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Cytotoxic Effect of *Drosophila* Deoxynucleoside Kinase Gene on Replicating Plasmid in HeLa Cells

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To enhance the levels of transgene expression from plasmid-based nonviral vectors, replicating plasmids containing the SV40 origin and the SV40 large T antigen gene, as a model replicating unit, were constructed. The replicating luciferase plasmid DNA produced the luciferase protein more efficiently than the non-replicating luciferase plasmid DNA, as expected. Surprisingly, the introduction of the replicating plasmid DNA containing the *Drosophila melanogaster* deoxyribonucleoside kinase (Dm-dNK) gene was highly cytotoxic and caused cell death without nucleoside analogs. Our results confirm that transgenes on a replicating plasmid represent an excellent tool for effective protein production and suggest that efficient production of the Dm-dNK protein in tumor cells could be an attractive cancer therapy.

Key words replicating plasmid; deoxynucleoside kinase; cell death

Gene delivery with nonviral vectors is a promising approach, due to its excellent safety profile in comparison to that of viral vectors.[1—5] However, low and transient transgene expression is a major concern for nonviral vectors. One of the keys to overcome this problem would be the establishment of an efficient and targeted delivery system, in which intracellular trafficking is considered. In addition, the controlled intranuclear disposition of the delivered plasmid would be quite important for achieving practical gene therapy.[5] Indeed, analyses of the intranuclear disposition of plasmid DNA have indicated that the reasons for the low and transient transgene expression are the decreases in the amount of the exogenous DNA and the expression efficiency from one copy of the transgene over time.[6—8] Thus, the maintenance of both the plasmid DNA and its expression efficiency is essential for the controlled intranuclear disposition.

We focused on a replicating plasmid, to prevent the decrease in the amount of the exogenous DNA. The SV40 origin-SV40 large T antigen system was used as a model. The large T antigen binds the origin, acts as helicase that unwinds DNA, and recruits DNA polymerase α-primase, which initiates replication.[9,10] The plasmid with the origin and the large T antigen gene is replicated in dividing cells, and the replication of the plasmid is conducted in the *S* phase of the cell cycle, along with the endogenous chromosomal DNA. The replicating plasmid could alleviate the decrease in transgene expression by maintaining the amount of the exogenous DNA. This type of plasmid DNA was previously used in extrachromosomal replication and transgene expression.[11,12]

In addition, we focused on the deoxyribonucleoside kinase from the fruit fly *Drosophila melanogaster* (Dm-dNK). Previously, we reported that the presence of the Dm-dNK gene on a plasmid enhanced the cytotoxicity of nucleoside analogs.[13] We expected that this gene on a replicating plasmid would be more effective than that on a non-replicating plasmid for nucleoside analog-induced cell death.

In this study, we transfected replicating plasmid DNAs containing the luciferase and Dm-dNK genes into human cells. Our results confirmed that the replicating plasmid is an excellent tool for efficient protein production. We serendipitously discovered that the Dm-dNK gene on the replicating plasmid was highly cytotoxic and caused cell death without nucleoside analogs.

MATERIALS AND METHODS

Materials Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) and Invitrogen Japan (Tokyo, Japan) in purified forms.

Construction of Plasmid DNAs pYK-CMV-luc (8.5 kb) and pCMV-dNK (7.5 kb), containing the luciferase and dNK genes, respectively, under the cytomegalovirus (CMV) promoter were used as the parental plasmids in this study.[6,13] The pCMV-luc-T plasmid (11.5 kb), containing the SV40 origin/promoter and the SV40 large T antigen gene as well as the CMV promoter-luciferase gene, was constructed by ligating the SV40 origin/promoter-large T antigen gene fragment (the 3.1-kb *Bam*HI fragment) of pMY189[14] into the *Bgl*II site of pYK-CMV-luc (Fig. 1). The orientation of the SV40 origin/promoter and the large T antigen gene was confirmed by restriction mapping. The pCMV-dNK-T plasmid (10.6 kb) was constructed by replacement of the luciferase gene of pCMV-luc-T with the dNK gene. These plasmid DNAs were amplified in the *Escherichia coli* strain DH5α and purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit.

