Gene-directed Enzyme Prodrug Therapy for Osteosarcoma: Sensitization to CPT-11 *in Vitro* and *in Vivo* by Adenoviral Delivery of a Gene Encoding Secreted Carboxylesterase-2[1]

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Abstract

Despite improvement in the treatment of osteosarcoma (OS), there are still many patients who cannot benefit from current treatment modalities. This warrants exploration of new treatment options. To that end, we investigated gene-directed enzyme prodrug therapy (GDEPT) with the use of human liver carboxylesterase-2 (CE2) and the anticancer agent CPT-11. CPT-11 is a clinically approved prodrug that needs to be metabolized into the active drug SN-38 by CEs, which occurs rather inefficiently in humans. GDEPT aims at high production of CE2 at the tumor site, resulting in efficient local conversion of CPT-11 into SN-38. Here, we show that OS cells transduced with an adenoviral vector containing the cDNA encoding a secreted form of CE2 (Ad-sCE2) expressed and efficiently secreted CE2. *In vitro*, transduction of a panel of OS cell lines with Ad-sCE2 resulted in sensitization up to 2800-fold to CPT-11 treatment. Primary OS short-term cultures, derived from patients suffering from a classic high-grade OS, demonstrated increased CPT-11 sensitivity up to 70-fold after transduction with Ad-sCE2 *in vitro*. When mice bearing s.c. MG-63 OS xenografts were intratumorally injected with Ad-sCE2 and CPT-11, this resulted in a significant difference in time to reach 2000 mm^3 in tumor volume as compared with animals receiving Ad-sCE2 or CPT-11 treatment ($P < 0.05$). Taken together, these data suggest that OS cells are sensitive for the combination of Ad-sCE2 and CPT-11.

Introduction

OS[4] is the most common primary bone tumor in children and young adults (1). Despite recent improvement in the treatment of OS, there are still too many patients who cannot benefit from current treatment modalities (2). The overall survival of primary OS with combined treatment, consisting of neoadjuvant chemotherapy and surgery, now varies between 50 and 65% (3–5). Attempts to treat OS with multiple new agents have not increased survival rates. Therefore, new treatment options have to be explored for this type of disease.

Camptothecin derivatives have been explored in Phase I–II trials, including patients with OS refractory to standard treatment protocols. In these trials, partial responses have been observed (6, 7). Pratesi *et al.* (8) have shown complete tumor response and cures in 70% of animals bearing s.c. U2OS OS. The tumor variant selected for resistance against cisplatin was still responsive to camptothecin treatment (8).

CPT-11 is a semisynthetic, water-soluble derivative of camptothecin that differs from other camptothecin analogues in that it is a prodrug that undergoes de-esterification to the much more potent topoisomerase I inhibitor SN-38 (9). A class of enzymes that converts CPT-11 to SN-38 is the human CEs. CE activity can be detected in human liver, intestines, and other sites (10). Although patients do express CE, the amount of CPT-11 administered systemically that is converted to the active drug SN-38 is in the range of only 5–10% (11). Furthermore, the usefulness of CPT-11 is hampered by dose-dependent toxicity, primarily diarrhea. A strategy to increase the antitumor effects of CPT-11, while decreasing the unwanted side effects, is to express the cDNA encoding CE specifically at the site of the tumor. After sys-

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[4] The abbreviations used are: OS, osteosarcoma; Ad-sCE2, adenoviral vector containing the cDNA encoding secreted human CE isoform-2; CE, carboxylesterase; CMV, cytomegalovirus; CPT-11, irinotecan or 7-ethyl-10[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin; CRAd, conditionally replicating adenovirus; GDEPT, gene-directed enzyme prodrug therapy; GFP, green fluorescent protein; MOI, multiplicity of infection; pfu, plaque forming unit; sCE2, secreted CE2; SN-38, 7-ethyl-10-hydroxy-camptothecin; TGD, tumor growth delay.

temic administration of CPT-11, this will lead to tumor-specific conversion and therefore tumor-specific toxicity. This approach is called GDEPT. Another GDEPT approach previously described for OS in experimental models uses the viral enzyme thymidine kinase in combination with the prodrug acyclovir. The efficacy of this approach was shown *in vitro* (12), as well as *in vivo* (13, 14). In the latter case, 80% of gene therapy-treated animals bearing s.c. OS xenografts survived, whereas survival rates increased to 100% when gene therapy was combined with methotrexate. The advantage of using CE/CPT-11 GDEPT approaches is that human isoforms of CE can be used that should not result in an immunological response and subsequent enzyme inactivation. Therefore, repeated administration should be possible.

