2-5A Antisense Telomerase RNA Therapy for Intracranial Malignant Gliomas

Shigehiko Mukai, Yasuko Kondo, Shoji Koga, Tadashi Komata, Barbara P. Barna, and Seiji Kondo

Center for Surgery Research [S. M., Y. K., S. Kog., T. K., S. Kon.] and Department of Neurosurgery [T. K., S. Kon.], The Cleveland Clinic Foundation, Cleveland, Ohio 44195; Department of Neurosurgery, The Mount Sinai School of Medicine, New York, New York 10029-6574 [Y. K., T. K., S. Kon.]; and RammelKamp Center for Education and Research, MetroHealth Medical Center, Cleveland, Ohio 44109 [B. P. B.]

ABSTRACT

Malignant gliomas are the most common intracranial tumors and are considered incurable. Therefore, exploration of novel therapeutic modalities is essential. Telomerase is a ribonucleoprotein enzyme that is detected in the vast majority of malignant gliomas but not in normal brain tissues. We, therefore, hypothesized that telomerase inhibition could be a very promising approach for the targeted therapy of malignant gliomas. Thus, 2-5A (5′-phosphorylated 2′-5′-linked oligoadenylate)-linked antisense against human telomerase RNA component (2-5A-anti-hTER) was investigated for its antitumor effect on an intracranial malignant glioma model. 2-5A is a mediator of one pathway of IFN actions by activating RNase L, resulting in RNA degradation. By linking 2-5A to antisense, RNase L degrades the targeted RNA specifically and effectively. Prior to the experiments using intracranial tumor models in nude mice, we modified the in vitro and in vivo treatment modality of 2-5A-anti-hTER using a cationic liposome to enhance the effect of 2-5A-anti-hTER. Here we demonstrate that 2-5A-anti-hTER complexed with a cationic liposome reduced the viability of five malignant glioma cell lines to 20–43% within 4 days but did not influence the viability of cultured astrocytes lacking telomerase. Furthermore, treatment of intracranial malignant gliomas in nude mice with 2-5A-anti-hTER was therapeutically effective compared with the control (P < 0.01). These findings clearly suggest the therapeutic potentiality of 2-5A-anti-hTER as a novel approach for the treatment of intracranial malignant gliomas.

INTRODUCTION

Malignant gliomas are the most common malignant brain tumors. The 5-year survival rate for patients with the most aggressive form of malignant glioma, glioblastoma multiforme, is less than 5% even with surgery, radiation therapy, and chemotherapy (1, 2). Certainly, new therapeutic strategies are necessary. Telomerase, a ribonucleoprotein enzyme, is thought to be a good candidate for targeted therapy of cancer (3–5) because approximately 90% of malignant tumors of various origins express telomerase, whereas most of normal tissues do not. As for malignant gliomas, the vast majority of tumors express telomerase activity (6–8). In contrast, the activity is not detected in normal brain tissues or cells. These observations indicate a therapeutic opportunity on telomerase-positive malignant gliomas by inhibiting telomerase.

Recently, using an antisense oligonucleotide against hTER linked to 2-5A (5′-phosphorylated 2′-5′-linked oligoadenylate), we demonstrated its effect on cultured cells and s.c. tumors in nude mice of malignant glioma cell lines (8). 2-5A is known as one pathway of IFN actions. 2-5A activates RNase L that is ubiquitous in mammalian cells, and this results in single-stranded RNA cleavage after UN (dinucleotide sequences beginning with a uridylate residue) sequences (9, 10). Antisense against a certain target linked to 2-5A causes the degradation of the targeted RNA in a highly specific manner and enhances the potency of antisense by at least 20-fold (11–13). In the previous study, 2-5A-anti-hTER degraded hTER 5 h after the treatment and reduced glioma cell viability to 20–30% 2 weeks later (8). The reduction of viability was mainly attributable to induction of apoptosis. Although the results were encouraging for the development of cancer therapy, our goal is to treat intracranial malignant gliomas with 2-5A-anti-hTER. Therefore, it is essential to investigate the effect of 2-5A-anti-hTER on intracranial tumors.