DNA Transfection DNA transfection was performed with the Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, U.S.A.), essentially according to the supplier's instructions. HeLa cells (5×10^4 cells/well) were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum in a 5% CO_2/air atmosphere at 37 °C for 24 h. The pYK-CMV-luc and pCMV-luc-T plasmids (1.4 fmol) were mixed with the pBR322 plasmid as "carrier DNA," to give a total amount of 400 ng. In the case of the 70-fmol experiments, the pBR322 plasmid was added to the pYK-CMV-luc plasmid to give a total amount of 500 ng DNA. The DNA mixture was combined with the "Plus" reagent and was then complexed with the lipids. The HeLa cells were treated with the lipid–DNA complex. After 3 h, the complex was removed and the cells were incubated in DMEM supplemented with

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10% serum. The medium was changed at 24 h intervals. The cells were washed with PBS before the luciferase assay and DNA extraction.

Luciferase Activity and Quantitative PCR Luciferase activity was measured with a Luciferase Assay System (Promega, Madison, WI, U.S.A.), according to the supplier's instructions.

The DNA was extracted with the SepaGene reagent (Sanko Jun-yaku, Tokyo, Japan). Quantitative polymerase chain reaction (Q-PCR) was performed using the ABI 7700 sequence detection system, and the 7500 real time PCR system (Applied Biosystems, Foster City, CA, U.S.A.), and SYBR-green chemistry. A portion of the recovered DNA was analyzed by Q-PCR. The luciferase gene in the cells was detected using the following primers: Luc (+), 5′-dGGTCCTATGATTATGTCCGGTTATG-3′; Luc (−), 5′-dATGTAGCCATCCATCCTTGTCAAT-3′.

Measurement of Cytotoxicity HeLa cells (5×10^4 cells/well) were transfected with 70 fmol of the pCMV-dNK-T, pCMV-dNK, and pCMV-luc-T plasmids, as described above. The total amount of transfected DNA was adjusted to 500 ng by the addition of pBR322. At 48 h after transfection, living cells were counted by excluding dead cells stained by trypan blue.

Caspase Activity HeLa cells (5×10^3 cells/well) were transfected with 14 fmol of the pCMV-dNK and pCMV-luc-T plasmids, as described above. The total amount of transfected DNA was adjusted to 100 ng by the addition of pBR322. The caspase 3/7 activities were measured by a Caspase-Glo 3/7 Assay kit (Promega), according to the supplier's instructions. Untreated cells and cells treated with etoposide (final concentration 10 μM) were used as the negative and positive controls, respectively.

Statistical Analysis Statistical significance was examined by the Student's *t*-test. Levels of $p<0.05$ were considered to be significant.

RESULTS

Efficient Luciferase Gene Expression from the Replicating Plasmid We constructed replicating plasmid DNAs with the SV40 large T antigen gene under the control of the SV40 promoter within the origin sequence (Fig. 1). Using this SV40 origin-SV40 large T antigen system, the plasmid could be replicated in dividing cells to increase its copy number. We used the luciferase gene to monitor the amounts of the plasmid DNA and its encoded protein.

We transfected the replicating luciferase plasmid (pCMV-luc-T, 1.4 fmol) into HeLa cells. As shown in Fig. 2A, the luciferase activity was significantly higher for the replicating plasmid DNA than for the non-replicating plasmid DNA (pYK-CMV-luc) at 48 and 72 h after transfection. We then quantitated the amounts of the luciferase DNA in the cells by quantitative PCR. The amount of the luciferase DNA was more abundant for the replicating plasmid than for the non-replicating plasmid at 48 h posttransfection (Fig. 2B), suggesting that the introduced plasmid DNA was replicated in the cells. A similar tendency was observed at 72 h after transfection, although the difference was statistically insignificant. Thus, the incorporation of the sequences necessary for replication (the SV40 origin and the SV40 large T antigen gene) improved the transgene expression.

The luciferase expression was also enhanced by the use of the replicating plasmid DNA, when the amount of the transfected plasmid DNA was increased to 70 fmol (Fig. 3). Increased luciferase expression was also observed in human HEK293 cells (Fig. 4).

Cytotoxicity of the Dm-dNK Gene on Replicating Plasmid The efficacy of nucleoside analog drugs was previously reported to be enhanced by transfection of a non-replicating plasmid DNA carrying the Dm-dNK gene, pCMV-

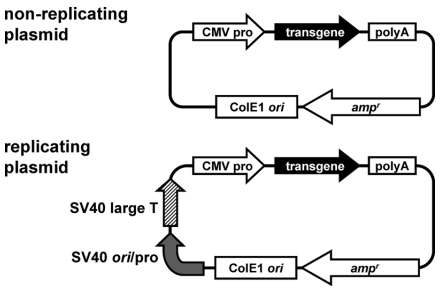

Fig. 1. Structures of the Plasmid DNAs Used in This Study

pro, promoter; polyA, polyA signal; *amp*^{r}, *E. coli* ampicillin resistance gene; *ori*, replication origin. The SV40 *ori*/pro functions as the replication origin and the promoter for the SV40 large T antigen gene in human cells. The ColE1 *ori* acts as the replication origin in *E. coli*.

