Application of the Cre Recombinase/loxP System Further Enhances Antitumor Effects in Cell Type-specific Gene Therapy against Carcinoembryonic Antigen-producing Cancer

Takashi Kijima, Tadashi Osaki, Kazumi Nishino, Toru Kumagai, Toshiki Funakoshi, Hiroyuki Goto, Isao Tachibana, Yoshiro Tanio, and Tadamitsu Kishimoto

Department of Molecular Medicine, Osaka University Medical School, Osaka 565-0871 [T. Kij., T. O., K. N., T. K., T. F., H. G., I. T.]; Department of Internal Medicine, Osaka Prefectural General Hospital, Osaka 558-0056 [Y. T.]; and Osaka University, Osaka 565-0871 [T. Kii.], Japan

ABSTRACT

A considerable number of studies of cancer have shown that the cell type-specific promoter is an effective tool for selective expression of foreign genes in tumor cells. However, few reports have demonstrated significant in vivo antitumor effects using this strategy thus far, possibly because the low activity of such a promoter results in insufficient expression of genes in cancer cells as well as in insignificant antitumor effects, even when the cells are infected by highly efficient gene transfer methods. To overcome this problem, we used the Cre/loxP system for the cell type-specific gene therapy against carcinoembryonic antigen (CEA)-producing cancer. We constructed a pair of recombinant Ads. One expresses the Cre recombinase (Cre) gene under the control of the CEA promoter (Ad.CEA-Cre). The other contains the herpes simplex virus thymidine kinase (HSV-TK) gene separated from the strong CAG promoter by insertion of the neomycin resistance (neo) gene (Ad.Lox-TK). The HSV-TK gene of the latter Ad is designed to be activated through excisional deletion of the neo gene by Cre enzyme released from the former one only when CEA-producing cells are infected simultaneously with these Ads. Coinfection by these Ads rendered a human CEA-producing cancer cell line 8.4-fold more sensitive to ganciclovir (GCV) compared with infection by Ad.CEA-TK alone, the HSV-TK gene of which is directly regulated by the CEA promoter. On the other hand, coinfection with these Ads did not significantly change the GCV sensitivity of non-CEA-producing cells. Intratumoral injection of Ad.CEA-Cre combined with Ad.Lox-TK followed by GCV treatment almost completely eradicated CEA-producing tumors established in the subcutis of athymic mice, whereas intratumoral injection of Ad.CEA-TK with GCV administration at most retarded the growth of inoculated tumors. These results suggest distinct advantages of the Cre/loxP system applied in the conventional cell type-specific gene therapy against cancer.

INTRODUCTION

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is an oncofetal protein that is often detected at high levels in the serum of cancer patients, including those with cancers of the lung, colon, stomach, and pancreas (1–4). Many cases of these cancers are found to be already advanced at initial diagnosis, are refractory to conventional chemoradiotherapy, and show poor prognosis (3, 5, 6). Many cases of these cancers are found to be already advanced at initial diagnosis, are refractory to conventional chemoradiotherapy, and show poor prognosis (3, 5, 6). Some researchers have introduced foreign genes into tumor cells. However, few reports have demonstrated significant in vivo antitumor effects using this strategy thus far, possibly because the low activity of such a promoter results in insufficient expression of genes in cancer cells as well as in insignificant antitumor effects, even when the cells are infected by highly efficient gene transfer methods.

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2 To whom requests for reprints should be addressed, at Department of Molecular Medicine, Osaka University Medical School, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan.

3 The abbreviations used are: CEA, carcinoembryonic antigen; CD, cytotoxic deaminase; HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; Ad, adenoviral vector; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; NLS, nuclear localization signal; moi, multiplicity of infection; pfu, plaque-forming unit(s); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; i.t., intratumoral(ly).