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MATERIALS AND METHODS

Tumor Cells. Human malignant glioma U373-MG, T98G, A172 cells were purchased from American Type Culture Collection (Rockville, MD). Human malignant glioma U251-MG and GB-1 cells were gifts from Drs. A. Nishiyama (University of Connecticut, Storrs, CT) and T. Morimura (National Utano Hospital, Kyoto, Japan). Human astrocyte PIN cells were used as a control (8). Cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.) 4 mm glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Synthesis of 2-5A-anti-hTER. The selection of the antisense against hTER was described previously (8). Briefly, we designed the 2-5A antisense oligonucleotide to the region between residues 76 and 94, because this part was predicted to be the most open by the MFOLD computer program. The sequence of the antisense is 5′-GGG GAG GCA AAA GCA C-3′. To investigate the effect of 2-5A-anti-hTER, we synthesized a test oligonucleotide (spA₃-anti-hTER) with complete homology to the targeted sequence and two controls (8). One control is spA₃-anti-hTER that has a nonfunctional chimeric 2-5A linked to the anti-hTER. The other control oligonucleotide is spA₃-anti-hTER, which contains functional 2-5A, but with six mismatched nucleotides in the antisense that would prevent homologous binding with the telomerase RNA. These chimeric oligonucleotides synthesized on solid supports and purified as described previously (10–13) were gifts from B. Banypashadray and Dr. R. H. Silverman (Cleveland Clinic, Cleveland, OH) or were synthesized at Hybriion (Milford, MA).

Cell Viability Assay. The cytotoxic effect of 2-5A-anti-hTER on tumor cells was determined by using a trypan blue dye exclusion assay as described.
previously (8). The cells were seeded at 5 × 10³ cells/well (0.1 ml) in 96-well flat-bottomed plates and incubated overnight at 37°C. Then, oligonucleotides (0.1–5.0 μM) with or without Lipofectamine (0.4–1.2 μl; Life Technologies, Inc.) were added into cells every 2 or 24 h. The first day of the treatment was counted as Day 0.

FITC-labeled 2-5A-anti-hTER. FITC-labeled 2-5A-anti-hTER was a gift from Gemini Technologies, Inc. (Cleveland, OH). Cells (5 × 10⁴) were seeded in each well of 4-well chamber glass slides (Nalge Nunc International, Naperville, IL). On the following day, the cells were treated with 0.5 μM FITC-2-5A-anti-hTER with or without Lipofectamine. After incubation for 3 h at 37°C, the cells were washed with PBS at 4°C. Then, the cells were fixed with 4% paraformaldehyde in PBS for 30 min on ice followed by washing with PBS overnight. The cells were covered with Vectashield (Vector, Burlingame, CA) and were sealed with coverslip. The slides were observed and the images were analyzed by the confocal laser scanning microscope (Aristoplan, Leica, Inc., Deerfield, IL) which belongs to the core-facility of the Cleveland Clinic Foundation.

Telomerase Activity (TRAP) Assay. The TRAP assay was performed with TRAPEZE Telomerase Detection kit (Oncor Inc., Gaithersburg, MD) according to the manufacturer’s instructions with some minor modifications (8, 19). Cells were washed once in PBS, and homogenized in 50 μl of ice-cold lysis buffer. After 30 min of incubation on ice, the lysates were centrifuged at 10,000 × g for 15 min at 4°C, and the supernatant was rapidly frozen and stored at −80°C. The concentration of protein was measured using the Bio-Rad Protein Assay (Richmond, CA). Equal amounts of protein extracts were used for telomerase assays. Two μl of each extract was assayed in a 50-μl reaction mixture containing: 5 μl of 10× TRAP reaction buffer, 50 μM deoxyxynucleotide triphosphates, 0.2–0.4 μl of [α-32P]dCTP (10 μCi/μl, 3000 Ci/mmole), 0.1 μg of TS primers, 1 μl of primer mix, 1 μg of T4 gene 32 protein (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 2 units of Taq DNA polymerase (Boehringer Mannheim). An internal telomerase activity standard (ITAS, 36 hp) was included in the primer mix. After a 30-min incubation at room temperature for telomerase extension, the reaction mixture was then subjected to PCR amplification in a thermal cycler for 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. The PCR product was electrophoresed in 0.5 × Tris-borate EDTA on a 6% polyacrylamide gel. The gel was then dried, and autoradiography was performed. Radioactivity was also quantitated with a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Flow-Cytometric Analysis of Apoptosis. When cells undergo apoptosis, one of the earliest changes is the membrane phospholipid PS translocated from the inner to the outer leaflet of the membrane. Thus, PS is exposed to the inner to the outer leaflet of the membrane. Therefore, PS can be detected using PS-binding protein such as annexin V (20). To provide a comparative assay of apoptosis by annexin V labeling, tumor cells (1 × 10⁶) treated with 2-5A-anti-hTER for 4 days were harvested, washed, fixed with ice-cold 70% ethanol (50 min, 4°C) and were resuspended in binding buffer [10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂]. Fifty μl of FITC-annexin V (R&D Systems, Minneapolis, MN) were added and were incubated for 15 min in the dark at room temperature before flow-cytometric analysis.

