Prolonged Stability and Sustained Prodrug Cell Killing Activity Using Receptor-mediated Delivery of Malarial Circumsporozoite-Cytosine Deaminase Fusion Protein into Liver Cancer Cells[1]

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Abstract

An effective strategy of delivering recombinant DNA or protein by nonviral vectors faces two major challenges: (*a*) the selective delivery to the specific target tissue; and (*b*) a long-term expression of the protein once inside the cells. The present study describes a receptor-mediated delivery strategy using recombinant fusion protein consisting of malaria circumsporozoite (CS) protein as a ligand and bacterial cytosine deaminase (CD), which catalyzes the production of 5-fluorouracil from its prodrug 5-fluorocytosine. We demonstrate that the CD-CS fusion protein can be internalized in a receptor-mediated manner, providing a target delivery. The internalized CD-CS is capable of synthesizing 5-fluorouracil from the exogenously added 5-fluorocytosine and elicits cell killing with bystander activities. Most importantly, the internalized recombinant protein is stable and remains functional for at least several days, probably because of the entrapment of the fusion protein in particular cytoplasmic compartments that are free from cytoplasmic degradation machinery. Thus, it is possible to use a simple recombinant fusion strategy to enhance intracellular protein stability for manufacturing biological active product in a cell type-specific manner. The application of this strategy in the treatment of liver cancers and liver metastasis of colorectal cancers is discussed.

Introduction

There are two major obstacles to the development of nonviral systems for recombinant DNA delivery into mammalian cells: the cell-type specificity and stability of the delivered recombinant products. DNA delivered by liposomes or other nonviral vectors can elicit very little cell-type specificity and usually supports transient expression of the recombinant DNA. A receptor-mediated gene/protein (1, 2) delivery approach takes advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, in principle, the delivery can be highly specific and avoid unwanted side effects to many nontargeted cells. Many ligands have been investigated for delivering recombinant DNA to target different cell types. However, one drawback of this strategy is that the recombinant DNA thus delivered is encapsulated in particular cellular compartments, most likely in the endosomal-lysosomal compartments. The entrapped recombinant DNA requires endosomal lysing agents, *e.g.,* adenovirus, for release into the cytoplasm en route to the nucleus for expression (3–5). Similar situations may be applicable to the delivery of recombinant proteins. Nonetheless, we envision that the entrapped DNA or protein would become stabilized by virtue of its sequestration away from cellular degradation machinery that normally operates in the cytoplasm. If so, one may take advantage of this prolonged stability of the internalized recombinant protein to manufacture biologically active compounds *in situ*. This principle, if proven, should be particularly useful for the delivery of recombinant protein within the context of prodrug strategy.

To prove this principle, we used a protein ligand derived from malarial sporozoite called CS[3] protein. Malaria is transmitted by the bite of infected female anopheles. Minutes after infection, the malarial sporozoites invade hepatocytes. This liver-specific invasion is mainly mediated by the CS protein, which densely coats the outer surface of sporozoites. Receptor for the CS protein is distributed predominantly at the basolateral domain of hepatocytes (6, 7). We demonstrated previously that CS protein could be used as a delivery vehicle to introduce recombinant DNA into hepatocytes in cultures with the aid of adenovirus as an endosomal lysing agent (8). In this communication, we prepared a recombinant protein by fusing the bacterial CD to the NH_2 terminus of CS protein. The CD enzyme catalyzes the conversion of nontoxic prodrug 5-FC to the toxic metabolite 5-FU. We demonstrate that

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[3] The abbreviations used are: CS, circumsporozoite; CD, cytosine deaminase; CD-CS, circumsporozoite-cytosine deaminase fusion protein; 5-FU, 5-fluorouracil; 5-FC, 5-fluorocytosine; HCC, hepatocellular carcinoma; MCC, liver metastases of colorectal cancer; IPTG, isopropyl thio-β-D-galactoside; RI, region I; RII+, region II+; ASOR, asialoorosomucoid.

the CD-CS fusion exhibits cell-type specificity similar to that of CS. More important, the CD enzyme remains stable in the cells and elicits sustained prodrug cell killing for at least several days. Because 5-FU is an antitumor agent commonly used for the treatment of HCC and MCC, we consider that this prodrug strategy may be potentially applicable for the treatment of these liver diseases.

Materials and Methods

Chemicals and Radiochemicals. 5-FC, 5-FC-6-[^{3}H] (4.1 Ci/mmol), and 5-FU were purchased from Sigma Chemical Company (St. Louis, MO). Cytosine-6-[^{3}H] (14.7 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Brea, CA). pQE-60 vector and Ni-NTA agarose were from Qiagen (Chatsworth, CA). [^{35}S]methionine and NCS II solubilizer were from Amersham Corporation (Arlington Heights, IL). Peptides E35 (EWSPCSVTCGNGIZVRIKPGSAN) and A128 (GNEIEPGNNAYGSQSDTDASELT) were synthesized by a Vega Coupler 250 C synthesizer and purified by high performance chromatography as described previously (8).