4 T. Kijima, unpublished observations.
MATERIALS AND METHODS

Cell Lines. Two human adenocarcinoma cell lines of the lung, VMRC-LCD and A549, and a human colon cancer cell line, LoVo, were all obtained from Japan Cancer Research Resources (Tokyo, Japan). A human embryonal kidney cell line, 293, was purchased from American Type Culture Collection (Manassas, VA). Three cancer cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. 293 cells were grown in DMEM containing 10% FBS and penicillin/streptomycin. CEA Production by Tumor Cells. Cells grown in culture medium were washed, suspended in PBS, and homogenized with a sonicator at 50 W for 2 min. The CEA content in the cell lysate was measured by solid-phase RIA. The lower limit of detection of CEA was 0.5 ng/ml. The total protein concentration was assayed by Lowry’s method (Special Reference Laboratory, Tokyo, Japan). Each value represents the former concentration divided by the latter one (ng/mg protein).

CAT Assay. The CAT assay was carried out as described previously (11). In brief, 5 μg of a plasmid with the SV40 promoter-CAT gene (pSV2CAT) or a plasmid with the CEA promoter-CAT gene (pCEACAT) was cotransfected with 10 μg of PSV-β-galactosidase, a plasmid with the LacZ gene driven by the SV40 promoter as an internal control for transfection, into each cell line. 14C-labeled chloramphenicol and its acetyl form were quantitated in duplicate by liquid scintillation counting in Aquasol (DuPont-NEN, Boston, MA). The ratio of the count for the acetyl form to total count was calculated as the CAT activity. The CAT activity of pCEACAT was expressed as a percentage of that of pSV2CAT, a positive control plasmid.

Construction of Ads. The recombinant replication-defective Ads were constructed by the cosmid-adenoviral DNA terminal protein complex method (21). The CATK fragment prepared as the BglII-HindIII fragment from pCEACAT (11) was ligated into the SmaI site of pAxCw cassette cosmid and designated pAxosCEATK. pAxosCASCk and pAxCALNLsTk were constructed by subcloning the TK fragment from the BglII-PvuII site of pTKS (11) into the SmaI site of pAxCw and pAxCALNLw cassettes (20), respectively. The BglII-HindIII CEA promoter fragment from pCEACAT (11) was also inserted into the SmaI site of pAxAvcNCre cassette to produce pAxCosCAEC (Cre). Cassette cosmid pAxCw, pAxCATw, pAxCALNLw, and pAxCwNCre were all gifts from Dr. I. Saito (Tokyo University, Tokyo, Japan). Each expression cosmid and adenoviral DNA terminal protein complex were cotransfected into 293 cells by the calcium phosphate precipitation method to produce Ad through homologous recombination, and each Ad was isolated from a single plaque. As shown in Fig. 1A, AxCosCAEsTK (designated Ad.CAG-TK here), an Ad that contains the HSV-TK gene directly driven by the CAG promoter (consisting of the cytomegalovirus immediate early enhancer and a modified chicken β-actin promoter; Ref. 22), strongly expresses the HSV-TK gene in all types of infected cells, regardless of whether they produce CEA. The Ads AxCosCEATK (Ad.CEA-TK) and AxCosCEANCre (Ad.CEA-Cre) are designed to express the HSV-TK and the Cre gene, respectively, exclusively in CEA-producing cells under the control of the CEA promoter. Ad.CEA-Cre possesses a SV40 poly(A) signal inserted upstream of the CEA promoter for prevention of nonspecific read-through transcription of the Cre gene and a NLS for keeping Cre recombinase inside the nucleus. AxCALNLsTK (Ad.Iox-LTK) is designed to express the HSV-TK gene under the control of the strong CAG promoter only after Cre excises the neomycin resistance gene in a loxP site-specific manner. AxCALNLcTk (Ad.CAG-LacZ), with NLS-tagged β-galactosidase (Nls-LacZ) gene driven by the CAG promoter, and AxCALNLNZ (Ad.Iox-LacZ), identical to Ad.Iox-TK, except that the HSV-TK gene is replaced by the Nls-LacZ gene, were also supplied by Dr. I. Saito (20, 23). Solutions of these Ads were prepared as described previously (24) and stored at −80°C until use.