We have screened a panel of human malignant cell lines originating of different tissues to explore which cell lines could be sensitized to CPT-11 by addition of CE into the culture medium. All OS cell lines tested became highly sensitive for CPT-11 in the presence of extracellular CE (unpublished results). To explore the effect of CPT-11 on OS cells in a GDEPT approach, we constructed Ad-sCE2. A secreted form of CE2 might have the advantage that it will likely spread through a solid tumor mass, resulting in extracellular conversion of CPT-11. This may lead to antitumor effects to untransduced neighboring tumor cells, also designated as the bystander effect.

In this study, we describe the construction and characterization of the Ad-sCE2 adenoviral vector and its ability to sensitize OS cell lines and primary OS cells to CPT-11 *in vitro*. In addition, we report on the usefulness of Ad-sCE2 in human OS xenografts grown in nude mice and treated with CPT-11.

Materials and Methods

Cells and Culture Conditions. MG-63 (Ref. 15; courtesy of Dr. Clemens Löwik, Leiden University Medical Center, the Netherlands), MNNG-HOS (16), SaOs-2 (Ref. 17; courtesy of Dr. Frans van Valen, Westfalische Wilhelms-Universität, Münster, Germany), CAL-72 (Ref. 18; courtesy of Dr. Jeanine Gioanni, Laboratoire de Cancerologie, Faculté de Medicine, Nice, France), and HEK293 (American Type Culture Collection, Manassas, VA) cell lines were maintained in DMEM supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (all from Life Technologies, Inc., B.V., Breda, the Netherlands) at 37°C in a 5% CO_2 humidified atmosphere.

Patient Material. Fresh tumor material was obtained from patients having a classic high-grade OS and was brought into culture as described previously (19). From patient 6, cells were cultured before (OS-6) and after (OS-6a) chemotherapy. From patient 1, only material after chemotherapy was cultured (OS-1a). All experiments were performed in cell cultures at passage 0–5.

Construction of Ad-sCE2. The adenoviral vector Ad-sCE2 was constructed using the AdEasy method (20). The plasmid pSTCF-sCE2 containing the sCE2 open reading frame with a myc-6xHis tag at the COOH-terminal ending (21) was digested with *Pme*I and *Nhe*I, and the sCE2-comprising fragment was cloned into *Xba*I and *Eco*RV-digested pAdTrack-CMV. The resulting plasmid was digested with *Pme*I and recombined with adenoviral backbone plasmid pAdEasy-1 in *Escherichia coli* BJ5183 cells to construct pAdEasy-sCE2. After linearization with *Pac*I, the plasmid was transfected into the adenovirus-packaging cell line HEK293, and virus was further propagated in HEK293 cells according to standard techniques. Purified virus stocks were prepared by two successive bandings on CsCl gradients. Viral particle titer and pfu titer was determined by $OD_{260\ nm}$ and limiting dilution, respectively. The pfu titer was 1.5×10^{12}, and the viral particle/pfu ratio was 18.1. For all experiments, the pfu titer was used, and Ad-GFP was taken along as a negative control (22).

Western Blot. Equivalent amounts of supernatant or cellular lysate from SaOs-2 cells infected with Ad-sCE2 or Ad-GFP (MOI 100) were dissolved in sample buffer (23) with 5% 2-mercaptoethanol and boiled at 95°C for 5 min. Samples were subjected to electrophoresis through a denaturing 10% SDS-polyacrylamide gel, and protein bands were electroblotted onto polyvinylidene difluoride protein membrane (Bio-Rad, Veenendaal, the Netherlands). Proteins were detected using mouse anti-myc antibody 9E10 (24) and horseradish peroxidase-conjugated rabbit antimouse IgG (DakoCytomation, Heverlee, Belgium). Blots were developed with enhanced chemiluminescence (Lumilight Plus; Roche Diagnostics, Almere, the Netherlands).