Construction of Recombinant DNA. pCS27I C6xHis encodes a CS protein with deletion of internal repeats but retains the receptor-binding domain (Region II+) and six histidine residues. This plasmid also contains an IPTG-regulatable promoter element followed by a ribosomal binding side in front of the CS gene and a transcriptional terminal signal from chloramphenicol acetyltransferase gene behind the translational termination codon. CD gene was synthesized by PCR using oligo (5′ AGT*GGATCC*ACGTTTGTAATCGASTGGC, underscored nucleotides contain *Bam*HI site) and oligo (5′ ACA*GGATCC*AATAACGCTTTACAAACA), and plasmid template of pSD112 (a gift of Dr. Jan Neuhard, University of Copenhagen, Copenhagen, Denmark), which contains the *Escherichia coli* CD gene (9). The PCR product was purified and digested by restriction enzyme *Bam*HI, and ligated into plasmid pCS27I C-6xHis at the *Bam*HI site, which is located at the 5′ end of the CS gene. The resulting recombinant, designated pCD5–73.15.5, encoded CD-CS fusion protein with the full-length CD inserted in frame after the third amino acid residue of the CS protein. Similarly, *Bam*HI-digested PCR product was cloned into the *Bam*HI site of a pQE-60 vector (Qiagen), and the resulting recombinant was designated as pCDP6, which also contains six histidine residues at the COOH terminus. All of the recombinant plasmids were verified by DNA sequencing. The plasmid DNA was transformed into *E. coli* SG13009(pREP4) hosts (2).

Expression and Purification of Recombinant CD-CS, CD, and CS Proteins. Bacterial cells carrying various recombinant plasmids were grown in 1 liter of 2× YT broth containing 16 g bactotryptone, 10 g yeast extract, 5 g NaCl, 100 mg ampicillin, and 25 mg kanamycin for 2–3 h. When the cultures reached an A600 of 0.6, 2 mM of IPTG was added to the medium. After induction (3 h), the cells were harvested by centrifugation and resuspended in 10 ml of buffer MCAC-0 [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 10% glycerol, freshly prepared 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin]. Cells were sonicated, and the resulting cell lysates were centrifuged at 12,000 rpm in the HB4 rotor of a Sorval high-speed RC5B centrifuge for 35 min. Clear lysate was mixed gently with 4 ml of Ni-NTA agarose in ice for 1 h. After washing the agarose with buffer MCAC-20 (MCAC-0 containing 20 mM imidazole), the recombinant protein was eluted with buffer MCAC-200 (MCAC-0 plus 200 mM imidazole). Fractions containing CD-CS, CD, or CS were analyzed by 10% PAGE, pooled, and dialyzed extensively in PBS. Proteins were concentrated and frozen in aliquots at −70°C until used. Typical yields (per liter) were 1–3 mg, and 4–5 mg of CD-CS and CD proteins, respectively.

[^{35}S]CD-CS-labeled protein was prepared by the method described by Giovane *et al.* (10). In brief, bacterial cultures harboring recombinant plasmids encoding CD-CS were growth in 2× YT medium until A600 of 0.6. Cells were pelleted and recultured in 100 ml of MEM (Life Technologies, Inc. Bethesda, MD) supplemented with 1 mM glutamine, 25 mM HEPES (pH 7.5), 1.5 mM IPTG, and 0.1 ml of L-[^{35}S]methionine (1000 Ci/mmol, 10 mCi/ml). After culturing cells at 37°C for an additional 2.5 h, cells were harvested, and the recombinant proteins were purified according to the procedure as described above.

CD Enzymatic Activity. Crude cell extracts were prepared by sonicating cells in a buffer [50 mM sodium phosphate, (pH 7.8) and 0.3 M NaCl] followed by centrifugation. CD activity was determined by measuring the production of uracil in a reaction mixture (50 μl) containing 5 mM 6-[^{3}H]cytosine (0.45 Ci/mol), 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 1 mM EDTA, and cellular crude extract (10–20 μg/ml) or eluted fractions. After 1–2 h of incubation at 37°C, 10 μl aliquots were withdrawn and analyzed by thin layer chromatography (Silica Gel 60; Selecto Scientific, Norcross, GA). Chromatograms were developed in 1-butanol/water (86/14, v/v). Positions of cytosine and uracil were identified by UV light. The corresponding spots for cytosine and uracil were cut out, and radioactivities were determined in a scintillation counter. In some cases, 6-[^{3}H]5-FC (4.0 Ci/mmol) was used as a substrate in the assay.

Cytotoxicity Assay. Human hepatoma (HepG2), rat hepatoma (H4IIE), and mouse colorectal carcinoma MCA-26 cells were grown in DMEM supplemented with 10% fetal calf serum (Life Technologies, Inc.) containing penicillin (100 units/ml) and streptomycin (100 μg/ml). HL-60 cells were maintained in RPMI 1640 containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). All of the cultures were maintained in a 37°C incubator maintained with 5% CO_2 in air. Cells were plated in Costar 24-well culture plate at a density of 1–2 × 10^5 cells/well. Cells were treated with different amounts of purified recombinant proteins for 4 h. The medium was then removed, and cells were washed and replaced with fresh DMEM containing 1 mM 5-FC. After 3–4 days of incubation, cells were gently detached by trypsin. Viable cells were determined by trypan blue exclusion staining and counted using hemacytometer.

Uptake of [^{35}S]CD-CS Recombinant Protein. Cultured cells were incubated with 30 μl of [^{35}S]CD-CS (5 × 10^4 cpm/μg). At different time intervals, cells and culture medium were collected. Aliquots of collected cells and medium were resuspended in 0.1 ml of NCS II solubilizer (Amersham Corporation) and incubated at 50°C in a water bath overnight.

Radioactivities were determined by a scintillation counter. Protein contents were measured by the Bio-Rad protein assay kit.

