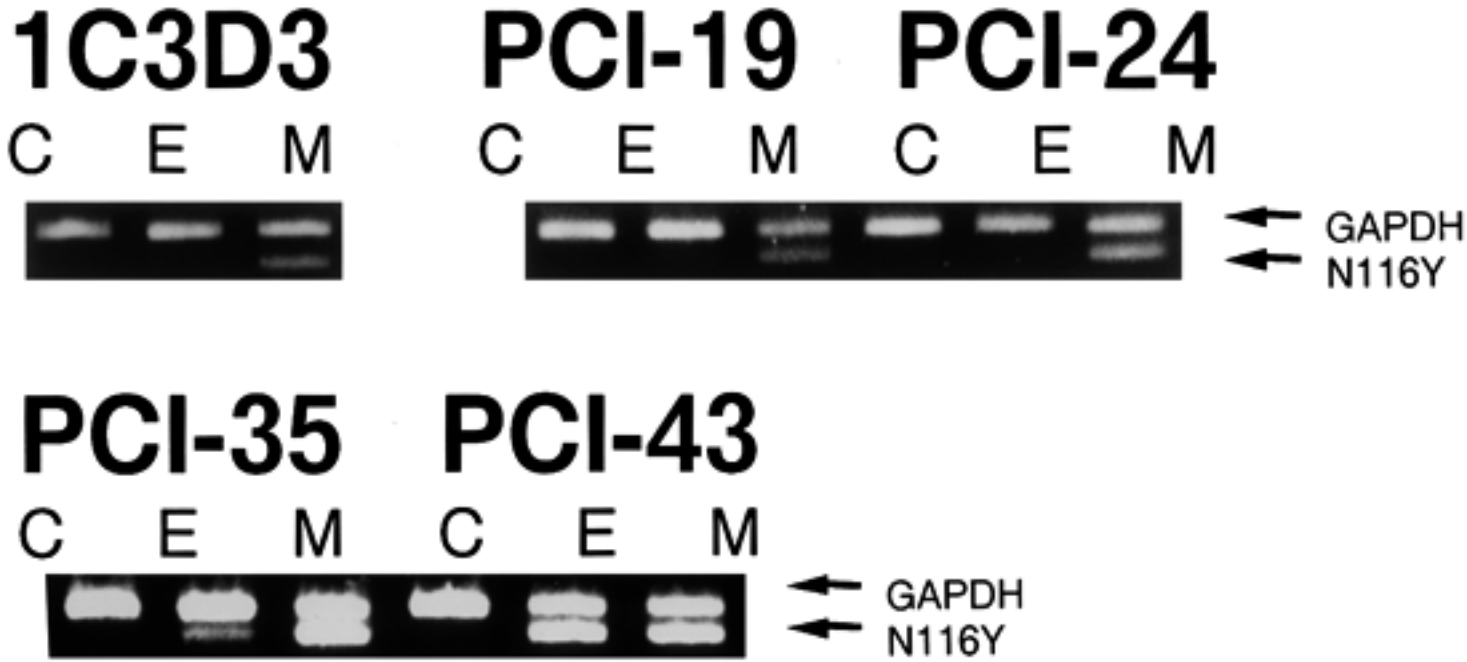
Figure 5: (a) Selective transgene expression in vivo. 1x106 of PCI-43 tumor cells were injected into the spleen of a nude mouse and five days later, 4x108 pfu of Ad CEA-Lac Z was injected through the same route. Three days after the inoculation, the liver was evaluated for the presence of the Lac Z product, beta-Galactosidase, using X-gal staining. Original magnifications, x400 (hematoxylin and eosin counterstain). (b) Suppressive effect of Ad CEA-N116Y on liver metastases in nude mice. 1x106 PCI-43 tumor cells were injected into the spleen and one day or five days later, 4x108 pfu of Ad CEA-N116Y was injected through the same route. Six weeks after the inoculation, the mean number of liver metastatic colonies from three liver slices was significantly reduced in Ad CEA-N116Y treated mice as compared with that of Ad CEA-Lac Z treated mice ($p < 0.01$). Data are presented as the mean determined from the result of six mice (one day) or four mice (five days) in each group. Black columns: Ad CEA-Lac Z, White column: Ad CEA-N116Y. Bars show SD.