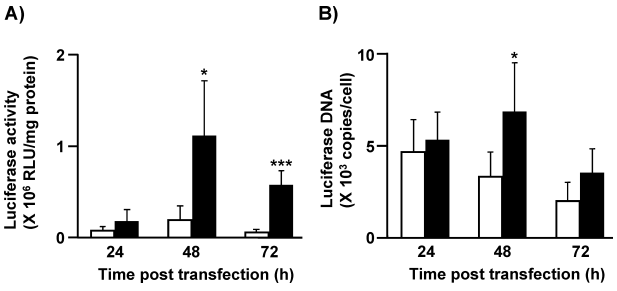

Fig. 2. Expression of the Luciferase Gene and Measurement of the Amounts of Luciferase DNA, Examined by Transfection with the Replicating Plasmid

The pYK-CMV-luc and pCMV-luc-T plasmids (1.4 fmol) were introduced into HeLa cells, as described in Materials and Methods. The cells were harvested, and the luciferase activities and the amounts of luciferase DNA were measured. (A) Expression of the luciferase gene. (B) Amounts of luciferase DNA. The values represent the averages of at least three separate experiments. Bars indicate S.D. (standard deviation). Open columns, pYK-CMV-luc; filled columns, pCMV-luc-T. $*p<0.05$, $***p<0.001$ (*vs.* pYK-CMV-luc).

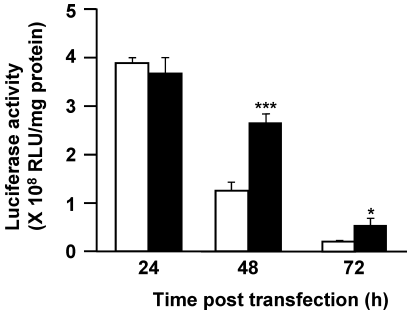

Fig. 3. Expression of the Luciferase Gene, Examined by Transfection of the Replicating Plasmid

The pYK-CMV-luc and pCMV-luc-T plasmids (70 fmol) were introduced into HeLa cells, as described in Materials and Methods. The cells were harvested, and the luciferase activities were measured. The values represent the averages of at least three separate experiments. Bars indicate S.D. (standard deviation). Open columns, pYK-CMV-luc; filled columns, pCMV-luc-T. $*p<0.05$, $***p<0.001$ (*vs.* pYK-CMV-luc).

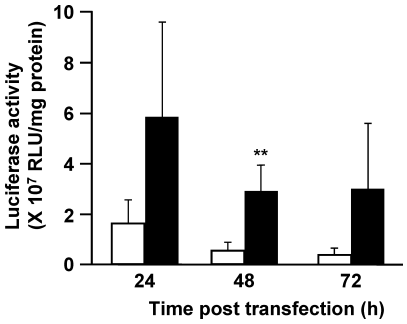

Fig. 4. Expression of the Luciferase Gene in HEK293 Cells, Examined by Transfection of the Replicating Plasmid

The pYK-CMV-luc and pCMV-luc-T plasmids (70 fmol) were introduced into HEK293 cells. The cells were harvested, and the luciferase activities were measured. The values represent the averages of four separate experiments. Bars indicate S.D. (standard deviation). Open columns, pYK-CMV-luc; filled columns, pCMV-luc-T. ** $p<0.01$ (*vs.* pYK-CMV-luc).

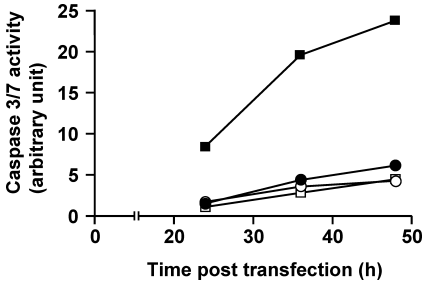

Fig. 6. Caspase 3/7 Activities in HeLa Cells Transfected with the Replicating Dm-dNK-Plasmid

The pCMV-dNK and pCMV-dNK-T plasmids (14 fmol) were introduced into HeLa cells, as described in Materials and Methods. The combined caspase 3/7 activity was measured at the time points indicated after the transfection. Four separate experiments were performed, and typical results are shown in this figure. Open circles, pCMV-dNK; filled circles, pCMV-dNK-T; open squares, untreated cells; closed squares, etoposide-treated cells.

