Secreted and tumour targeted human carboxylesterase for activation of irinotecan

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Inirinotecan (CPT-11) is an anticancer agent for the treatment of colon cancer. CPT-11 can be considered as a prodrug, since it needs to be activated into the toxic drug SN-38 by the enzyme carboxylesterase. An approach to achieve tumour specific activation of CPT-11 is to transduce the cDNA encoding carboxylesterase into tumour cells. A secreted form of carboxylesterase may diffuse through a tumour mass and may activate CPT-11 extracellularly. This could enhance the antitumour efficacy by exerting a bystander effect on untransduced cells. In addition a secreted tumour-targeted form of carboxylesterase should prevent leakage of the enzyme from the site of the tumour into the circulation. We have constructed a secreted form of human liver carboxylesterase-2 by deletion of the cellular retention signal and by cloning the cDNA downstream of an Ig kappa leader sequence. The protein was secreted by transfected cells and showed both enzyme activity and efficient CPT-11 activation. To obtain a secreted, tumour-targeted form of carboxylesterase-2 the cDNA encoding the human scFv antibody C28 directed against the epithelial cell adhesion molecule EpCAM, was inserted between the leader sequence and carboxylesterase-2. This fusion protein showed CPT-11 activation and specific binding to EpCAM expressing cells. Importantly, in combination with CPT-11 both recombinant carboxylesterase proteins exerted strong antiproliferative effects on human colon cancer cells. They are, therefore, promising new tools for gene directed enzyme prodrug therapy approaches for the treatment of colon carcinoma with CPT-11.

Keywords: cancer; carboxylesterase; chemotherapy; CPT-11; prodrug; SN-38

Conventional chemotherapy lacks specificity for tumour cells. This results in dose-limiting side effects and insufficient concentrations of the drugs in the tumour, through which efficacy is limited and drug resistant cellular subpopulations may emerge. These problems may be overcome by expressing an enzyme that is capable of converting a non-toxic prodrug into a toxic drug specifically in tumour cells. This so-called gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy aims to increase the concentration of the drugs in the tumour while reducing the systemic toxicity. The gene encoding the prodrug-activating enzyme is delivered to the tumour cells by, for example, an adenoviral vector, followed by systemic administration of the prodrug. In this regard, several prodrug-convertible enzymes have been extensively studied, such as the herpes simplex virus thymidine kinase enzyme that converts ganciclovir (GCV) into the active compound GCV-P and bacterial cytosine deaminase that activates 5-FC to the anticancer drug 5-FU (Moolten, 1986; Austin and Huber, 1993).

A prodrug for the treatment of colon carcinoma is irinotecan (CPT-11 or 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin). CPT-11 is converted by carboxylesterases (CE) into the toxic drug SN-38 (7-ethyl-10-hydroxycamptothecin) by cleavage of the bulky dipiperidino side chain at the carbon position (Tsuij et al., 1991; Satoh et al., 1994). CPT-11 has demonstrated antitumour activity in immune deprived animals bearing human tumour xenografts (Houghton et al., 1995, 1996; Thompson et al., 1997a,b) and is approved for use in the treatment of metastatic colorectal cancer in humans. Although SN-38 can be detected in the plasma of cancer patients only minutes after the administration of CPT-11 (Gupta et al., 1997a,b), 90% of the administered CPT-11 is not converted to SN-38 (Rivory et al., 1997).

CEs are a ubiquitously expressed class of enzymes. High levels of enzyme activity are found in human liver and lung (Satoh and Hosokawa, 1998). Different isoforms of human CE have been described. CE1 is found in liver only, whereas CE2 is also found in the intestines and CE3 is found in brain cells (Hojiring and Svensmark, 1977; Kettermann et al., 1989). Furthermore, it has been shown that human alveolar macrophages release a serine esterase that is identical to liver CE1 (Munger et al., 1991). CE1 is found in liver only, whereas CE2 is also found in the intestines and CE3 is found in brain cells (Hojiring and Svensmark, 1977; Kettermann et al., 1989). Furthermore, it has been shown that human alveolar macrophages release a serine esterase that is identical to liver CE1 (Munger et al., 1991).