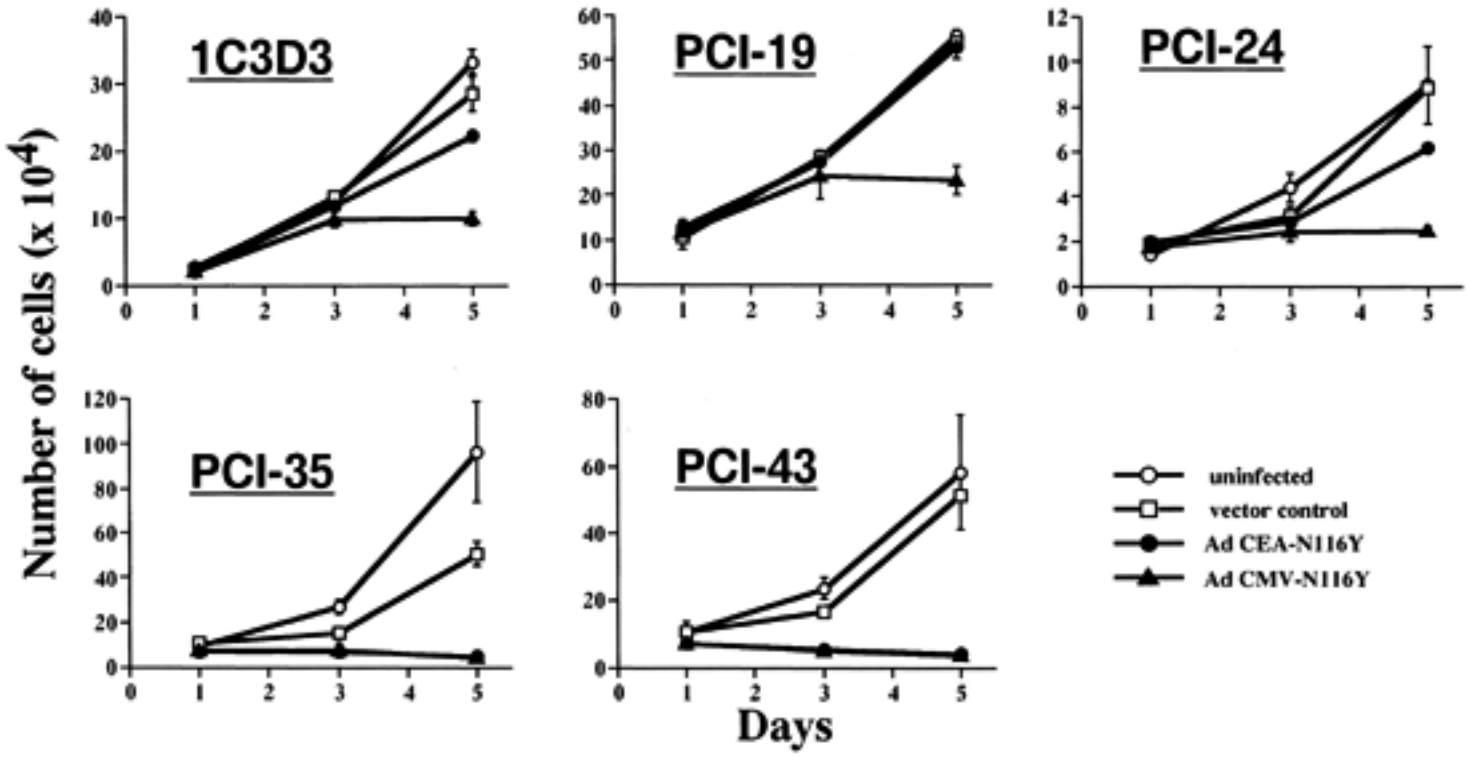
Table 1. CEA secretion in a panel of cell lines