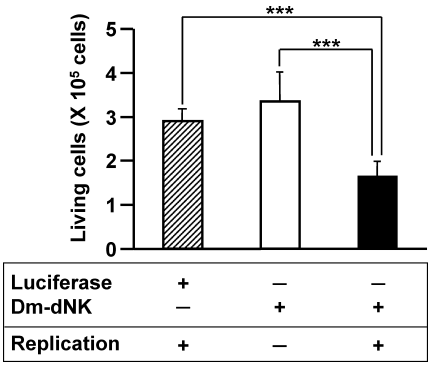

Fig. 5. Cytotoxicity of the Dm-dNK Gene Encoded by the Replicating Plasmid in HeLa Cells

The pCMV-luc-T, pCMV-dNK, and pCMV-dNK-T plasmids (70 fmol) were introduced into HeLa cells, as described in Materials and Methods. The cells were harvested, and the viable cells were counted at 48 h after the transfection. The values represent the averages of at least three separate experiments. Bars indicate S.D. (standard deviation). Hatched column, pCMV-luc-T; open column, pCMV-dNK; filled column, pCMV-dNK-T. *** $p<0.001$.

dNK.[13] A further increase in the cytotoxicity of these drugs may be achieved by the efficient production of Dm-dNK from replicating plasmid DNA. To extend our study to cancer gene/chemo therapy, the replicating plasmid DNA carrying the Dm-dNK gene, pCMV-dNK-T (Fig. 1), was transfected into HeLa cells. The non-replicating plasmid, pCMV-dNK, and the replicating plasmid carrying the luciferase gene, pCMV-luc-T, were also transfected as controls. Since transfection with cationic lipids itself could be toxic, the total amounts of DNA were adjusted by the addition of carrier DNA, and the same amounts of transfection reagents were used.

Unexpectedly, the number of viable HeLa cells was reduced at 48 h after the transfection of pCMV-dNK-T, without nucleoside analog treatment (Fig. 5). Upon the transfection of 70 fmol of the plasmid, *ca.* 3×10^5 cells were alive in the cases of the non-replicating pCMV-dNK and replicating pCMV-luc-T plasmids. On the other hand, *ca.* 1.5×10^5 cells were viable after the transfection of pCMV-dNK-T. This reduction in the number of living cells was due to cell death, since more trypan blue-positive cells were observed in the pCMV-dNK-T experiment (data not shown). Thus, *ca.* 50% of the HeLa cells seemed to die after the introduction of the replicating plasmid DNA carrying the Dm-dNK gene. Since the non-replicating pCMV-dNK plasmid did not cause cell death, the amount of the Dm-dNK protein seems to be critical. Under similar experimental conditions, introduction of the replicating plasmid DNA carrying the Dm-dNK gene induced cell death after 24 and 72 h (data not shown).

No apparent increases in the efficacies of nucleoside analog drugs (no decreased 50% inhibitory concentration values) were obtained by transfection of the replicating pCMV-dNK-T plasmid (data not shown). This could be a consequence of cell death, when a significant amount of the Dm-dNK protein was produced. The expression of the protein would be low in the surviving cells.

Caspase 3/7 Activities Were Not Enhanced by Transfection of the Dm-dNK Gene on a Replicating Plasmid To examine whether the cell death induced by the transfection of the replicating plasmid DNA containing the Dm-dNK gene occurred by apoptosis, the caspase 3 and 7 activities were measured. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells.[15,16] The combined caspase 3/7 activity was elevated by etoposide, a known apoptosis inducer (Fig. 6).[17] The caspase 3/7 activity for the HeLa cells transfected with the replicating Dm-dNK plasmid DNA was much lower than that for the etoposide-treated cells and similar to that for the cells transfected with the control plasmid DNA, although the activity was slightly higher for the replicating plasmid DNA than for the control plasmid DNA. Thus, the typical apoptosis pathway, involving caspases 3 and 7, was not the major pathway for the induced cell death. Necrosis could be the pathway since we did not observe morphological changes of the transfected cells (data not shown).