Results

Preparation of Recombinant CD-CS, CD, and CS Proteins. The amino acid sequence of CS protein can be divided into three domains: (from the NH_2-terminal) RI, repeat and RII+ (2). The function of RI is not clear. The repeat domain, which consists of three copies of NANPNVDP and 21 copies of NANP, is immunodominant and confers the major antigenicity of CS protein (7, 11). RII+ contains an evolutionarily conserved stretch of 23 amino acid residues that interact with receptors (6). We prepared recombinant plasmid DNA encoding CD-CS fusion protein using the repeat-deleted CS version (7). The repeat-deleted CS protein and CD were similarly prepared and used in parallel in the CD-CS experiments (Fig. 1*B*).

The expression of these proteins was under the control of the *lac* repressor. On induction by IPTG, these bacterial hosts produced recombinant proteins consisting of 10–30% of total cellular proteins (data not shown). These recombinant proteins contained six histidine tags at their respective COOH termini. Therefore, purification of these proteins could be readily achieved by affinity column chromatography through a Ni-NTA column. A one-step fractionation by passing the crude extracts from the induced cultures through the column resulted in 120–130-fold purification (data not shown). The purified proteins were then analyzed by SDS-PAGE. Single bands corresponding to the molecular mass of 45 kDa and 34 kDa were observed for the CD and CS preparations, respectively (Fig. 1*A*). A major band corresponding to molecular mass of 87 kDa was found in the purified CD-CS sample. This molecular mass is consistent with that for the fusion between CD and CS. CD-CS preparations also contained a minor component with molecular mass of 65 kDa. The identity of this minor band remains unknown; it may represent a degradation product of CD-CS. The specific activity of the CD-CS fusion protein, as measured by the conversion of [^{3}H]cytosine to [^{3}H]uracil, was 2493 nmol/min/mg protein, ~75% that of the CD protein (3308 nmol/min/mg). This reduction in specific activity was presumably because of the fusion. These results indicated that the recombinant fusion protein retained the enzymatic activity of CD.

Cell Type-specific Killing Activities of CD-CS in the Presence of 5-FC. To determine whether CD-CS could be internalized by hepatoma cells, we treated HepG2 cells with ^{35}S-labeled CD-CS protein. At different time intervals, cells were extensively washed with regular medium. Uptake of the label was determined by scintillation counting. The kinetics of protein uptake reached a plateau ~4 h after the treatment (Fig. 2). In a parallel experiment, the treated cells were additionally washed with a solution containing 0.25 M acetic acid. The kinetics of CD-CS uptake in the mild acid-washed cells followed the kinetics similar to that of regular washed conditions, suggesting that the CD-CS proteins were internalized, because these mild acid-washing conditions should have removed cell surface-associated ligand (12). The uptake of recombinant protein can be competitively inhibited by a peptide (E35) containing the 23 amino acid sequence in the RII+ region that recognizes CS receptor (Fig. 2, *triangles*) but not the unrelated sequence (A128; not shown). These results demonstrated that CS-CS could be internalized by hepatoma cells by a receptor-mediated mechanism. Similar results were obtained with mouse colorectal metastatic cells (MCA26).

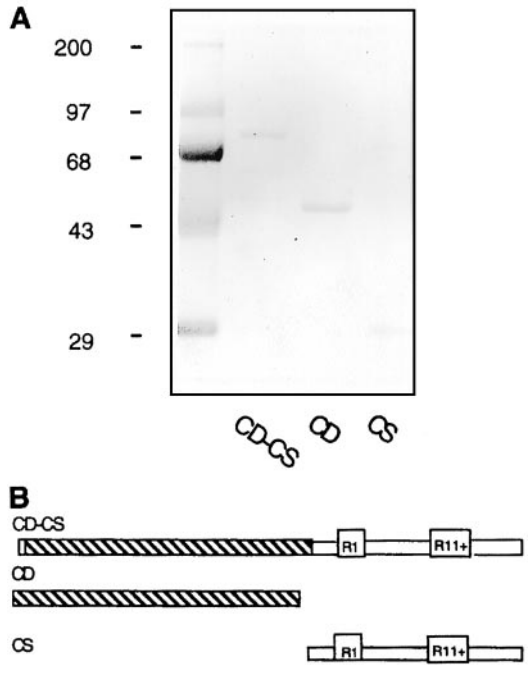

Fig. 1. A, SDS-PAGE analyses of recombinant CD-CS, CD, and CS proteins purified from bacterial hosts. *B,* schematic diagrams showing the structures of these proteins. RI and RII+ refer to different domains as described in the text.

To determine whether the CD-CS protein could exert cell type-specific killing activities in the presence of exogenously added 5-FC, we performed the following experiments using a pair of cell lines, *i.e.,* HepG2, which contains receptors for the CS protein, and HL60, which does not (8, 13). First, HepG2 and HL60 cells were treated with recombinant proteins CD-CS, CS, and CD for 4 h. Each set of treated cells was divided into two parts: one part was extensively washed with the regular medium to remove the recombinant proteins; the other part was not. Cells in both parts were then treated with 5-FC. As expected, unwashed HepG2 cells treated with CD-CS or CD alone were sensitive to the subsequent 5-FC treatments because of the manufacture of cytotoxic 5-FU from 5-FC by the CD activities in the medium; the CS-treated HepG2 cells were not sensitive to 5-FC (Fig. 3*A, hatched bars*). In the washed cultures, only CD-CS-pretreated cells were sensitive to the treatment of 5-FC (Fig. 3*A, solid bars*). These results suggested that CD-CS could be taken up by HepG2 cells and exerted cell killing activity, whereas CD could not. However, in similar experiments with HL-60 cells (Fig. 3*B*), removal of CD-CS or CD from the treated cells failed to induce cell death on subsequent addition of 5-FC.