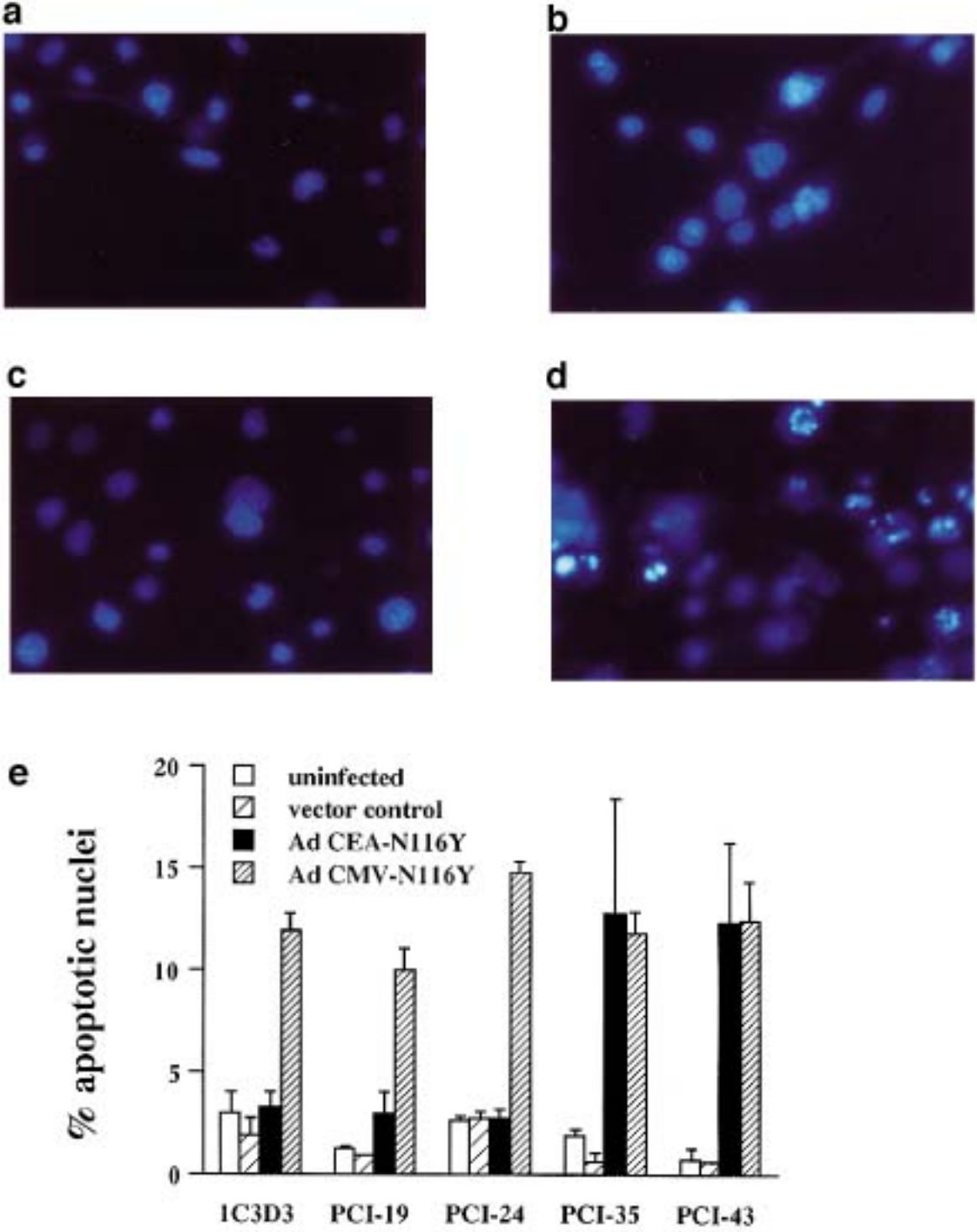
Cell lines	Origin	CEA secretiona
1C3D3	human embryonic pancreas	NDb
PCI-19	human pancreatic cancer	ND
PCI-24	human pancreatic cancer	ND
PCI-35	human pancreatic cancer	10.5 ± 2.3
PCI-43	human pancreatic cancer	34.0 ± 3.4