β-Galactosidase Assay. One hundred thousand LoVo or A549 cells were coinfected with Ad.CEA-Cre and Ad.Iox-LacZ at various ratios of moi, the number of pfu of viral vectors used for infection per cell. Three days after infection, cell lysates were prepared, and the β-galactosidase activity was measured using o-nitrophenyl-β-D-galactopyranoside as the substrate (25).

In Vitro GCV Sensitivity of Tumor Cells Infected with Recombinant Ads. One thousand A549 or 3 × 104 LoVo or VMRC-LCD cells were seeded in triplicate onto 96-well culture plates. Twenty-four h later, the culture medium was removed, and suspensions of Ad.CEA-TK alone, Ad.CAG-TK alone, or Ad.Iox-TK in combination with Ad.CEA-Cre were distributed onto cell monolayers at a total moi of 30. After another 24 h of incubation, the medium was replaced with that containing various concentrations of GCV (F Hoffmann-La Roche, Basel, Switzerland). After cultivation for an additional 6 days, MTT assay was carried out as described previously (11). The 50% growth inhibitory concentration (IC50) of GCV for each cell line was calculated using a curve-fitting parameter, and the results are represented as the means ± SD from three independent experiments. Student’s t test was used in the statistical analysis of IC50 in individual conditions. The difference was considered statistically significant at P < 0.05.

Animal Experiments. On day 0, CEA-producing LoVo cells were inoculated s.c. into the flank of male BALBi/c nu nu mice at 5–7 weeks of age (Clea Japan, Tokyo, Japan). Mice were divided into eight groups: group I, Ad.CEA-Cre and Ad.Iox-TK injection with GCV treatment; group II, Ad.CEA-Cre and Ad.Iox-TK injection with 0.9% NaCl (saline) treatment; group III, Ad.CEA-TK injection with GCV treatment; group IV, Ad.CEA-TK injection with saline treatment; group V, PBS injection with GCV treatment; group VI, PBS injection with saline treatment; group VII, Ad.CEA-Cre and Ad.Iox-LacZ injection with GCV treatment; and group VIII, Ad.CEA-Cre and Ad.Iox-LacZ injection with saline treatment. Groups VII and VIII were excluded in the experiment shown in Fig. 4B. When the average area of the inoculated tumors reached 20 mm2, Ads were injected i.t. Each recombinant Ad was injected at a ratio of 1:1 in the coinfection groups (groups I, II, VII, and VIII). From the day after the first viral injection, mice received i.p. administration of 50 mg/kg body weight of GCV or saline twice a day for 2 weeks. The tumor size was measured every 3–4 days and expressed as the product of the perpendicular diameters of individual tumors. The treatment schedule including the number of inoculated cells and the dosage of Ads is specified in each experiment. The number of mice in each group is five in Fig. 4A and seven in Fig. 4B. Each animal experiment was repeated twice. Results are presented as the means ± SD. Student’s t test was used in the statistical analysis of the size of tumors in individual groups. The difference was considered statistically significant at P < 0.05.

RESULTS

CEA Production and CEA-specific Promoter Activity in Human Cancer Cell Lines. The amount of CEA protein in cell homogenate was measured (Table 1). LoVo and VMRC-LCD cells produced significant amounts of CEA protein (77.2 ± 12.7 and 204.1 ± 43.5 ng/mg protein, respectively), whereas secretion from A549 cells was below the detectable level. The activity of the CEA promoter was also analyzed using CAT assay (Table 1). The CAT activities of pCEACAT in LoVo and VMRC-LCD were 98.2 ± 0.7 and 61.7 ± 3.4% of that of pSV2CAT, respectively, whereas it was very low in A549 cells (4.2 ± 0.4%). A549 produced a significant amount of CEA in our previous experiments (11), but it became a non-CEA-producing cell line during a long-term cultivation. These results suggest that the CEA promoter shows selective activity in CEA-producing cancer cells, although the activity is not so strong.