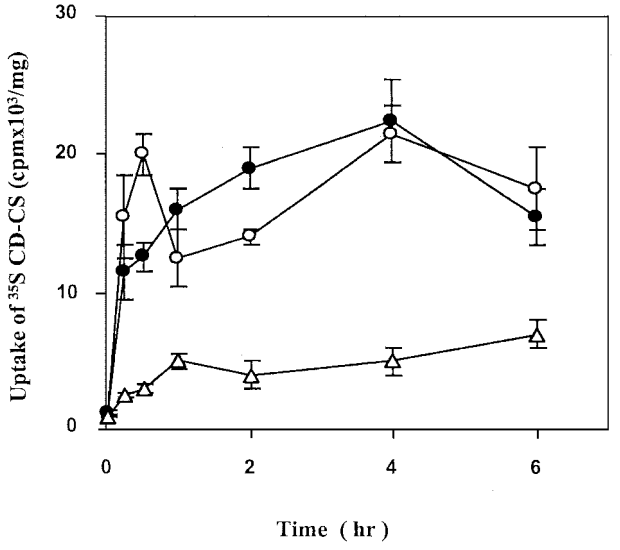

Fig. 2. Uptake of CD-CS in HepG2 cells. Purified [^{35}S]CD-CS protein (0.2 μM; 5 $\times$ 10^4 cpm/μg) was incubated with 1 $\times$ 10^5 HepG2 cells. At the different time intervals indicated, cells were harvested and lysed with tissue solubilizer. The radioactivity was measured by a liquid scintillation counter. ● and ○ represent cells harvested without acid wash and with acid wash, respectively. △ is from a control in which different amounts of 1 μM E35 peptide, which contains RII+ amino acid sequence, was added.

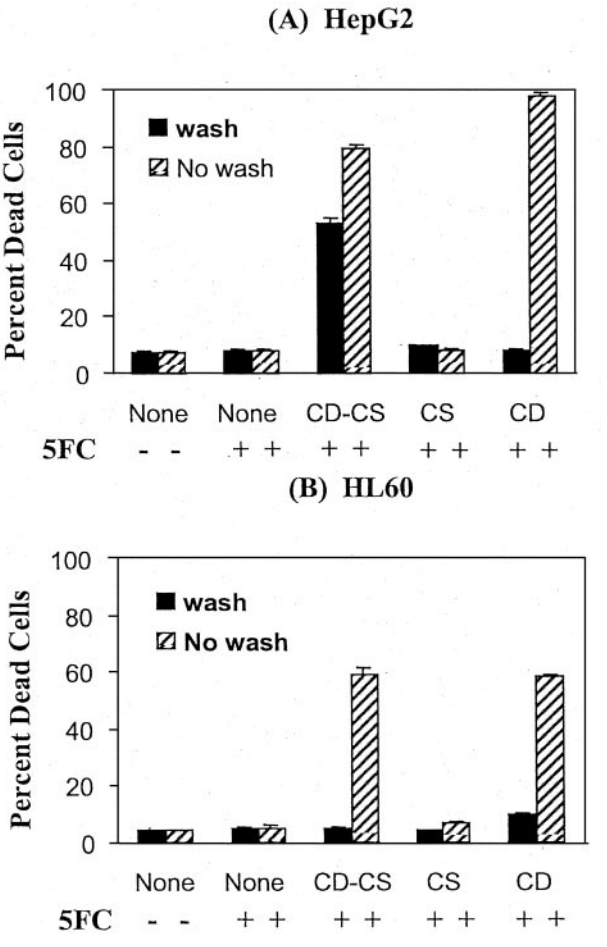

Fig. 3. Effects of CD-CS, CS, and CD on cell viability of HepG2 cells (*A*) and HL60 cells (*B*) in the presence of 5-FC. Cells (1 $\times$ 10^5) were incubated with 0.64 μM each of CS, CD-CS, or CD for 4 h. Each culture was then divided into two sets: one set was washed extensively with regular medium and grown in regular medium containing 1 mM 5-FC. The other set of cells was not washed and maintained in the same medium containing 1 mM 5-FC. Cell viability was counted 4 days thereafter; *bars,* ± SD.

The failure of cell killing was not because of intrinsic resistance of HL-60 cells to 5-FU, because cell death was observed in the unwashed control. These results indicate that both CD-CS and CD could not be taken up by HL-60 cells, consistent with the finding that these cells lacks a CS receptor. CD-CS-mediated cell killing was concentration-dependent with an LD_{50} for HepG2, rat hepatoma H-4-II-E, and MCA26 cells of ~0.22 μM (data not shown).

To demonstrate that the observed cell killing was related to the production of 5-FU from 5-FC, HepG2 and HL60 cells were treated with CD-CS protein for 4 h. Cells were then washed and incubated in regular medium supplemented with [^{3}H]5-FC. Later (2 d), cell extracts were prepared. The amounts of [^{3}H]5-FC and [^{3}H]5-FU in the cell extracts were measured using thin-layer chromatography. Likewise, the amounts of these radioactively labels in the medium was measured. As shown in Fig. 4, >30% [^{3}H]5-FU conversion were found in the cultured medium and in cell extracts derived from HepG2 cells, whereas only <5% conversion was seen in HL60 cells. The percentages of conversion indicated here were normalized by cell numbers. It is not likely that the substantial amounts of [^{3}H]5-FU found in the medium were contributed significantly by cell lyses, because no apparent cell death was observed under these conditions. These results indicate that the internalized CD-CS in HepG2 cells was functional, *i.e.,* capable of synthesis 5-FU from 5-FC. More important, the synthesized 5-FU could leak into the medium, which could exert bystander effects of cell killing (14, 15).