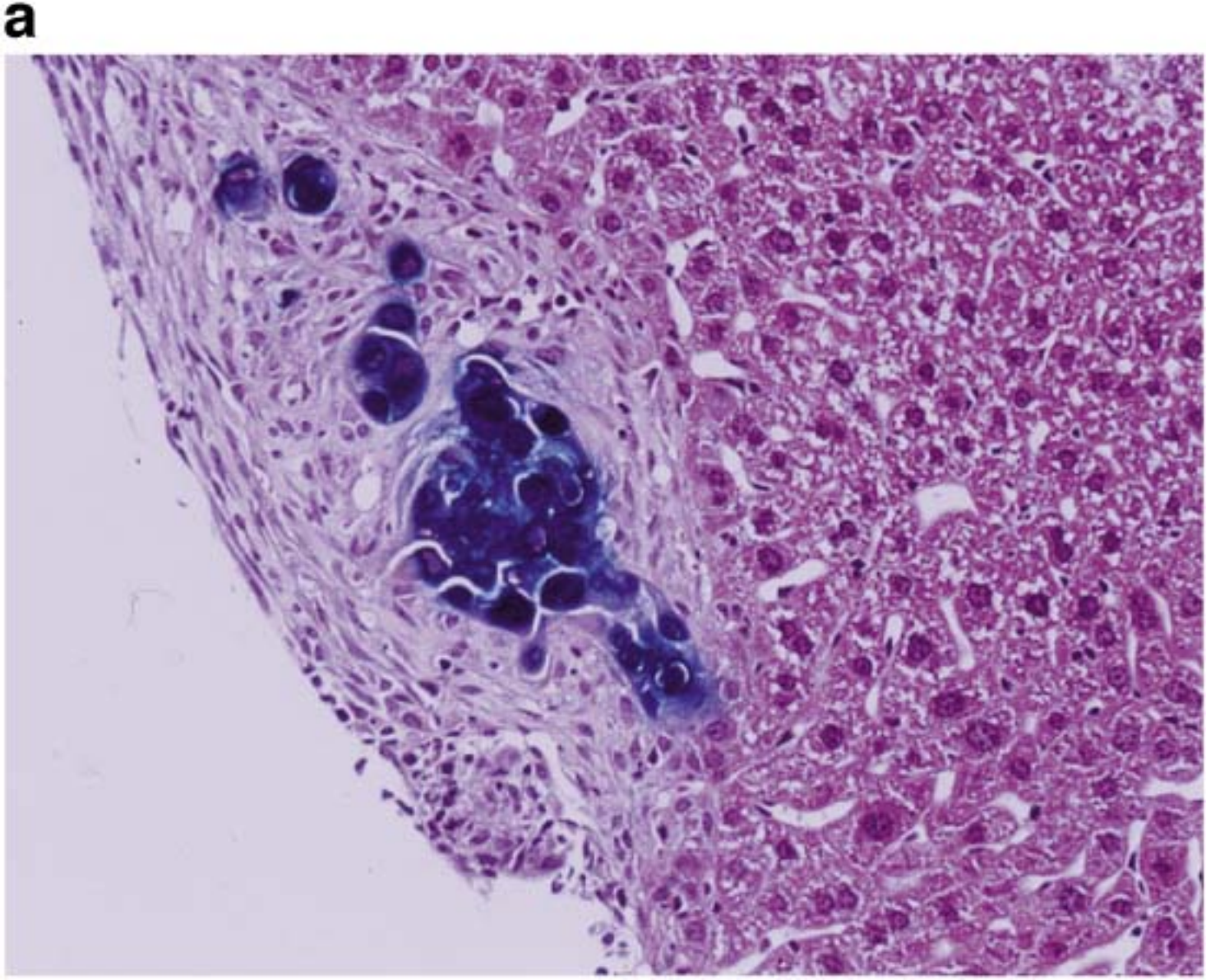
a CEA produced by the cell lines was measured by EIA (ng/lO6cells/48h). The results are presented as the mean ± SD determined from three separate experiments.
b Not detected