Mechanisms Activating the HSV-TK Gene Specifically in CEA-producing Cancer Cells by the Cre/IoxP System. Fig. 1B illustrates the mechanisms activating the HSV-TK gene specifically in CEA-producing cancer cells. When CEA-producing cells are infected simultaneously with Ad.CEA-Cre and Ad.Iox-TK, the former Ad produces Cre recombinase under the control of the CEA promoter. Then

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CEA productiona (ng/mg protein)</th>
<th>CEA promoter activityb (% of SV40 promoter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoVo</td>
<td>77.2 ± 12.7</td>
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</tr>
<tr>
<td>VMRC-LCD</td>
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<td>61.7 ± 3.4</td>
</tr>
<tr>
<td>A549</td>
<td>Not detectable</td>
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</table>

a Level of CEA production was calculated as the concentration of CEA protein divided by that of the total protein in cell lysates. Results represent the means ± SD of three isolated measurements.
b The CAT activity of pCEACAT is expressed as a percentage of that of pSV2CAT. Results represent the means ± SD of three independent experiments. Each experiment was performed in duplicate.
In Vitro Enhanced GCV Sensitivity of CEA-producing Cancer Cells by Coinfection with Ad.CEA-Cre and Ad.lox-TK. To yield maximum expression of the HSV-TK gene in CEA-producing cancer cells using the Cre/loxP system, we coinfected LoVo, VMRC-LCD, and A549 cells with Ad.CEA-Cre and Ad.lox-TK at various ratios. There was no significant difference in transduction efficiency of Ads among these three cell lines, as determined by infection with Ad.CAG-LacZ (data not shown). GCV sensitivity was evaluated using MTT assay (Fig. 3). The lowest IC₅₀ was obtained in both LoVo and VMRC-LCD cells when coinfected with the paired Ads was performed at a ratio of 1:1. These results were compatible with those obtained by infection with Ad.CEA-Cre and Ad.lox-LacZ (Fig. 2). The IC₅₀ of each cell line (0.17 ± 0.04 and 1.40 ± 0.50 μM, respectively) were 8.4- and 3.3-fold lower than that obtained by infection with Ad.CEA-TK alone (1.43 ± 0.21 and 4.63 ± 0.71 μM, respectively; P < 0.01) and 2488- and 316-fold lower than the values for nontransduced cell lines (423.4 ± 40.4 and 443.3 ± 55.1 μM, respectively). The IC₅₀ of these CEA-producing cells conferred by the Cre/loxP system were comparable with those obtained by infection with Ad.CAG-TK, which rendered all of these cell lines highly sensitive to GCV, regardless of CEA production. In LoVo and VMRC-LCD cells, the lowest IC₅₀ was only 2.1- and 1.5-fold higher than the value for those infected with Ad.CAG-TK (0.17 ± 0.04 and 0.08 ± 0.02 μM, respectively). Infection with Ad.lox-TK alone did not significantly change GCV sensitivity in CEA-producing cells. Moreover, coinfection with Ad.CEA-Cre and Ad.lox-TK did not induce significant change in sensitivity of non-CEA-producing A549 cells. These results show that infection with the produced Cre excises the neomycin resistance gene between a pair of loxP sites out of the latter Ad and activates the HSV-TK gene driven by the strong CAG promoter.

**Determination of the Ideal Ratio of the Paired Ads to Achieve Maximum Expression of the LacZ Gene Using the Cre/loxP System.** To maximize the expression of the LacZ gene using the Cre/loxP system in vitro, we analyzed the ideal ratio of Ad.CEA-Cre to Ad.lox-LacZ. Cre released from Ad.CEA-Cre activates the LacZ gene driven by the CAG promoter. Human adenocarcinoma cell lines, LoVo and A549, were infected simultaneously with these coupled Ads at various ratios at a total moi of 30. The LacZ gene expression was quantified by β-galactosidase assay (Fig. 2). In CEA-producing LoVo cells, the strongest β-galactosidase activity (7.1 ± 0.3 μunits/cell) was obtained with Ads at a ratio of 1:1. No nonspecific β-galactosidase activity was detected on infection with Ad.lox-LacZ alone. Moreover, in non-CEA-producing A549 cells, the β-galactosidase activity remained at very low levels, regardless of the ratios of the paired Ads. The β-galactosidase activity at each ratio increased in proportion to the total moi and reached the maximum level at a total moi of 30 (data not shown). Significant cell toxicity caused by infection with Ads was observed at a moi higher than 30 (data not shown). These results show that the Cre/loxP system allows specific expression of the LacZ gene in CEA-producing cells and confers the highest level of expression when these coupled Ads are used at a ratio of 1:1.