Esterase Activity. Cellular lysates or supernatants of SaOs-2 cells infected with Ad-sCE2 or Ad-GFP were incubated with 200 μl of 100 mM TRIS-HCl (pH 8.0) containing 1 mM *p*-nitrophenyl-acetate (Sigma-Aldrich, Zwijndrecht, the Netherlands), a substrate for CE. Conversion to paranitrophenol was monitored over a 10-min period using an ELISA plate reader (Bio-Rad) at a wavelength of 415 nm at room temperature.

***In Vitro* Proliferation Assay.** OS cell lines and primary OS cell cultures were plated in a 96-well microtiter plate (Greiner Bio-One B.V., Alphen aan den Rijn, the Netherlands) at 5.10^3 cells/well in 100 μl. After 24 h, medium was removed and cells were infected with Ad-sCE2 at a MOI of 1, 10, or 100 in 50 μl in culture medium with 2.5% FCS. After 1 h, virus was removed, and 200 μl culture medium with or without CPT-11 (Aventis, Strasbourg, France) in a range of concentrations was added to separate wells in triplicate. Control experiments were performed in which the OS cells were infected with Ad-GFP and treated with CPT-11, or cells were only treated with CPT-11 or SN-38 (Aventis). After an incubation period of 6 days, growth was determined by incubating the cells with the cell proliferation reagent WST-1 (Roche Diagnostics) at 37°C. After 2 h, the absorbance was measured at a wavelength of 450 nm using an ELISA plate reader. The antiproliferative effects of treated cells were expressed as percentages of growth from uninfected, untreated control cells.

Crystal Violet Staining. In a separate assay, cytotoxicity in OS cell lines and primary OS cell cultures was measured by crystal violet staining. Cells were treated as described for the antiproliferative assay. At the end of the experiment, medium was aspirated from cell cultures, and adherent cells were fixed with 4% formaldehyde in PBS for a 10-min period

Table 1 Antiproliferative effects of CPT-11 and SN-38 and for CPT-11 after transduction with Ad-sCE2 or Ad-GFP in OS cells

Name	Cell type	CPT-11 (IC_{50} in μM)	SN-38 (IC_{50} in μM)	Ratio[a]	Ad-GFP + CPT-11 (IC_{50} in μM)	Ad-sCE2 + CPT-11 (IC_{50} in μM)	d.o.s.[b]
SaOs-2	Cell line	2	0.0025	800	2	0.0007	2857
MG-63	Cell line	3.5	0.003	1166	2.8	0.02	175
CAL-72	Cell line	2.5	0.004	625	4	0.03	83.3
MNNG-HOS	Cell line	4	0.004	1000	4	0.4	10[c]
OS-1A	Primary	3	0.1	30	5	0.4	7.5
OS-2	Primary	>100	3	>33.3	>100	9	>11.1
OS-6	Primary	>100	2	>50	>100	30	>3.3
OS-6A	Primary	>100	1.5	>66.6	>100	1.5	>66.6
OS-7	Primary	100	0.8	125	100	11	9
OS-8	Primary	20	0.2	100	50	0.3	66.6

[a] The ratio is expressed as IC_{50} for CPT-11 divided by the IC_{50} for SN-38.
[b] The degree of sensitization (d.o.s.) is expressed as IC_{50} for CPT-11 divided by the IC_{50} for CPT-11 after cellular transduction with Ad-sCE2.
[c] The transduction efficiency of the MNNG-HOS cell line was <5%, which could explain the moderate d.o.s.

at room temperature. After fixation, cells were washed and incubated for 15 min with 1% crystal violet dissolved in 70% ethanol. Hereafter, cells were washed with water, air-dried, and scanned on a Bio-Rad GS690 scanner.