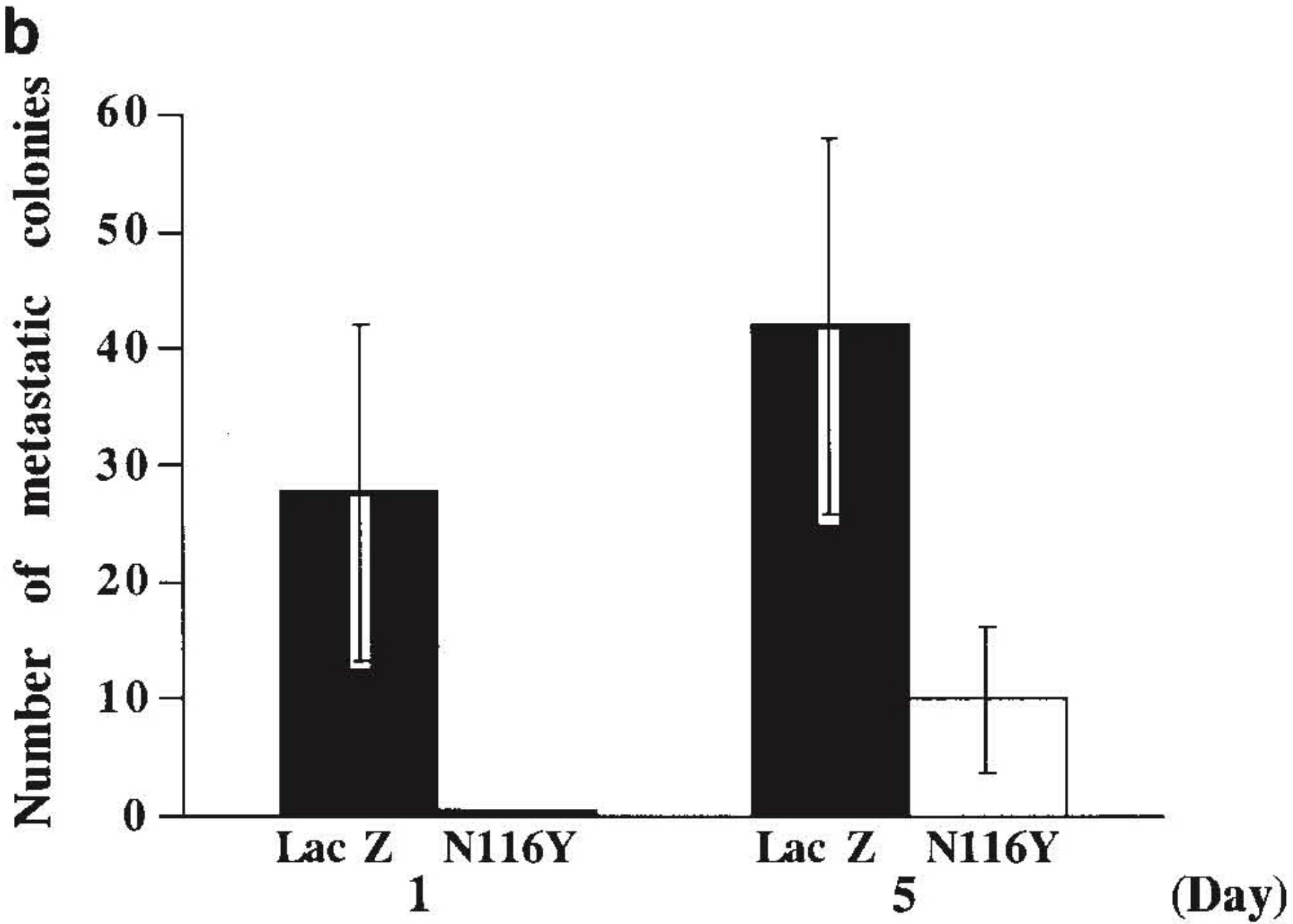

% pGL3-Control
120
100
80
60
40
20
0
1C3D3
PCI-19
PCI-24
PCI-35
PCI-43


1C3D3
PCI-19
PCI-24
C E M
C E M C E M
GAPDH
N116Y
PCI-35
PCI-43
C E M C E M
GAPDH
N116Y


1C3D3
PCI-19
PCI-24
PCI-35
PCI-43
Number of cells (x 10^4)
Days
uninfected
vector control
Ad CEA-N116Y
Ad CMV-N116Y


a
b
c
d
e
% apoptotic nuclei
20
15
10
5
0
uninfected
vector control
Ad CEA-N116Y
Ad CMV-N116Y
1C3D3
PCI-19
PCI-24
PCI-35
PCI-43


a


b
Number of metastatic colonies
60
50
40
30
20
10
0
Lac Z
N116Y
Lac Z
N116Y
1
5
(Day)