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Ad.CEA-Cre and Ad.lox-TK remarkably augments selective GCV sensitivity in CEA-producing cancer cells compared with infection by Ad.CEA-TK alone and that the magnitude of enhancement in GCV sensitivity correlates with the activity of the CEA promoter rather than the amount of CEA protein produced (Table 1).

Immediate Rejection of Established CEA-producing Tumors by Coinfection with Ad.CEA-Cre and Ad.lox-TK Followed by GCV Treatment. To evaluate in vivo antitumor effects on CEA-producing tumors by coinfection with Ad.CEA-Cre and Ad.lox-TK, we injected tumors of LoVo cells established in the subcutis of athymic mice i.t. with Ads, followed by GCV administration when the average tumor area reached 20 mm² (Fig. 4). When a low dose of Ads (2 × 10⁸ pfu per injection) was injected (Fig. 4A), significant regression of tumor growth was observed only in the mice treated with Ad.CEA-Cre and Ad.lox-TK followed by GCV (group I; group I versus groups III-VIII, P < 0.005). In three of five mice (60%) in this group, tumors were completely eradicated, and two other mice...
showed transient regression of tumor during GCV treatment. Nevertheless, the other treatment including Ad.CEA-TK/GCV (group III) did not have any significant effects on tumor growth (group III versus group VII, P > 0.05). To further augment antitumor effects, we administered an increased dose of Ads to the mice (5 × 10⁶ pfu per injection; Fig. 4B). All mice treated with Ad.CEA-Cre and Ad.Iox-TK followed by GCV injection (group I) showed immediate regression of inoculated tumors. Six of seven mice (86%) became free of palpable tumor by the end of the treatment schedule, and the tumor in the rest of the mice in this group remained a small nodule. On the other hand, Ad.CEA-TK/GCV (group III) as well as Ad.CEA-Cre and Ad.Iox-TK/saline treatment (group II) slightly retarded the growth of tumors but did not eradicate them (group III versus group IV, P < 0.05, from day 24 to day 44, and group II versus group VI, P < 0.01, from day 24 to day 44, respectively). Thus, antitumor effects observed in the Ad.CEA-Cre and Ad.Iox-TK/GCV treatment group (group III) were more remarkable compared with those of the Ad.CEA-TK/GCV treatment group (group III; group I versus group III, P < 0.0001). We did not observe any apparent side effects including body weight loss or death in these treatment cycles. These results suggest that the Cre/loxP system remarkably enhances antitumor effects on CEA-producing tumors in this cancer gene therapy model.

DISCUSSION

This is the first report demonstrating that application of the Cre/lox P system remarkably enhances selective expression of the HSV-TK gene in CEA-producing tumor cells and subsequent in vivo antitumor effects.

