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Specific targeting of cytosine deaminase to solid tumors by engineered *Clostridium acetobutylicum*

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The presence of severe hypoxia and necrosis in solid tumors offers the potential to apply an anaerobic bacterial enzyme/prodrug approach in cancer treatment. In this context the apathogenic *C. acetobutylicum* was genetically engineered to express and secrete *E. coli* cytosine deaminase (CDase). Considerable levels of functional cytosine deaminase were detected in lysates and supernatants of recombinant *C acetobutylicum* cultures. After administration of the recombinant *Clostridium* to rhabdomyosarcoma bearing rats used as a model, cytosine deaminase could be detected at the tumor site. Moreover, following administration of the vascular targeting agent combretastatin A-4 phosphate significantly increased levels of cytosine deaminase were detected at the tumor site as a consequence of enlarged tumor necrosis and subsequently improved growth of *C. acetobutylicum*. The results provide evidence for the potential application of *Clostrisdium*-based therapeutic protein transfer to tumors in anticancer therapy. **Cancer Gene Therapy (2001) 8, 294–297**

Key words: Clostridium; gene transfer; cytosine deaminase; hypoxia.

Despite the improvement of conventional radiotherapy and chemotherapy, lengthy survival rates and prognosis remain poor for many cancer patients. This led to a strong interest in the investigation of anticancer protocols based on gene therapy. Herein, the use of suicide genes has been put forward to create artificial differences in sensitivity between normal and malignant cells, with specifically the cytosine deaminase (CDase)/5-FC suicide gene/prodrug system being well investigated. The major hurdle, however, remains the tumor-specific transfer of the gene encoding the therapeutic protein.

A fundamental difference between normal and tumor tissues is the presence of extensive hypoxia responsible for, e.g., resistance to radiotherapy and chemotherapy. Hypoxia can, however, also be appreciated as an opportunity for tumor-selective therapy.[1] Apathogenic clostridia have been proven to selectively proliferate in the hypoxic/necrotic regions of solid tumors, and the potential use of *Clostridium* as an antitumor agent has been indicated earlier in clinical studies. With the current availability of recombinant DNA technology for clostridia and the possibility to transform *Clostridium*, the use of these strictly anaerobic bacteria has attracted renewed interest. As such, recombinant apathogenic clostridia may be used as a selective vector system in an enzyme/prodrug approach for the treatment of cancer.

In the present report, we describe the development of recombinant *Clostridium acetobutylicum* strains secreting high amounts of CDase in the medium. The *Escherichia coli* CDase was therefore placed under transcriptional control of the *C. histolyticum* clostripain promoter, preceded by the clostripain signal sequence, and cloned in a stable *E. coli–Clostridium* shuttle vector. We also document the expression and secretion of functional enzyme at the tumor site, when recombinant clostridia were administered to rhabdomyosarcoma-bearing WAG/Rij rats. The use of vascular targeting treatment with Combretastatin A-4 phosphate in combination with the suicide gene transfer system enabled a dose intensification of CDase activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, DNA manipulations, and transformation procedures

C. acetobutylicum DSM792 and NI4082 were cultivated in 2× YT medium or RCM (supplemented with erythromycin when needed) at 37°C in an anaerobic cabinet. Transformation procedures and DNA manipulations were carried out essentially as described.[2] The *E. coli*/*C. acetobutylicum* shuttle plasmid pKNT19 was used as a cloning vector. The *E. coli* CDase gene (*cod*A) was present on the pSD112 plasmid, kindly provided by S. Danielsen (University of Copenhagen, Denmark). The clostripain regulatory sequence was available on Cl60-23, a gift from Weisheimer Malz (Andernach, Germany).

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Plasmid construction

Mutations were carried out using the "Altered Sites™ *in vitro* Mutagenesis kit" (Promega). An *Eco*RI site (oligonucleotide 5′-ATTATCCTTGGATTGAATTCCTGGATTTGCATA-3′) was introduced four codons upstream of the signal sequence cleavage site of the clostripain regulatory sequence. The resulting 1.04-kb *Hin*dIII/*Eco*RI fragment was cloned in pBluescript, leading to pBsclos*Eco*RI. A *Sca*I site was introduced two codons downstream of the GTG initiation codon of the *cod*A-cDNA with oligo 5′-GCGTTATTCGA-CACAGTACTCCTCCACATGC-3′. pBsclos*Eco*RI was digested with *Eco*RI, treated with Klenow polymerase, digested with *Bam*HI, and treated with alkaline phosphatase prior to *in-frame* fusion with the 1.5-kb *Sca*I/*Bam*HI fragment containing the codA-cDNA. Finally, the closcodA fragment was isolated by *Hin*dIII/*Bam*HI digestion and inserted into the *Bam*HI/*Hin*dIII–digested pKNT19 shuttle vector.