To demonstrate the bystander effect, we treated HepG2 cell with CD-CS for 4 h. Cells were harvested and extensively washed with the regular medium. The pretreated cells were mixed in different ratios with freshly prepared HepG2 or HL60 cells. The populations were cultured in fresh DMEM containing 5-FC for 3 days. In parallel, CD-CS-untreated cells were similarly mixed with fresh cells as controls. As shown in Fig. 5, mixed populations containing 0, 10, 50, and 100% CD-CS-treated HepG2 cells resulted in 8, 50, 85, and 100%, respectively, of total HepG2 cell death (Fig. 5, *solid bars*) whereas the controls showed <10% total cell death in all of the cases (Fig. 5, *hatched bars*). Similar results were obtained using HL60 cells for mixing (data not shown). Together, these results demonstrated the bystander effect of the CD-CS/5-FC strategy.

Stability of Internalized CD-CS in Cultured Cells. Two experiments were carried out to determine the stability of the internalized CD-CS. First, confluent HepG2 cells were treated with [^{35}S]CD-CS or with [^{35}S]CS for 4 h. Cells were washed to remove the radioactive labels and replenished with fresh medium. At different time intervals from 2 h to 7 days, cell extracts were prepared, and total proteins were

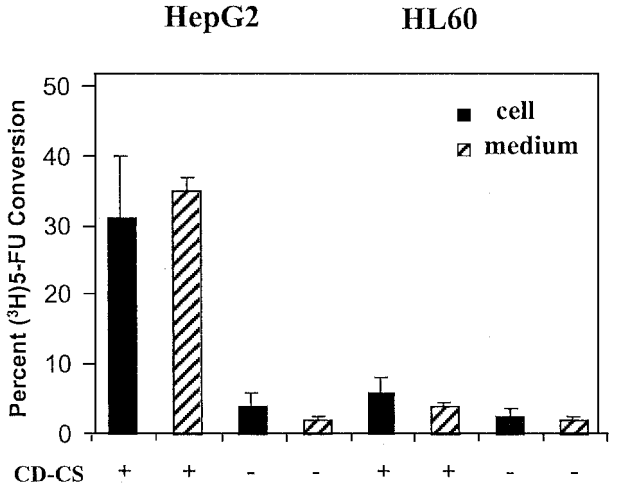

Fig. 4. Conversion of 5-FU in HepG2 or HL-60 cells after treatment with CD-CS and [^{3}H]5-FC. HepG2 or HL-60 cells were treated with 0.64 μM of CD-CS for 4 h. Cells were washed extensively and then cultured in regular medium containing [^{3}H]5-FC. Two days later, cell extracts were prepared, and medium was collected. [^{3}H]5-FC and [^{3}H]5-FU were separated by thin-layer chromatography. Data were expressed as percentage of uracil conversion from three experiments; *bars,* ±SD.

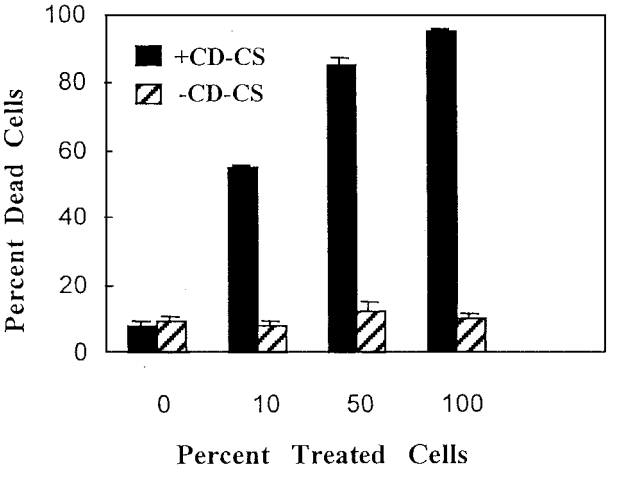

Fig. 5. Bystander effect of CD-CS/5-FC treatment. Different percentages of CD-CS-treated HepG2 cells (as indicated) were mixed with the untreated cells. Cells were treated with 1 mM 5-FC, and percentage of dead cells was measured 4 days after treatment. As a control, untreated HepG2 cells were also used in the mixing experiment; *bars,* ±SD.

separated by SDS-PAGE. Gels were stained with Coomassie Blue to view the protein loading in different samples (Fig. 6, *C* and *D*) and then autoradiographed to view the stability of the labeled protein (Fig. 6, *A* and *B*). Densitometric analyses were used to quantify the remaining intracellularly labeled proteins using the 2-h time point as reference of 100% (Fig. 6*E*). These analyses revealed that the internalized CD-CS proteins were reduced ~50% 2 days after the delivery and remained stable thereafter for at least 7 days, whereas the internalized CS protein continued to decline to ~5% at 7 days after delivery. These results demonstrated that the recombinant CD-CS protein exhibited enhanced intracellular stability.

Second, to demonstrate that the internalized CD-CS remains functional several days after delivery, we carried out cell-killing experiments. HepG2 cells were treated with 0.5 μM of CD-CS or CD for 4 h, and the recombinant proteins were removed from the medium and the cells were maintained in fresh medium thereafter. At different time intervals, cells were treated with 5-FC. Sustained cell killing was observed even 28 days after the removal of the recombinant protein (Fig. 7). Because CD was not internalized, only basal level of cell killing (<10%) was observed in the CD-treated cells. These results, collectively, indicated the sustained stability and prolonged cell-killing properties of the recombinant fusion protein.