***In Vivo* Experiments.** The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act (1977) and the published "Guidelines on the Protection of Experimental Animals" by the council of the European Community. The protocol was approved by the committee on Animal Research of the Vrije Universiteit.

Female athymic nu/nu mice, weighing 25–35 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were housed under pathogen-free conditions and were fed *ad libitum*. Tumor pieces (3 × 3 × 3 mm), derived from s.c. injected MG-63 cells in previous recipients, were implanted under the skin of one flank in anesthetized mice. Mice were weighed, and tumor size was monitored twice/week using digital calipers. The tumor volume was calculated from the average of tumor length and width according to the formula: $4/3\pi r^3$. The volume doubling time of untreated MG-63 tumors was 1.6 days.

To determine the highest dosage of CPT-11 that had no effect on tumor growth, mice were divided in four groups of three animals each when the tumor nodules reached 200–300 mm^3. Mice in each group were intratumorally injected with 0.143, 1.43, or 14.3 μg CPT-11 in 25 μl of PBS or PBS alone for 7 consecutive days. Tumors were measured for a period of 3 weeks.

In the next experiment, mice were divided into three groups when the tumor nodules reached 200–300 mm^3 (designated as day 0). Tumors were injected with 1.10^9 pfu of Ad-sCE2 in 25 μl of PBS followed by intratumoral injection with 1.43 μg CPT-11 in 25 μl of PBS for 7 consecutive days (nine animals). Tumors of control mice received PBS on day 1 followed by injection with 1.43 μg CPT-11 for 7 consecutive days (six mice) or 1.10^9 pfu of Ad-sCE2 on day 1 followed by injection with PBS for 7 days (six mice). The mice were euthanized when the tumors reached a size of ≥ 2000 mm^3. The different treatment modalities were evaluated by comparing TGD (time required to reach five times the initial tumor volume) and comparing the time for the tumors to reach a volume of ≥ 2000 mm^3, which was regarded as the end point of the Kaplan-Meier curves.

Statistical analyses were performed using SPSS (SPSS, Inc., Chicago, IL). The ANOVA test was used to compare the growth delay between the different treatment groups. The Kaplan-Meier curves were calculated and differences in time for the tumors to reach ≥ 2000 mm^3 between different treatment groups were analyzed using the log-rank test.

Results

Antiproliferative Effects of CPT-11 and SN-38. To assess the sensitivity of OS cells to CPT-11 and SN-38, IC_{50}s (the concentrations of prodrug or drug that results in 50% growth inhibition) were determined. OS cell lines and short-term cultures, derived from tumors of patients with classic high-grade OS, were subjected to a range of concentrations of CPT-11 or SN-38 for 6 days. The IC_{50}s for CPT-11 and SN-38 and the ratios of these values are shown in Table 1. The OS cell lines tested were 625-1200-fold more sensitive to SN-38 than to CPT-11. In three primary OS cultures, this varied between 30- and 125-fold. For the other three short-term primary cultures tested, the ratio was at least >33 times but could not be determined exactly because the highest concentration of CPT-11 used was not toxic to these cells. These results indicate that efficient activation of CPT-11 to SN-38 would result in considerable antiproliferative effects in OS cells.

Construction and Characterization of Ad-sCE2. A secreted form of human liver CE2 (sCE2) was obtained by deletion of the cellular retention signal HTEL and by cloning it downstream of an IgGκ leader sequence. At the COOH-terminus a myc-6xHis tag was cloned for easy detection and purification. The expression of sCE2 was driven by the CMV promoter (21). The adenovirus vector Ad-sCE2 was constructed by inserting the sCE2 expression cassette adjacent to a GFP expression cassette in place of the adenovirus E1 region (Fig. 1).