Several studies have been performed using CPT-11 in combination with human CE1 in a GDEPT approach. Kojima et al. (1998a,b) described the construction of a replication deficient adenoviral vector containing the human liver CE1 gene driven by the CMV promoter. In vitro results showed that several tumour cell lines infected with this virus express CE1 and in the presence of CPT-11 tumour growth was effectively suppressed. However, on many other tumour cell lines only minimal effects were observed. This underscored the notice that the success of a GDEPT approach for CPT-11 requires an enzyme with a high efficiency of converting...
CPT-11 to SN-38. The rabbit CE was found to be 100–1000-fold more efficient in converting CPT-11 than human liver CE1 and was 12–55-fold more efficient in sensitising transfected cells to CPT-11 (Danks et al, 1999). Therefore, an adenoviral vector expressing rabbit CE was constructed and transduction of human tumour cells led to sensitisation to CPT-11 (Wierdl et al, 2001). The disadvantage of rabbit CE, however, is that expression of a nonhuman protein in patients may lead to an immunological response and subsequent enzyme inactivation. A human enzyme with higher affinity and higher efficiency than CE1 may overcome these limitations. It was shown that human CE2 has a higher affinity and a higher conversion velocity for CPT-11 than CE1 (Humerickhouse et al, 2000). Therefore, we envisaged that CE2 would be a candidate to employ in a GDEPT approach to treat human tumours.

To achieve efficient kill of all tumour cells, a bystander effect is required, whereby CPT-11 is cleaved to SN-38 that not only kills the tumour cells in which CE2 is formed, but also neighbouring tumour cells that do not express CE2. We hypothesised that extra-cellular conversion of CPT-11 would lead to a larger bystander effect than intracellular conversion and, furthermore, that a fusion protein consisting of secreted CE2 fused to a tumour specific antigen antibody will be retained in the tumour thereby preventing leakage of the enzyme into the circulation and therefore further reducing unwanted side effects.

In this study we describe the construction of a secreted form of CE2 (sCE2) by deletion of a C-terminal cellular retention signal and by adding the Ig kappa leader sequence. Furthermore, a secreted targeted form of human CE2 (C28-sCE2) was constructed by fusing sCE2 to a human scFv directed against Epithelial Cell Adhesion Molecule (EpCAM), a tumour-associated antigen. The binding specificity and enzyme activity of the secreted form of CE2 and the fusion protein and their ability to sensitise human tumour cell lines to CPT-11 is determined and compared to wild-type intracellularly expressed human CE2 (CE2).

MATERIALS AND METHODS

Chemicals

Two polymerase, PCR buffer and dNTPs were obtained from Roche (Almere, The Netherlands). Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA) and Life Technologies (Breda, The Netherlands). The kits used for DNA isolation, purification and extraction from agarose gel were from Qiagen (Hilden, Germany). The substrate p-nitrophenyl-acetate was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The prodrug CPT-11 and the drug SN-38 were obtained from Sigma-Aldrich (Sone and Wang, 1997), 659-664.

Cell lines

The COS-7 and the human colon cancer SW1398 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Paisley, UK) supplemented with 5% (COS-7) or 10% foetal calf serum (Life Technologies, Paisley, UK) with 100 units/ml penicillin (Life Technologies) and 50 μg/ml streptomycin (Life Technologies) in a humidified atmosphere containing 5% CO₂ at 37 °C.

Construction of psCE2 and pC28-sCE2

The pBluescript vector containing the CE2 open reading frame (Sone and Wang, 1997) was digested with EcoRI and the CE2 encoding fragment was ligated into the EcoRI linearised eukaryotic expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands). This construct was called pCE2. To construct a secreted form of CE2 (psCE2), two primers (sense 5’-GACGCGGCC- CAGGCCCCCG2AAGCTTGAGCAGCCATCC-3’ and antisense 5’-GACTCGAGGCGCCGTCTCCTTCCAGGTCCTGACG-3’) were designed to introduce an Sfi restriction site (italic) at the beginning of the sequence encoding the mature protein and a NotI restriction site (italic) before the retention signal, as shown in Figure 1. After performing the PCR, the SfiI/NotI digested fragment was isolated, purified, and ligated into the eukaryotic expression vector pSTCF. A flexible (Gly4Ser)2 linker was introduced downstream of CE2, as described previously (de Graaf et al, 2002). To allow insertion of sCE2 downstream of the (Gly,Ser)₂ linker, a PCR was performed to obtain a DNA fragment encoding a secreted form of CE2 starting from the mature CE protein and ending just before the cellular retention signal. Both primers used in the PCR (sense 5’-GTGTCGCGCCCCGGCAGACT- CAGCCAATCCCATC-3’ and antisense primer as described above) contained a NotI site (italic). The PCR product was digested with NotI and inserted into the NotI sites of the vector containing C28 with the (Gly,Ser)₂ linker to obtain pC28-sCE2.