Discussion

Our results demonstrated that, when fused with CS protein, the "suicide gene" product CD could be cointernalized and elicited cell killing activity with bystander properties on the subsequent application of 5-FC. Most importantly, the cointernalized CD maintains sustained stability. These findings demonstrate that it is possible to use a fusion strategy to enhance protein stability. The exact reasons for the prolonged intracellular stability of CD-CS are currently not known and require additional investigations, but explanations can be offered. Because uptake of CD-CS is receptor-dependent, it is reasonable to assume that the internalized CD-CS is entrapped in endosomal-lysosomal compartments. This speculation is supported from our previous finding that an endosomal releasing agent (adenovirus) could enhance the expression of recombinant DNA delivered by CS protein (8). The endosomally compartmentalized CD-CS may be sequestered away from cytosolic proteolytic degradation machinery, thereby prolonging its stability. It has been demonstrated recently that recombinant CS constructs introduced into the cytoplasm by liposome fusion or by transient transfection lead to inhibition of protein synthesis in mammalian cells (16). We did not find that the internalized CD-CS or CS exhibited cytotoxic effects, consistent with the compartmentalization of the recombinant protein after internalization.[4]

We believe that the described CD-CS/5-FC prodrug strategy has broad applications in protein therapy research: (*a*) similar strategy could be used to deliver other prodrug converting enzymes, *e.g.,* human β-glucuronidase for the prodrug epirubicin-glucuronide (17, 18); and (*b*) stabilization of the internalized ligand may cause alterations of normal receptor-mediated endocytotic physiology. Many malignant cells overexpress receptors for various growth factors (19). It may be possible to use fusion strategy analogy to the one described here to intervene the normal receptor physiology of these overexpressed receptors, thereby controlling the growth of cancer cells.

Aside from these potential applications, we consider that the CD-CS/5FC described here may have value in the treat-

[4] Y-C. Lin-Lee and M. T. Kuo, unpublished observations.

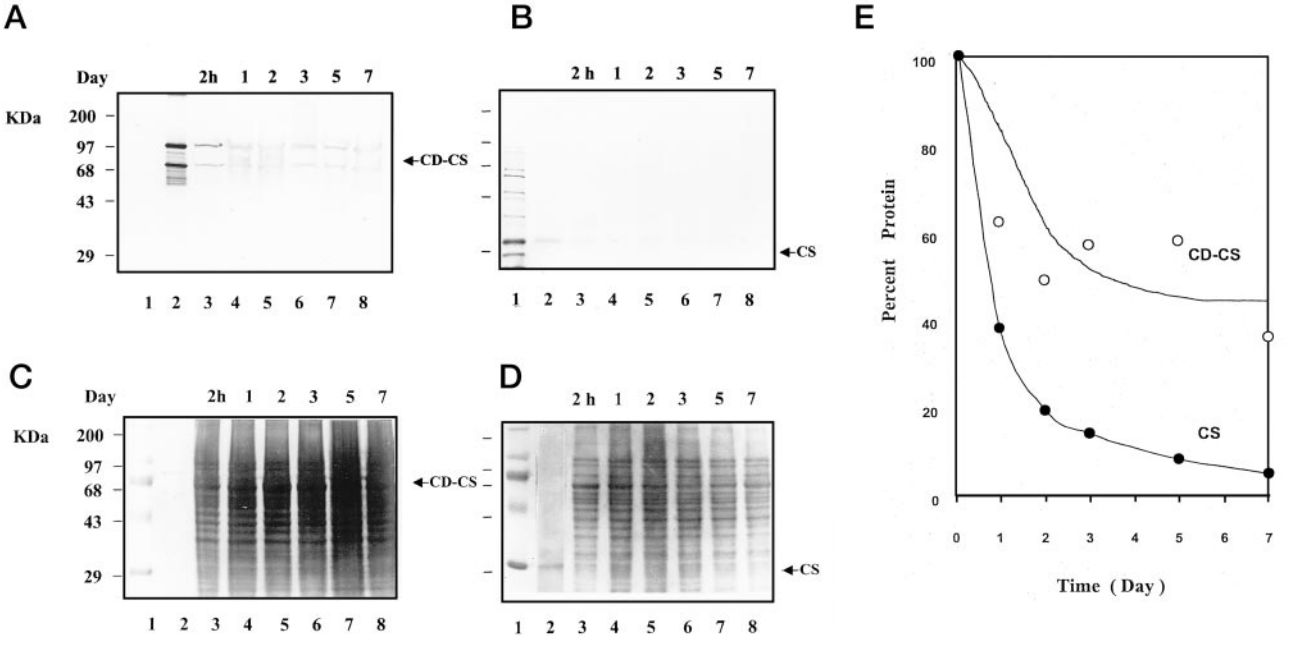

Fig. 6. Stability of CD-CS and CS proteins in HepG2 cells. HepG2 cells were treated with [^{35}S]CD-CS protein (4.3×10^4 cpm/μg) or CS (5×10^4 cpm/μg) for different time intervals as indicated. Cells were harvested and cell extracts were prepared. Proteins were separated by 10% SDS-PAGE. Gels were stained by Coomassie Blue (*C* and *D*) followed by autoradiography (*A* and *B*). *Lane 1*, molecular weight markers; *Lane 2*, purified labeled CD-CS (*C*) or CD (*D*) proteins; *Lanes 3–8,* cell extracts obtained at 2 h, and 1, 2, 3, 5, and 7 days, respectively. *E,* densitometric analysis of autoradiographs shown in *A* and *B* (data represent the average of two independent determinations).