SaOs-2 cells were infected with either Ad-sCE2 or control virus Ad-GFP (MOI 100), and after 6 days, supernatant and cellular lysates were analyzed for sCE2 expression by Western blot. Fig. 2*A* shows that almost all sCE2 was detected in the supernatant of infected cells. Functional activity of sCE2 at day 6 after transduction was demonstrated by an esterase activity assay (Fig. 2*B*). Again, most of the CE activity was

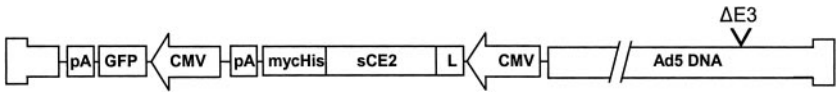

Fig. 1. Schematic presentation of the adenovirus Ad-sCE2 that was constructed by inserting the sCE2 expression cassette adjacent to a GFP expression cassette in place of the adenovirus E1 region. The sCE2 expression cassette includes the CMV promoter (CMV), an IgG κ leader sequence for secretion (L), a COOH-terminal myc- and 6xHis-tag for detection and purification (mycHis), and an SV40 polyadenylation signal (PA).

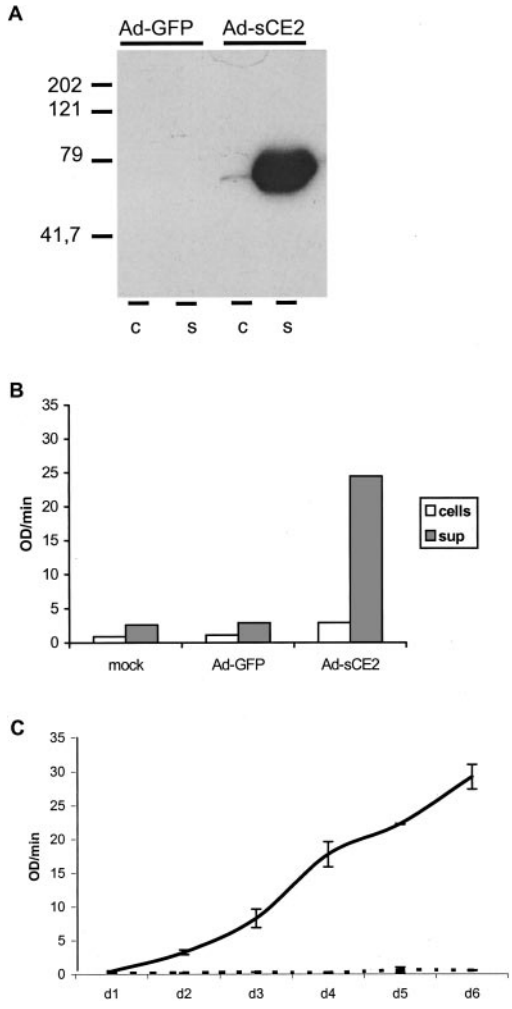

Fig. 2. Characterization of Ad-sCE2 infected SaOs-2 cells by Western blot and by esterase activity assay. *A*, Western blot of the cellular lysates and supernatants of SaOs-2 cells infected with Ad-sCE2 at MOI 100. As a negative control, cells were infected with Ad-GFP at MOI 100. sCE2 was detected using an antibody directed against the myc tag. In *Lanes 1* and *3*, cellular lysates (c), and in *Lanes 2* and *4*, supernatants (s), of SaOs-2 cells infected with Ad-GFP (*Lanes 1* and *2*) or Ad-sCE2 (*Lanes 3* and *4*) are shown. sCE2 migrated with an apparent molecular weight of M_r 75,000 and almost all protein was secreted. *B*, CE activity in cellular lysates and supernatants of SaOs-2 cells untransduced or transduced with Ad-sCE2 or Ad-GFP at MOI 100. Samples were incubated with 1 mM *p*-nitrophenyl acetate and conversion was measured over a 10-min period. Ad-GFP-infected cells did not show enhanced CE activity in cellular lysates and culture medium compared with mock-infected SaOS-2 cells, whereas Ad-sCE2-infected cells clearly expressed the CE enzyme, which was detected mainly in the supernatant. *C*, CE activity in supernatants of SaOs-2 cells transduced with Ad-GFP (*dotted line*) or Ad-sCE2 (*bold line*) at MOI 100 at different time points postinfection. In time, CE activity in supernatants of Ad-sCE2-transduced cells gradually increased.