In Vivo Treatment. For s.c. tumors, U373-MG cells (5 × 10⁶ cells in 0.05 ml of serum-free DMEM and 0.05 ml of Matrigel) were inoculated s.c. into the right flank of 8–12-week-old female BALB/c nude mice (five mice for each treatment group). Tumor growth was monitored by using calipers every 12 or 24 h, the percentage of cell viability of malignant glioma cells to about 20–30% after treatment every 12 h for 2 weeks. To develop this treatment for clinical studies in the future, we should optimize the administration protocol. Is it possible to reduce both the treatment frequency (e.g., from every 12 h to every day or every other day) and the concentration of oligonucleotides? And can the new treatment modality induce the antitumor effect at earlier stages than 2 weeks? Recently, it has been well demonstrated that a cationic liposome enhances the intracellular incorporation rate of oligonucleotides (16–19). To improve the cytotoxic effect of 2-5A-anti-hTER, we, therefore, modified the treatment modality using Lipofectamine.

First, we optimized the concentration of Lipofectamine to determine its appropriate dose that, by itself, would not be cytotoxic to cells. As shown in Fig. 1A, we added Lipofectamine (0–1.2 μl/100 μl DMEM) to U373-MG cells every 24 h for 4 days, and the cell viability of tumor cells was determined. The cell viability of tumor cells was decreased by the addition of Lipofectamine in a dose-dependent manner. An amount of 0.8 or 1.2 μl of Lipofectamine reduced cell viability to 50 or 30%, respectively. However, the cytotoxic effect of 0.4 μl of Lipofectamine was minimal. This, then, was the concentration chosen for additional experiments.

To determine the optimal ratio of 2-5A-anti-hTER to Lipofectamine, we mixed 0.4 μl of Lipofectamine with 2-5A-anti-hTER in the range between 0.1 and 5.0 μM in advance, and we treated U373-MG cells every 12 or 24 h for 4 days. As shown in Fig. 1B, treatment with 2-5A-anti-hTER (2.0 or 5.0 μM) without Lipofectamine every 12 h reduced cell viability to 75 or 45%, respectively. However, when the concentration of 2-5A-anti-hTER was 0.1 or 0.5 μM, there was no antitumor effect 4 days after the treatment of every 12 h. When 2-5A-anti-hTER (0.1–5.0 μM) was added every 24 h in the absence of Lipofectamine, the cytotoxic effect was not observed. When Lipofectamine was mixed with 2-5A-anti-hTER (2.0 or 5.0 μM) and the cells were treated every 24 h, the percentage of cell viability was 45 or 40%, respectively. Interestingly, the lack of linearity of
response of tumor cells to increasing amounts of 2-5A-anti-hTER was observed, and the best effect on cell viability was obtained when 0.5 μM 2-5A-anti-hTER mixed with Lipofectamine was added every 24 h. This finding strongly suggested that to the extent that a cationic liposome is used, the careful optimization of transfection conditions is essential for the highest effect in 2-5A-anti-hTER as well as in conventional DNA or antisense oligonucleotide.