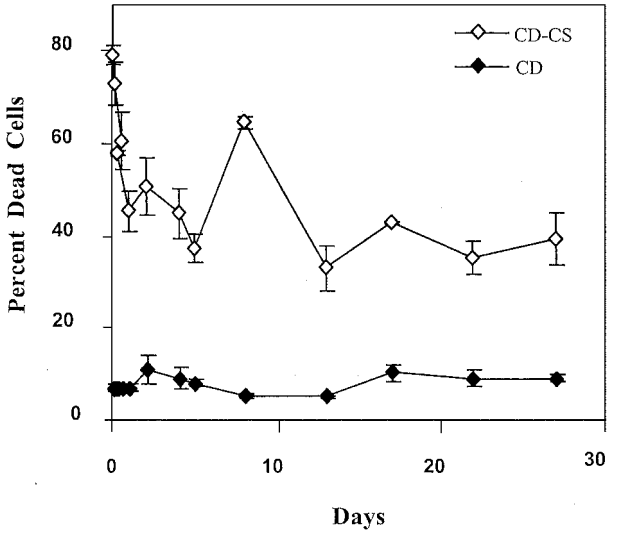

Fig. 7. Persistent effect of CD-CS on cell killing of HepG2 cells. HepG2 cells were treated with 0.8 μM of CD-CS or CD as a control for 4 h. Cells was washed with fresh medium and cultured in the regular medium. Cultures were split once every 6 days. At different time intervals, cells were treated with 1 mM 5-FC. Cell viability was counted 4 days thereafter. Each point represents an average of three independent experiments; *bars,* ±SD.

ment of HCC and MCC, because receptors for CS protein are also present in these malignancies as determined by immunohistochemistry (2) using anti-CS protein antibody (20). At present, the discriminating nature of CD-CS binding between normal livers and liver cancers, if any, is unknown. In a clinical setting, the CD-CS recombinant could be delivered through the circulation and the prodrug 5-FC through i. p. injection. MCC or HCC and normal livers will be the primary targets of 5-FU production. This would reduce the side effects of 5-FU to many organs, *e.g.,* bone marrow and gastrointestinal tracts, if 5-FU is otherwise delivered systematically. Although normal livers in addition to MCC and HCC are the sites of 5-FU manufacture, the relative sensitivity of 5-FU to the malignant cells as compared with the nonproliferative normal liver cells may render preferential killing to the cancer cells. The cytotoxic effects of 5-FU are mainly contributed by its active metabolites, which inhibit thymidine synthase and interference with DNA and RNA syntheses through incorporations of nucleotide analogues (21). Although other factors may contribute to the overall efficacy of 5-FU (22), cells with high proliferative activity (for example, bone marrow and gastrointestinal tracts) are generally more sensitive to the toxicity of 5-FU. In this regard, the CD-CS/5-FC strategy may at least eliminate the toxicities to these organs. Alternatively, because MCC and HCC receive blood supply mainly from the hepatic artery, whereas normal hepatocytes derive the majority of nutrient blood supply from the portal vein, CD-CS could be delivered through hepatic arterial infusion to enhance additional targeting specificity.

Although many previous studies on receptor-mediated gene/drug delivery to livers have focused on the use of ASOR receptors (3), we believe that CS protein has the additional advantages for hepatic gene/protein targeting. ASOR receptor recognizes sugar (galactose) moiety in the ligand (23),

whereas CS protein recognizes a peptide ligand. Thus fusion CS protein can be prepared through recombinant DNA technology. Additionally, ASOR receptor synthesis are often down-regulated in individual with hepatitis (23), liver cirrhosis (24), and HCC (24, 25), whereas we found that CS bindings to HCC and MCC remain suggesting that the CS protein carrier may be particularly useful for individuals who suffer from these malignancies.

From the pharmacokinetic point of view, the half-life of 5-FU in the circulation is rather short (~30 min; Ref. 26) in comparison with that of 5-FC (>6 h; Ref. 13), the CD-CS/5-FC prodrug system may improve the bioavailability of 5-FU as compared with the direct injection of 5-FU. Moreover, the bystander effects of the strategy may have the added advantage of eradicating cancer cells that do not express receptor or lack of angiogenesis because of tumor cell heterogenicity.

In conclusion, we have presented a receptor-mediated protein delivery system that is technologically simple but has great potential for biomedical application. We are now investigating the application of this strategy to other model systems.

References

1. Curiel, D. T., Wagner, E., Cotton, M., Birnstiel, M. L., Agarwal, S., Li, C. M., Loechel, S., and Hu, P. C. High efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes. Hum. Gene Ther., *3:* 147–154, 1992.

2. Hall, S. J., Chen, S. H., and Woo, S. L. The promise and reality of cancer gene therapy. Am J. Hum. Genet., *61:* 785–789, 1997.

3. Smith, R. M., and Wu, G. Y. Hepatocyte-directed gene delivery by receptor-mediated endocytosis. Semin. Liver Dis., *19:* 83–92, 1999.

4. Zatloukal, K., Wagner, E., Cotton M., Phillips, S., Plank, C., Steinlein, P., Curiel, D. T., and Birnstiel, M. L. Transferrinfection: a highly efficient way to express gene constructs in eukaryotic cells. Ann N Y Acad. Sci., *660:* 136–153, 1992.