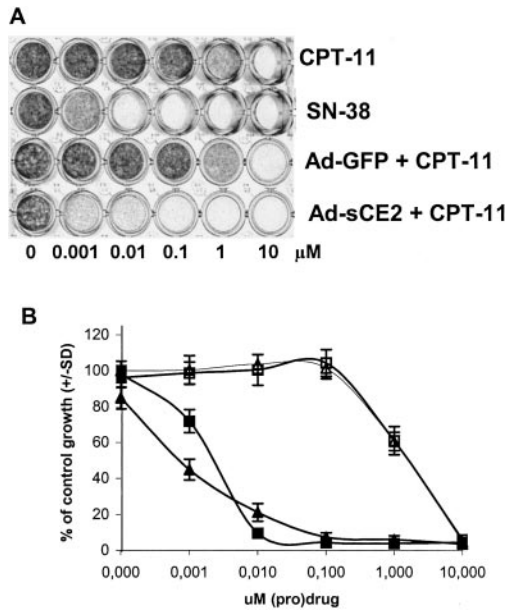

Fig. 3. Cytotoxicity assay on SaOs-2 cells infected with AdsCE2 or Ad-GFP. Cell survival was determined with crystal violet staining (*A*) or WST-1 proliferation assay (*B*). *A*, the SaOs-2 cells were transduced with Ad-GFP or Ad-sCE2 at MOI 100 as indicated and treated for 6 days with CPT-11 or SN-38 at the indicated concentration. Subsequently, adherent cells were stained with crystal violet and photographed. *B*, Ad-GFP-transduced SaOs-2 cells (□) did not show additional inhibition of growth when compared with CPT-11-treated untransduced cells (△). Transduction with Ad-sCE2 in combination with CPT-11 (▲) resulted in toxicity comparable with exposure to SN-38 (■). Data are presented as mean of triplicates ± SD.

detected in the supernatant of SaOs-2 cells infected with Ad-sCE2, confirming the results of the Western blot.

To follow sCE2 secretion in the supernatant of Ad-sCE2-transduced cells in time, SaOs-2 supernatants were harvested at days 1–6 after transduction and a CE activity assay was performed (Fig. 2*C*). A gradual increase of CE activity in time after transduction was observed.

Antiproliferative Effects of CPT-11 Activation in Ad-sCE2-transduced OS Cells. To show improved conversion of the prodrug CPT-11 to the toxic drug SN-38 by Ad-sCE2 encoded sCE2, OS cell lines or primary OS cells were transduced with Ad-sCE2 or Ad-GFP as a control, followed by exposure to CPT-11. Six days after transduction, surviving cells were visualized by crystal violet staining. Antiproliferative effects were quantified with the WST-1 assay. Ad-sCE2 exhibited a dose-dependent sensitization to CPT-11 (data not shown). Fig. 3 shows the results obtained for the OS cell

line SaOs-2 transduced at MOI 100. Ad-sCE2 enhanced the sensitivity of SaOs-2 cells to CPT-11 ~2800-fold, resulting in toxic effects comparable with treatment with SN-38 alone. Table 1 summarizes the results of the proliferation assay on all OS cell lines and primary OS cell cultures transduced at MOI 100. All OS cells tested could be sensitized to CPT-11 after infection by Ad-sCE2, ranging from 10- to 2800-fold for OS cell lines, whereas primary OS cells were up to two orders more sensitive. Interestingly, also primary cell cultures from patients resistant against cisplatin and doxorubicin treatment, *i.e.* OS-1a and OS-6a, were sensitized to CPT-11 by infection with Ad-sCE2. In general, in this experimental setting, where not all cells were transduced with Ad-sCE2, CPT-11 prodrug conversion was incomplete, yielding 7–70% cytotoxicity compared with SN-38 treatment.