The idea that a greater or lesser amount of 2-5A-anti-hTER or Lipofectamine than the optimized ratio would impair its effect was also supported by the findings obtained from s.c. tumor models as described below. These results indicated that treatment with 2-5A-anti-hTER in the presence of Lipofectamine reduced the treatment frequency to every 24 h and induced a better antitumor effect at one-tenth of the concentration of oligonucleotides used previously.

To confirm that Lipofectamine enhanced the incorporation of 2-5A-anti-hTER into tumor cells, U373-MG cells were treated with FITC-labeled 2-5A-anti-hTER. As shown in Fig. 1C, incorporation of FITC-2-5A-anti-hTER into U373-MG cells. Cells (5 × 10⁴) were seeded in each well of 4-well chamber glass slides and incubated at 37°C overnight. The cells were treated with 0.5 μM FITC-labeled spA₄-anti-hTER with or without Lipofectamine (4.0 μl/ml). After being incubated for 3 h at 37°C, the cells were washed and fixed, and the specimens were analyzed by the confocal laser scanning; a and c, phase contrast; b and d, fluorescent; ×400.

**Effect of 2-5A-anti-hTER on Malignant Glioma Cells in Vitro.** We further investigated whether 2-5A-anti-hTER mixed with Lipofectamine was effective for other malignant glioma cells. Prior to the cell viability assay, we performed the TRAP assay to ensure that all of the malignant glioma cells that we used were telomerase-positive and that cultured astrocytes were telomerase-negative. As shown in Fig. 2A, all of the malignant glioma cells (U373-MG, U251-MG, GB-1, T98G, and A172) expressed telomerase activity. In contrast, cultured astrocytes P1N were telomerase-negative as described previously (8). The internal telomerase activity standard (ITAS) excluded the possibility of a false-negative result attributable to Taq polymerase inhibitors.

Next, we treated tumor cells and astrocytes with 2-5A-anti-hTER. As shown in Fig. 2B, spA₄-anti-hTER treatment with Lipofectamine every 24 h for 4 days reduced the cell viability of tumor cells to 20–43%. In contrast, the cytotoxic effect of control oligonucleotides (spA₂-anti-hTER and spA₄-anti-(M₆)hTER) was not significant. On the other hand, P1N astrocytes were resistant to those three types of oligonucleotides even in the presence of Lipofectamine. Furthermore, the flow-cytometric analysis using annexin V labeling was performed to determine the extent of apoptosis quantitatively. As shown in Fig. 2C, after the treatment with 2-5A-anti-hTER for 4 days, the percentage of annexin V-binding apoptotic U373-MG cells was 3.88% (a, Lipofectamine alone), 11.80% (b, control oligonucleotide, spA₂-anti-(M₆)hTER, and Lipofectamine), 14.92% (c, test oligonucleotide, spA₄-anti-hTER alone), or 99.82% (d, spA₄-anti-hTER and Lipofectamine), respectively. The discrepancy of results between cell viability assays (20% of tumor cells were viable) and apoptosis detection assays (99.82% of tumor cells showed apoptosis) may...
derive from the possibility that some of treated cells still remained viable while exhibiting apoptotic features. These results indicated that the cytotoxic effect of 2-5A-anti-hTER in the presence of Lipofectamine was specifically for telomerase-positive cells through induction of apoptosis, which suggests that 2-5A-anti-hTER could work as a tool of targeted therapy for telomerase-positive tumors.