5. Cristiano, R. J., Smith, L. C., and Woo, S. L. C. Hepatic gene therapy: adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. Proc. Natl. Acad. Sci. USA, *90:* 2122–2126, 1993.

6. Cerami, C., Frevert, U., Sinnis, P., Takacs, B., Clavijo, P., Santos, M. J., and Nussenzweig, V. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of Plasmodium falciparum sporozoites. Cell, *70:* 1021–1033, 1992.

7. Nussenzweig, V. Malaria sporozoites and chylomicron remnants compete for binding sites in the liver. Behring Inst. Mitt., *99:* 85–89, 1997.

8. Ding, Z. M., Cristiano, R. J., Roth, J. A., Takacs, B., and Kuo, M. T. Malarial circumsporozoite protein is a novel gene delivery vehicle to primary hepatocyte cultures and cultured cells. J. Biol. Chem., *270:* 3667–3676, 1995.

9. Danielsen, S., Kilstrup, M., Barilla, K., Jochimsen, B., and Neuhard, J., Characterization of the *Escherichia coli* codBA operon encoding cytosine permease and cytosine deaminase. Mol. Microbiol., *6:* 1335–1344, 1992.

10. Giovane, C., Schwalbach, G., and Weiss, E. *In vivo* labeling of overexpressed recombinant proteins in *E. coli.* Biotechniques, *22:* 796–798, 1997.

11. Miller, L. H., Goods, M. F., and Milon, G. Malaria pathogenesis. Science (Wash. DC), *264:* 1878–1883, 1994.

12. Runegar, M., Wei, X., Berndt, N., and Hamm-Alvarex, S. Transferrin receptor recycling in rat hepatocytes is regulated by protein phosphatase 2A, possibly through effects on microtubule-dependent transport. Hepatology, *26:* 176–185, 1997.

13. Rich, K. A., George, F. W., IV, Law, J. L., and Martin, W. J. Cell-adhesive motif in region II of malarial circumsporozoite protein. Science (Wash. DC), *249:* 1574–1577, 1990.

14. Huber, B. E, Austin, E. A., Good, S. S., Kick, V. C., Tibbels, S., and Richards, C. A. *In vivo* activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. Cancer Res., *53:* 4619–4626, 1993.

15. Huber, B. E., Austin, E. A., Richards, C. A., Davis, S. T., and Good, S. S. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc. Natl. Acad. Sci. USA, *91:* 8302–8306, 1994.

16. Frevert, U., Galinski, M. R., Hugel, F. U., Allon, N., Schreier, H., Smulevitch, S., Shkaibaei, M., and Clavijo, P. Malaria circumsporozoite protein inhibits protein synthesis in mammalian cells. EMBO J., *17:* 3816–3826, 1998.

17. Murdter, T. E., Sperker, B., Kivisto, K. T., McClellan, M., Fritz, P., Fridel, G., Linder, A., Booslet, K., Toomes, H., Dierkesmann, R., and Kroemer, H. K. Enhanced uptake of doxorubicin into bronchial carcinoma: β-glucuronidase mediates release of doxorubicin from a glucoronide prodrug (HMR 1826) at the tumor site. Cancer Res., *57:* 2440–1445, 1997.

18. Houba, P. H. J., Leenders, R. G. G., Boven, E., Scheeren, J. W., Pinedo, H. M., and Haisma, H. J. Characterization of novel anthracycline prodrugs activated by human γ-glucuronidase for use in antibody-direct enzyme prodrug therapy. Biochem. Pharmacol., *52:* 455–463, 1996.

19. Fan, Z., and Mendelsohn, J. Therapeutic application of anti-growth factor receptor antibodies. Curr. Opin. Oncol., *10:* 67–73, 1998.

20. Burkot, T. R., Da, Z. W., Geysen, H. M., Wirtz, R. A., and Saul, A. Fine specificity of monoclonal antibodies against the Plasmodium falciparum circumsporozoite protein: recognition of both repetitive and non-repetitive regions. Parasite Immunol. (Oxf.), *13:* 161–170, 1991.

21. Weclbecler, G. Biochemical pharmacology and analysis of fluorophrimidines alone and in combination with modulators. Pharmacol. Ther., *50:* 367–424, 1991.

22. Chu, E., and Allegra, C. J. Mechanisms of clinical resistance to 5-fluorouracil chemotherapy. Cancer Treat. Res., *87:* 175–195, 1996.

23. Shia, M. A., and Lodish, H. F. The two subunits of the human asialoglycoprotein receptor have different fates when expressed alone in fibroblasts. Proc. Natl. Acad. Sci. USA, *86:* 158–162, 1989.

24. Arima, T., Motoyama, Y., Yamamoto, T., Nagata, K., and Kondo, T. Serum glycoproteins in the liver disease. III. Desialylated glycoprotein in the acute hepatitis. Gastroenterol. Jpn., *12:* 39–42, 1977.

25. Marshall, J. S., Green, A. M., Pensky, J., Williams, D., Zinn, A., and de Carlson, D. M. Serum dialylated glycoproteins in patients with hepatobiliary dysfunction. J. Clin. Investig., *54:* 555–562, 1974.

26. Marshall, J. S., Williams, S., Jones, P., and Hepner, G. W. Serum desialyated glycoproteins in patients with hepatobiliary dysfunction. J. Lab. Clin. Med., *92:* 30–37, 1978.

27. Saltz, L. Drug treatment of colorectal cancer: current status. Drugs, *42:* 616–627, 1991.