Detection and activity measurement of CDase in vitro

Detection of CDase was performed using Western blot analysis with mAb 16D8F2.[3] For quantification of CDase activity, bacteria were sampled at various stages of growth. To 45 μL of Tris–HCl (pH 7.5) buffered lysates and supernatants, 5-FC (15 mM) was added and the solution was incubated for 24 hours at 37°C. Five-microliter aliquots of the samples and 5-FC/5-FU controls were then applied to TLC plates (Silica gel 60 F_{254}, Merck) that were developed in 86% butanol in water. Sections of the plates that contained the 5-FU UV-absorbing spots (254 nm)

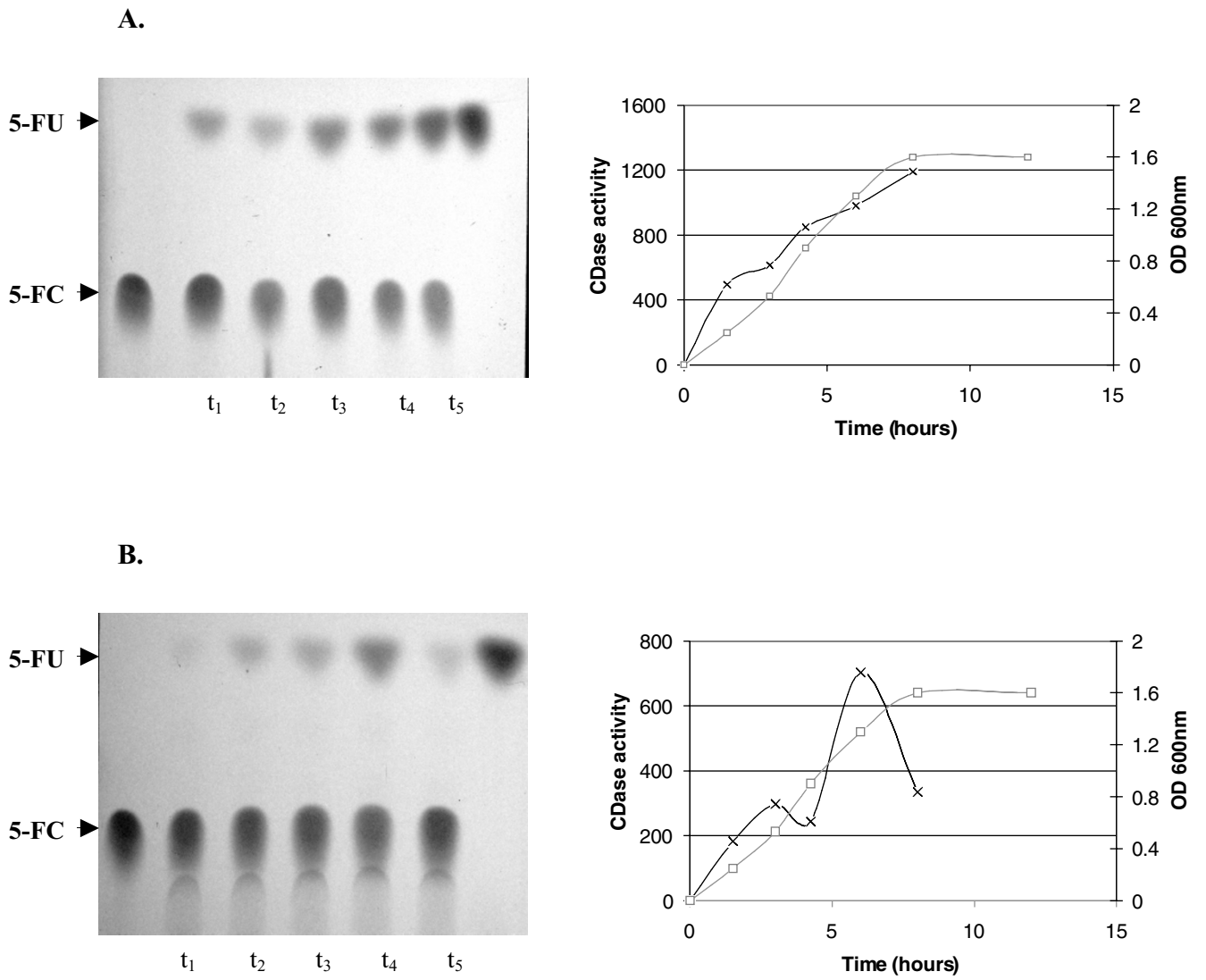

Figure 1. CDase activity in lysates **(A)** and supernatant **(B)** of *C. acetobutylicum* NI4082 transformed with pKNT19closcodA as a function of growth stage (□). Samples were taken at various time points during growth ($t_1 \rightarrow t_5$) and applied to thin layer chromatography plates (left panel). The amount of 5-FU on the corresponding TLC plates was quantified as described in *Materials and Methods* and the CDase activity (×) was calculated as picomoles 5-FU produced per minute per milliliter of cell culture (right panel).