DISCUSSION

In this study, we used the SV40 origin and large T antigen system, a good model system for replication in mammalian cells. We expected that the incorporation of the replication unit into the plasmid DNAs would facilitate the replication of the DNAs and the efficient production of the mRNAs encoded by the transgenes, as described previously.[11,12]

As shown in Figs. 2A and 3, the replicating plasmid DNA produced more luciferase protein in the transfected HeLa

cells. The orientation of the SV40 origin-SV40 large T antigen gene did not affect the luciferase expression (data not shown). Increased luciferase expression was also observed in human HEK293 cells (Fig. 4). Thus, the replicating plasmid effectively facilitated efficient transgene expression. However, increase in luciferase expression was larger than that in amount of luciferase DNA (Fig. 2). This might be due to improved intranuclear disposition of plasmid DNA after replication. Histones could be a key factor for the intranuclear disposition of exogenous DNA[18,19] and replication-dependent loading of histones would enable properly regulated expression from plasmid DNA. In agreement with this speculation, SV40 DNA binds with histones to form minichromosome.[20] Alternatively, the presence of the SV40 enhancer in the SV40 origin/promoter and/or production of the SV40 large T antigen might increase transcription.

The luciferase activities at 72 h were lower than those at 48 h, even in the case of the replicating plasmid DNA (Figs. 2A, 3). Moreover, the amount of the replicating plasmid DNA decreased from 48 to 72 h (Fig. 2B). This decrease might be because of degradation of the plasmid DNA. However, the amplified plasmid DNA would be stable since SV40 DNA binds with histones to form minichromosome.[20] Alternatively, this result could be due to silencing of the exogenous DNA, which would reduce the expression of both the SV40 large T antigen and luciferase protein,[6—8] although the mechanism of the silencing was still unknown. The decreased expression of the SV40 large T antigen would result in reduced plasmid replication.

Cell death induced by transfection of the replicating plasmid DNA containing the Dm-dNK gene was serendipitously discovered (Fig. 5). This cell death did not occur *via* typical apoptosis, since the activities of caspases 3 and 7 in the HeLa cells transfected with the replicating Dm-dNK plasmid DNA were similar to those of the HeLa cells transfected with the control plasmid DNAs (Fig. 6). The cell death would be elicited by the presence of large amounts of the Dm-dNK protein in the cells. Dm-dNK is a multisubstrate enzyme that phosphorylates the four pyrimidine and purine 2′-deoxyribonucleosides, and the catalytic rate of 2′-deoxyribonucleoside phosphorylation by Dm-dNK is four- to 20000-fold higher than any of the mammalian deoxyribonucleoside kinases.[21,22] Thus, the enhanced production of Dm-dNK could unbalance the intracellular 2′-deoxyribonucleoside 5′-triphosphate (dNTP) pool, which is usually tightly regulated. This interpretation is supported by previous reports that some nucleoside analogs, such as 5-fluoro-2′-deoxyuridine and 2-chloro-2′-deoxyadenosine, disrupted the balance of the dNTP pools, and consequently induced cell death.[23,24] The results shown in Fig. 5 suggest that the efficient production of the Dm-dNK protein in tumor cells could be an attractive cancer therapy by disturbing nucleotide metabolism, which is essential for cellular viability. Moreover, production of the SV40 large T antigen might contribute to the cell death although cell death was not induced by transfection of the replicating luciferase plasmid DNA. Further studies are necessary to address molecular mechanism(s) of the induced cell death.

When we consider the *in vivo* application of the Dm-dNK gene, the tumor-specific expression of the Dm-dNK protein would be necessary. To accomplish this, the tumor-specific delivery of the gene by carriers with ligand molecules for tumor-targeting, for example, transferrin,[25] and the use of a tumor-specific promoter, such as the RAD51 promoter,[26] would be required.

The wild type SV40 large T antigen used in this study has a transformation property that is considered to be primarily mediated by binding to host tumor suppressor gene products, including p53, retinoblastoma (RB), and RB-related proteins, such as p107.[27—29] However, Cooper *et al.* reported that a mutant large T antigen substituted at the 107th and 402nd amino acid residues did not bind significantly to RB, p107, and p53, and retained the replication ability.[12] Thus, this mutant large T antigen gene, instead of the wild type gene, would be useful for safe gene therapy in the near future.

Previously, Jenke *et al.* reported another type of replicating plasmid DNA, containing a tetramer of a 155-bp minimal nuclear scaffold/matrix region between the coding and polyA signal regions.[30] Although the precise replication mechanism for the plasmid is unknown, this type of plasmid DNA might be a reasonable alternative for the plasmid DNAs used in this study.

In this study, the cytotoxic effect of the Dm-dNK gene encoded by a replicating plasmid was discovered. This result suggests the potential of this type of plasmid DNA for cancer therapy, since continuous cell division will allow plasmid DNA replication.

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