***In Vivo* OS Tumor Growth Inhibition.** To investigate if OS tumors could be sensitized to CPT-11 after transduction with Ad-sCE2 *in vivo*, nude mice bearing well-established s.c. MG-63 tumors of 200–300 mm^3 were treated by intratumoral injection with 1.10^9 pfu Ad-sCE2 or PBS. Subsequently, tumors were injected on 7 consecutive days with 1.43 μg of CPT-11 because this was the highest concentration of CPT-11 that did not by itself result in tumor growth inhibition as compared with PBS treatment (data not shown) or with PBS. CPT-11 was injected intratumorally because mice, in contrast to humans, have high esterase activity in their plasma (25). Tumor growth was monitored until a volume of $\geq$ 2000 mm^3 was reached. Tumors treated with Ad-sCE2 and PBS showed a tumor growth rate (time required to reach five times the initial tumor volume $\pm$ SD) of 4.8 $\pm$ 1.5 days. For tumors treated with PBS and CPT-11, the tumor growth rate was 4.6 $\pm$ 1.3 days. A significant increase in TGD was observed for the combination treatment with Ad-sCE2 plus CPT-11 (7.1 $\pm$ 1.7 days) as compared with Ad-sCE2 alone ($P = 0.021$) and CPT-11 alone ($P = 0.011$; data not shown).

Fig. 4 shows in Kaplan-Meier curves the time of the tumors to reach a volume of $\geq$2000 mm^3. At a volume of 2000 mm^3, the animals had to be sacrificed according to animal welfare guidelines for these experiments. Curve comparison with log-rank analysis showed a significant delay in time to reach $\geq$2000 mm^3 tumor volume between GDEPT-treated animals compared with Ad-sCE2 or CPT-11 alone ($P < 0.05$).

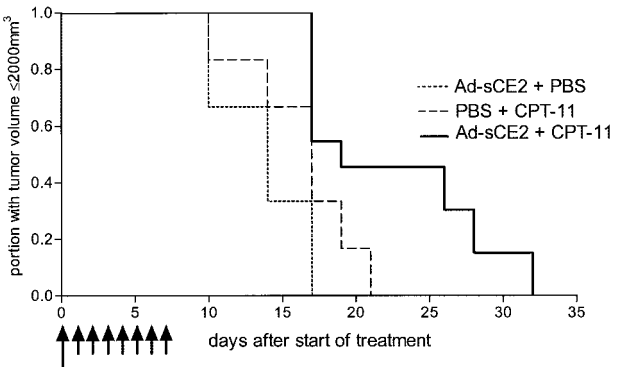

Fig. 4. Kaplan-Meier curves showing the time for the tumors to reach a volume of $\geq$2000 mm^3. Curve comparison with log-rank analysis showed a significant difference in time to reach $\geq$2000 mm^3 between GDEPT-treated animals compared with Ad-sCE2 or CPT-11 alone ($P < 0.05$).

Discussion

Despite improvement of the treatment of primary OS, the overall survival of patients varies between 50 and 65%. Therefore, new treatment modalities are warranted. In several studies using the enzyme-prodrug combination of herpes simplex virus thymidine kinase and ganciclovir, an antitumor response was observed in OS models. Both local treatment and systemic treatment of lung metastasis resulted in long-term survivors (12–14). In this study, we investigated the utility of an adenoviral vector containing the cDNA encoding a secreted form of human liver CE2, Ad-sCE2, to sensitize OS cells to the clinically approved prodrug CPT-11. OS cells transduced with Ad-sCE2 indeed secreted functional sCE2. Transduction of OS cells and primary cell cultures with Ad-sCE2 sensitized OS cells to CPT-11 *in vitro*.