were cut out and 5-FU was extracted with methanol. The extract was dried in a vacuum centrifuge (Hetovac) and the residue was resuspended in 50 μL of methanol. A 10-μL aliquot was then added to 190 μL HCl 0.1 M and the concentration of 5-FU was assayed spectrophotometrically at $OD_{266\ nm}$. This approach made quantitative monitoring of CDase activity possible without the need for radioactive substrates. Before incubation with 5-FC, the pH of the buffered reaction mixture was controlled and proven to be pH 7.2–7.4 for all samples taken at different stages during growth.

In vivo *studies*

WAG/Rij rats with subcutaneously implanted rhabdomyosarcomas of 6–20 cm^3 were used. Recombinant *C. acetobutylicum* DSM792 and NI4082 spores were administered intratumorally. Injection of *C. acetobutylicum* having no or empty vector was used as a control. Four to 5 days following injection, tumors and normal tissues were excised and grinded to look microscopically for the presence of *Clostridium* vegetative cells following Gram staining. CDase activity in tumors was measured following homogenisation of the tumor ($\sim$350 mg) in 450 μL of phosphate-buffered saline containing 1 μg/mL aprotinin, 30 μg/mL phenylmethylsulfonyl fluoride, and 5 mM EDTA. Following incubation for 24 hours at 37°C with 15 mM 5-FC, 50 μL aliquots were taken, quenched in methanol, and applied on Silica gel 60 F_{254} sheets that were developed in 86% butanol/water. After drying, 5-FU spots were visualized under short-wave UV illumination.

RESULTS AND DISCUSSION

In vitro *analysis of recombinant CDase activity in Clostridium*

The recombinant constructs in *E. coli* were quality-controlled by DNA sequence analysis and Western blotting. pKNT19closcodA was subsequently electroporated into *C. acetobutylicum* DSM792 and NI4082. After Western blotting of proteins present in supernatants and lysates of early logarithmic growth phase recombinant *Clostridium* cultures, mAb 16D8F2 strongly reacted with a protein of about 52 kDa, which corresponds to *E. coli* CDase. No CDase could be detected in supernatants or lysates of plasmid-free *C. acetobutylicum* cultures, or in cultures carrying the empty pKNT19 plasmid alone. The functionality of the clostripain regulatory sequences preceding the CDase coding sequence was herewith evidenced, not only for its expression but also for the secretion of this large protein by *Clostridium*. Our results parallel the observation that shuttle vectors based on the replicon of the *Bacillus* plasmid pIM13 showed structural and segregational stability. We previously demonstrated this with the *E. coli–C. acetobutylicum* pKNT19 and pIMP1 shuttle vectors developed for the secretion of the antitumor and radiosensitizing agent tumor necrosis factor-α. Significant amounts of biologically active tumor necrosis factor-α were measured in both lysates and supernatants of recombinant clostridia.[2]

Because of the potential importance of CDase and 5-FC/5-FU conversion in antitumor therapy, we focused on the expression and secretion of CDase by *Clostridium* at various stages of growth. Maximum enzyme activity in lysates (1084.5±189.5 pmol 5-FC converted to 5-FU/min/mL cell lysate) was obtained as the bacteria reached the stationary growth phase ($OD_{600\ nm}\sim$1.5) (Fig 1A). This activity level was maintained within the follow-up period of 20 hours (data not shown). In supernatants of recombinant *C. acetobutylicum* NI4082, the CDase activity reached a maximum (701.9±104.3 pmol 5-FC converted into 5-FU/min/mL supernatants) at the early logarithmic growth phase at $OD_{600\ nm}\sim$1.2 (Fig 1B). Subsequently, CDase activity decreased. This might be a consequence of the acidification of the culture medium resulting in denaturation of CDase. Experiments with the recombinant *C. acetobutylicum* DSM792 strain resulted in similar CDase activity patterns in both lysates and supernatants (data not shown). When high levels of active CDase could be delivered at the tumor site, this would result in significant amounts of 5-FU in the tumor, which could

Table 1. Overview of the *In Vivo* Experiments with Clostridia Recombinant for CDase