Effect of 2-5A-anti-hTER on s.c. Tumors. Obviously the goal in this study was to treat intracranial malignant gliomas in mice. Before the experiments, we optimized the ratio of 2-5A-anti-hTER to Lipofectamine using s.c. tumors. At first, we treated s.c. tumors with 5 nmol of 2-5A-anti-hTER, which was the same dose that we injected previously (8). As shown in Fig. 3A, treatment with 2-5A-anti-hTER (5 nmol) in the absence of Lipofectamine reduced the tumor volume to 80% after 1 week. Next, s.c. tumors were treated with 1 nmol of 2-5A-anti-hTER in the presence of Lipofectamine (0 to 3 μl/each). As shown in Fig. 3A, the tumor volume was decreased to 47% by the treatment with 1 nmol of spA4-anti-hTER and 0.3 μl of Lipofectamine (P < 0.01, compared with treatment with 5 nmol of spA4-anti-hTER alone). The concentration of 1 nmol of spA4-anti-hTER might be estimated to correspond to about 20–30 times that used in cultured cells if spA4-anti-hTER injected into the tumor should cover the entire tumor. Although the situation between in vitro and in vivo experiments is often different, it might be necessary to investigate the in vivo antitumor effect of the same concentration of 2-5A-anti-hTER as used in cultured cells. Interestingly, the treatment at the ratio of spA4-anti-hTER:Lipofectamine (1:0 or 1:3) showed some inhibitory effects on tumor growth but did not regress the tumor size.

These results indicated that the ratio of 2-5A-anti-hTER:Lipofectamine was very important to yield the expected results and that the treatment at the 1:0.3 ratio of 2-5A-anti-hTER:Lipofectamine showed a better antitumor effect than any other modalities that we tried. Furthermore, we compared the effect of spA4-anti-hTER with that of control oligonucleotide with mismatches using this ratio. As shown in Fig. 3B, there was no significant difference in treatment effect between control oligonucleotide, spA4-anti-(M6)hTER, and Lipofectamine alone. Less frequent treatments with 2-5A-anti-hTER would be desirable, especially for treating intracranial tumors, because direct treatments are invasive. To investigate this possibility, spA4-anti-hTER (3 nmol) mixed with Lipofectamine (1 μl) was injected directly into the s.c. tumors every other day (a total of four times) over a period of 7 days. As expected, this less frequent treatment with higher concentration reduced the tumor volume to 50%
U373-MG cells were inoculated s.c. in nude mice. When the size of the tumors reached 60–120 mm³, 2-5A-anti-hTER (1.0–5.0 nmol/tumor) with Lipofectamine (0–3.0 µl) was directly injected into the tumors (n = 5 for each group). A, the ratio of 2-5A-anti-hTER:Lipofectamine. spA4-anti-hTER (1.0 or 5.0 nmol/tumor) with Lipofectamine (0–3.0 µl) was directly injected into the tumors every 24 h for 1 week. Results shown are the means ± SD. B, effect of 2-5A-anti-hTER mixed with Lipofectamine on s.c. tumors. spA4-anti-hTER or spA4-anti-(M6)hTER (1.0 nmol/tumor) with Lipofectamine (0.3 µl) was directly injected into the tumors every 24 h for 1 week. Results shown are the means ± SD.

Effect of 2-5A-anti-hTER on Intracranial Tumors. On the basis of the results of the s.c. tumors treatment, we decided to treat intracranial tumors in mice with 3 nmol of 2-5A-anti-hTER and 1 µl of Lipofectamine by intracranial injections every other day (a total of five times). Because intracranial tumors were clearly detected 2 weeks after the inoculation with malignant glioma cells (data not shown), we started the treatment on day 14. We chose to treat the animals after the establishment of the tumor to simulate the clinical situation. As shown in Fig. 4A, all of the control mice (the mice that received no treatment or received Lipofectamine alone) with intracranial tumors died of their tumors within 8–11 weeks. Intracranial tumors treated with Lipofectamine alone grew extensively and invaded the other side of the brain (Fig. 4B, a and b). Although one of five mice treated with spA4-anti-(M6)hTER survived up to 14 weeks after intracranial inoculation with tumor cells, there was no statistical difference between control groups and spA4-anti-(M6)hTER treatment. As shown in Fig. 4A, one of five mice treated with spA4-anti-hTER died of intracranial tumors 11 weeks after tumor inoculation, but four of five mice survived up to 15 weeks (P < 0.01, compared with control groups). In brain tissues harvested from mice 15 weeks after treatment with spA4-anti-hTER, the place where tumor cells were inoculated and oligonucleotides were injected was detected, but tumor tissues were not detected (Fig. 4B, c and d). To investigate whether apoptotic cells were detected in the corresponding region, the TUNEL assay was performed using the consecutive specimens. However, TUNEL-positive cells were not detected (data not shown), which indicated the possibility that apoptotic cells induced by 2-5A-anti-hTER treatment might be removed by phagocytic cells until 15 weeks. These results indicated that the treatment of mice bearing intracranial malignant gliomas with 2-5A-anti-hTER was therapeutically effective.