Several isoforms exist from the human enzyme CE. Kojima *et al.* (26) have described the construction of an adenovirus containing the cDNA encoding intracellular human liver CE isoform 1. Only 3 of 11 cancer cell lines did show a marked (>5 fold) decrease in IC_{50} after transduction and CPT-11 treatment. Humerickhouse *et al.* (27) demonstrated that human CE2 has a higher affinity and a higher conversion velocity for CPT-11 than CE isoform 1. On the basis of the latter studies, we decided to use CE2 for our studies. Recently, Wierdl *et al.* (28) described the construction of an adenoviral vector containing the cDNA encoding a secreted form of rabbit liver CE, which is probably the most efficient isoform of CE (29). Because a nonhuman enzyme such as rabbit CE might elicit an immune response in humans and repeated administration of Ad-sCE2 is anticipated in clinical applications, we preferred to express a secreted form of a human CE in our study. Therefore, it was decided to use a secreted form of human liver CE2, and as we show herein, the adenovirus expressing human sCE2 was highly effective in GDEPT with CPT-11 for OS *in vitro*. We put particular emphasis on including primary short-term cultures. These primary OS cells were directly brought into culture after the tumor (piece) was surgically removed. Experiments were performed between passages 1 and 5 of these cultures. Therefore, these cells can be considered as reliable primary tumor cells. Although Coxsackie Adenovirus Receptor expression was very low on these primary cells (19), they could still be sensitized to CPT-11 by transduction with Ad-sCE2 at a relatively high MOI. Sensitization was not complete, which could be partly attributed to differences in transduction efficiency. Interestingly, heavily pretreated primary OS cells (OS-1a and OS-6a), which were resistant to doxorubicin and cisplatin, could still be sensitized to CPT-11 by Ad-sCE2 infection.

An *in vivo* experiment in which MG-63 tumors were infected with Ad-sCE2 and treated with CPT-11 showed a significant TGD and a significant difference in the time to reach a tumor volume of $\geq$2000 mm^3 compared with tumors treated with either CPT-11 or Ad-sCE2 alone. Although the results differed significantly between the experimental groups, the *in vivo* treatments did not result in cures. The modest effects *in vivo* could be explained by either low

transduction efficiency or a relatively low concentration of the prodrug.

The primary receptor for adenoviral entry Coxsackie Adenovirus Receptor is expressed in low amounts on the cell surface of the cell line MG-63 (19). During the *in vitro* experiments, only 25% of the MG-63 cells were transduced with Ad-sCE2 as determined by GFP expression. It is hypothesized that the MOI used to transduce MG-63 tumors *in vivo* was lower (1.10^9 pfu of Ad-sCE2/200 mm^3 tumor). So probably, only a very small percentage of tumor cells was indeed transduced by Ad-sCE2, and this might explain the poor therapeutic effect *in vivo*. To improve this, several possibilities can be explored. First of all, the efficacy of Ad-sCE2 and CPT-11 treatment *in vivo* can perhaps be improved by redirection of the adenoviral vector to tumor antigens. We previously demonstrated that targeting an adenoviral vector toward the epidermal growth factor receptor via a bispecific antibody improved gene transfer to OS cells *in vitro* (19). Another improvement of this approach could be the combination of enzyme prodrug therapy with CRAds. CRAds have shown promising preliminary results in clinical trials, especially in combination with chemotherapy. Preliminary experiments in which OS cells were transduced with Ad-sCE2 and a CRAd and treated with CPT-11 showed increased anticancer efficacy as compared with viral or enzyme prodrug therapy alone (unpublished results). Finally, we expect that a higher dose of CPT-11 could additionally enhance the efficacy of this approach. Unlike humans, however, mice have high esterase activity in their plasma, which precludes proper xenograft studies with higher doses of CPT-11 (30).

In clinical applications of GDEPT for human cancer, the adenoviral vector should be injected into the tumor lesion, whereas CPT-11 is given by the i.v. route. In this respect, it is questionable whether the tumor concentration of CPT-11 will be sufficient for enhanced SN-38-mediated tumor cell damage. This might perhaps be investigated in esterase-deficient mice (30). High intratumor concentrations of CPT-11 in humans may, however, also be reached in inoperable or recurrent OS injected with Ad-sCE2, after which, CPT-11 is given by intra-arterial infusion such as in isolated limb perfusion.

Altogether, our data suggest that the combination of Ad-sCE2 and CPT-11 could be further improved and developed into a new treatment modality for OS.

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