Strain	Tissue	CombreAp*	Presence† of CDase	Incidence (%)	CDase level‡
NI4082/pKNT19closcodA	tumor	–	yes	10/18 (55)	+
	liver	–	no	0/5 (0)	NA§
	spleen	–	no	0/5 (0)	NA
NI4082/pKNT19	tumor	–	no	0/6(0)	NA
DSM792/pKNT19closcodA	tumor	+	yes	9/9 (100)	++
	liver	+	no	0/5 (0)	NA
	spleen	+	no	0/5 (0)	NA
DSM792/pKNT19closcodA	tumor	–	yes	4/6 (66)	+
	liver	–	no	0/3(0)	NA
	spleen	–	no	0/3(0)	NA
DSM792/pKNT19	tumor	+	no	0/4 (0)	NA
	tumor	–	no	0/6 (0)	NA

*Single intraperitoneal administration, 25 mg/kg.
†Presence of CDase was evaluated using TLC analysis, measuring conversion of 5-FC to 5-FU.
‡(+) Low; (++) moderate–high.
§NA: not applicable.

lead to tumor control by 5-FU without systemic toxicity. Interestingly, it has been reported that 5-FU at the tumor site does not return rapidly into the systemic circulation, offering the opportunity for the therapeutic agent to exert locally its antineoplastic effects.

Detection of functional CDase in vivo

The results of the *in vivo* observations are summarized in Table 1. Gram staining revealed the presence of vegetative *Clostridium* cells in tumor specimen, but not in samples of liver or spleen. CDase activity was present only in tumors injected with recombinant bacteria. As expected, no functional enzyme was present in tumors injected with plasmid-free bacteria, in tumors treated with bacteria carrying the empty plasmid, or in the normal tissues (liver, spleen) that were investigated. Animals, concomitantly treated with CombreAp, showed higher incidence of CDase-positive tumors (100% *vs.* 55%). Moreover, the level of active CDase in these tumor specimens was considerably higher (mean conversion efficiency of 5-FC to 5-FU ~11%) as compared to tumors not treated with CombreAp (mean conversion efficiency of 5-FC to 5-FU ~3%). We already showed that targeting the tumor vasculature in rhabdomyosarcoma-bearing WAG/Rij rats using CombreAp resulted in extensive necrosis, observed already at 4 days postinjection.[4] The present results with recombinant clostridia confirmed the hypothesis that combining the administration of clostridia with CombreAp treatment should result in an increased therapeutic dose intensity. This should obviously also be expected in combinations with other strategies that induce tumor necrosis, such as radiotherapy or chemotherapy. The engineered *Clostridium* strains can thus be considered as *in situ* "cell factories" that produce and secrete antitumor therapeutics specifically at the tumor site, restricting activity to the tumor being targeted. As a consequence, this strategy holds promise for circumventing the limited therapeutic window of 5-FU treatment.

To show that the conversion of 5-FC to 5-FU was a consequence of the CDase present in the tumor and was not due to the metabolic activity of the *Clostridium* during the incubation, metronidazole (50 μg/mL) was added to rhabdomyosarcoma tumor homogenates prior to the incubation to kill all bacteria. No difference in the 5-FC/5-FU conversion pattern was observed whether or not metronidazole was added. Moreover, CDase activity was also detected when only supernatants of tumor homogenates were incubated with 5-FC. Conversion efficiencies of 5-FC to 5-FU after incubation with supernatants were similar as obtained after incubation with the whole tumor homogenates. Taken together, this clearly indicates that the CDase activity, recovered from the intratumoral tissue, originates from active enzyme that is secreted by metabolically active recombinant clostridia during the *in vivo* colonization of the tumor.

Separated from its antineoplastic effects, 5-FU also sensitizes tumor cells to irradiation, and significant improvements in tumor response can therefore be anticipated when the approach described in the present manuscript would be combined with radiotherapy. We recently showed that sufficient 5-FU should be available if the conversion efficiency from 5-FC is 1–3%, enabling sensitization enhancement ratios of 1.1–1.2 with daily 2 Gy fractionated radiotherapy.[5] Based on the obtained conversion efficiencies both *in vitro* and *in vivo* using the plasmids discussed above, it is reasonable to expect that clinically significant radiosensitizing effects can be achieved using the recombinant clostridia.

Besides its exquisite tumor selectivity, the application of the clostridial delivery vehicle is very safe. It elicits only a minor host immune response and the clostridial cells can be removed from the body at any time by the administration of metronidazole.

Overall, the present *in vivo*/*in vitro* investigations provide strong evidence for the potential use of apathogenic clostridia genetically engineered to express CDase in anticancer therapy.

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