The activity of the CEA promoter in CEA-producing LoVo cells was comparable with that of the SV40 promoter determined by CAT assay (Table 1). Ad.CEA-TK rendered LoVo cells 296-fold more sensitive to GCV in vitro in comparison with uninfected cells (Fig. 3). However, repeated i.t. injection of Ad.CEA-TK followed by administration of GCV at most retarded the growth of the inoculated tumors (Fig. 4). On the other hand, i.t. injection of Ad.CAG-TK, which has the strong CAG promoter, with GCV treatment completely eradicated s.c. LoVo tumors in all animals. It is conceivable that expression of the HSV-TK gene at a higher level is necessary for complete rejection of tumors. To augment selective expression of the HSV-TK gene in CEA-producing cells, we applied the Cre/loxP system. Coinfection with Ad.CEA-Cre and Ad.Iox-TK significantly enhanced the sensitivity of CEA-producing LoVo and VMRC-LCD cells to GCV. The IC₅₀ of these cells were only 2.1- and 1.5-times as high as those of Ad.CAG-TK-transfected LoVo and VMRC-LCD cells, respectively. Moreover, infection with Ad.Iox-TK alone did not induce nonspecific GCV sensitivity in CEA-producing cells, nor did coinfection with these Ads significantly change GCV sensitivity in Non-CEA-producing cells (Fig. 3). GCV treatment following i.t. injection of Ad.CEA-Cre combined with Ad.Iox-TK induced immediate regression of established CEA-producing LoVo tumors and resulted in complete tumor rejection in six of seven animals (Fig. 4B). This treatment significantly suppressed the growth of the other CEA-producing VMRC-LCD tumors as well in all mice but did not show any antitumor effects on non-CEA-producing A549 tumors (data not shown). The following reasons might account for the enhanced antitumor effects. (a) A limited amount of induced Cre recombinase effectively processes a large number of molecules on the target Ad genome (23, 26). (b) The activity of the CAG promoter is far stronger than that of the CEA promoter. In fact, Ad.CAG-TK made LoVo cells 18-fold more sensitive to GCV compared with Ad.CEA-TK (Fig. 3). (c) Cre is efficiently activated inside the nucleus by NLS with an effective initiator codon of Ad.CEA-Cre (23).

Marginal retardation of tumor growth was observed in mice injected i.t. with a higher dose of Ad.CEA-Cre combined with Ad.Iox-TK then administered saline (Fig. 4B). When LoVo cells were coinfected with Ad.CEA-Cre and Ad.Iox-TK or infected with Ad.CAG-TK alone in vitro at a total moi of 30, the viability of the infected cells decreased to 70 or 50% of that of uninfected cells, respectively (data not shown). Other reports also showed that i.t. injection of Ads containing the CAG promoter significantly retarded the growth of s.c. tumors (16). We speculate that injection by a higher dose of Ads with the CAG promoter interferes with the transcription of genes necessary for cell survival and induces apoptosis (12, 16).

Death related to severe hepatic toxicity is reported when animals receive administration of Ad expressing the HSV-TK gene driven by the cytomegalovirus promoter followed by GCV treatment (27, 28). In another report, reverse transcriptase-PCR analysis detected expression of transduced genes predominantly in the liver, the kidneys, and the lung, and histological examination revealed zonal necrosis in the periporal area of the liver when an Ad expressing the CD gene under the control of the CAG promoter was i.p. administered then 5-fluorocytosine given (29). We did not observe any side effects including elevation of transaminase or creatinine in the serum of animals treated with i.p. injection of Ad.CEA-Cre and Ad.Iox-TK followed by GCV administration (data not shown). Thus, application of the Cre/loxP system is expected to not only enhance antitumor effects at a reduced dosage of Ads but also avoid damage to normal tissues. Another advantage in application of this system is that it might be no longer needed to specify the enhancer or silencer sequences in the genome for each cell type-specific promoter. Our ultimate goal is to control metastasized tumor cells without affecting normal cells. We are now planning to evaluate the efficacy and side effects of this system in the peritonitis carcinomatosa model in mice, mimicking pleuritis for lung cancer and peritonitis for gastrointestinal cancers in future clinical applications to human beings.

Recently, a combination of HSV-TK/GCV gene therapy with cytokines, including interleukin 2 (30, 31), interleukin 4 (32), and granulocyte-macrophage colony-stimulating factor (31), has been shown to enhance antitumor effects. Modified Ad.Iox-TK in which the neomycin resistance gene is replaced by these cytokine genes would enhance antitumor effects. When these Ads are used simultaneously with Ad.CEA-Cre, not only is the HSV-TK gene expressed specifically in CEA-producing tumor cells, but the cytokines released nonspecifically from surrounding normal cells also induce antitumor immunity.

In conclusion, the Cre/loxP system augments possible clinical application of gene therapy specific for CEA-producing cancer.

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REFERENCES


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Application of the Cre Recombinase/loxP System Further Enhances Antitumor Effects in Cell Type-specific Gene Therapy against Carcinoembryonic Antigen-producing Cancer

Takashi Kijima, Tadashi Osaki, Kazumi Nishino, et al.


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