DISCUSSION

This study shows that treatment with 2-5A-anti-hTER mixed with a cationic liposome was specifically effective for telomerase-positive tumor cells and protected normal cells without telomerase from its cytotoxicity. The treatment modality described here was therapeutically effective for intracranial malignant gliomas, a fatal disease.

It is expected that the inhibition of telomerase is a promising therapy for cancers because telomerase is detected in the vast majority of tumor cells but not in most normal cells (3–5, 22). Recent investigations show that telomerase activity is inhibited by treatment with the antisense oligonucleotides, hammerhead ribozyme, or peptide nucleic acids against hTER (23–25). Actually, the above reports showed some effects on telomerase inhibition but not on cell viability. To investigate whether telomerase inhibition is available for cancer treatment, it is important to assess its effect on tumor growth using in vitro and in vivo systems. As demonstrated in this study, we have succeeded in treating tumor models in vitro and in vivo with 2-5A-anti-hTER. More recently, telomerase is reported to be essential for the formation of human tumor cells (26). Therefore, our approach is expected to be effective for the treatment of a wide range of tumors including malignant gliomas.

Recently, three components of the human telomerase have been identified: the RNA component (hTER; Ref. 27), the telomerase-associated protein (TEP1; Refs. 28, 29), and the telomerase catalytic subunit (hTERT; Refs. 30, 31). Although both hTER and hTERT are necessary for telomerase activity, the expression of hTERT is well associated with telomerase activity (32, 33). That is to say, to treat telomerase-positive tumors with inhibition of telomerase, it is likely that the hTERT is more attractive as a target than the hTER. Actually, the expression of the dominant-negative mutants of hTERT resulted in complete inhibition of telomerase activity, reduction in telomere length, and death of tumor cells (34, 35). This cell death was telomere-length dependent, inasmuch as cells with a long telomere were viable but exhibited telomere shortening at a rate similar to that of mortal cells (34, 35). The cell death in cells with a short telomere was...
induced 5–10 days after the induction of a dominant-negative hTERT mutant (35). In contrast, the present treatment with 2-5A-anti-hTERT induced massive apoptosis just 4 days after the treatment. What is the molecular mechanism by which treatment with 2-5A-anti-hTERT induces apoptosis in tumors within only 4 days? To answer the question, the following two observations, which are still under study, may be useful: (a) it took approximately 1 month to induce apoptosis in more than 50% of cells within 4 days after 2-5A-anti-hTERT treatment. This raises the possibility that 2-5A-anti-hTERT may directly or indirectly stimulate the apoptotic pathways regardless of telomere length in tumors; and (b) we have obtained evidence, using the caspase inhibition assays, that the caspase family is involved in the cytotoxic effect of 2-5A-anti-hTERT on malignant glioma cells4 or on prostate cancer cells (36). Caspases play a major role in the transduction of apoptotic signals and in the execution of apoptosis in mammalian cells (37–41). Therefore, caspases are thought to be a key factor in the function of 2-5A-anti-hTERT. Although additional studies are necessary to ascertain precisely the molecular mechanism underlying the effect of 2-5A-anti-hTERT, we will be able to conclude that its effect is attributable to the activation of the apoptosis pathway involving the caspase family irrespective of telomere length.

In summary, the treatment with 2-5A-anti-hTERT that was modified by the use of a cationic liposome effectively suppressed growth of cultured cells and s.c. tumors of malignant gliomas. Furthermore, treatment of intracranial malignant gliomas with 2-5A-anti-hTERT was therapeutically effective. This is the first report demonstrating the feasibility of antisense telomerase therapy for intracranial tumors. The present study strongly indicates that 2-5A-anti-hTERT is a promising experimental therapeutic agent for the treatment of malignant gliomas with telomerase activity.

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4467
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