Expression of CE2, sCE2 and C28-sCE2 fusion protein

COS-7 cells (2×10⁵) were transfected with 2 μg pCE2, psCE2 or pC28-sCE2 by Lipofectamine Plus reagent (Life Technologies) according to instructions of the manufacturer. Cells were grown in 3.5 ml DMEM containing 5% FCS and antibiotics. After 48 h, supernatants were removed and cells were harvested by trypsinization. Cellular lysates were obtained by three times freeze thawing in 350 μl PBS. For cytotoxicity assays and HPLC analysis proteins present in supernatants of transfected COS-7 cells were 10× concentrated using a Biomax-10 centrifugal filter (Millipore, Bedford, USA). Supernatants and cellular lysates were analysed for the presence of functional CE enzyme or C28-sCE2 fusion protein by Western blotting, esterase activity assay and cytotoxicity assays. Binding of proteins in supernatants of COS-7 cells transfected with pC28-sCE2 or psCE2 to EpCAM positive cells was determined by FACS analysis.

Western blot analysis

Proportional amounts of supernatant or cellular lysates from COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2 were dissolved in sample buffer (Laemmli, 1970) with 5% 2-mercaptoethanol and heated at 95°C for 5 min. Samples were electrophoresed through a denaturing 10% sodium dodecyl sulphate-polyacrylamide gel and protein bands were electroblotted onto PVDF protein membrane (BioRad). Proteins were detected using anti-myc antibody 9E10 (Chan et al, 1987) and HRP-conjugated rabbit anti-mouse IgG (Dako) or with rabbit-anti-CE2, an antibody directed to the C-terminal retention signal of CE which was a kind gift of Dr Yan, University of Rhode Island (Zhu et al, 2000), and HRP-conjugated swine anti-rabbit IgG (Dako). Blots were developed with enhanced chemo luminescence reagent (Lumilight Plus, Roche).

Esterase activity assay

Supernatants or cellular lysates of transfected COS-7 cells were incubated with 200 μl 100 mM Tris-HCl pH 8.0 containing...
Expression and characterisation of CE2, sCE2 and C28-sCE2

COS-7 cells were transfected with pCE2, pSC28-sCE2 and expressed proteins in supernatant and cellular lysates were analysed by Western blotting, FACS analysis, esterase activity assay and cytotoxicity assays. To assess the size of the expressed proteins and determine the amount of secreted protein, SDS–PAGE was performed followed by Western blotting and detection with antimyc antibody for sCE2 and C28-sCE2 or anti CE2 antibody for CE2 (Figure 2). The CE2 protein appeared to remain intracellular since it was only detected in the cellular lysate (Figure 2, lane 1) of transfected COS-7 cells. Like CE2, the sCE2 monomers migrated with an apparent molecular weight of 75 kDa. As expected, the majority of sCE2 was detected in the supernatant of transfected COS-7 cells, proving that deletion of the C-terminal retention signal and fusing the Ig kappa leader, indeed directed the protein into the secretory pathway (Figure 2, lane 3). The C28-sCE2 fusion protein, with an apparent molecular weight of 100 kDa, was also found mainly in the supernatants of transfected COS-7 cells (Figure 2, lane 6).

Functional enzyme activity of CE2, sCE2 and C28-sCE2 was demonstrated by an esterase enzyme activity assay (Figure 3). In cells transfected with pCE2 esterase activity remained intracellular, while in cells transfected with pSC28 or pC28-sCE2 almost all activity was detected in the culture medium. These results confirmed the results of the Western blotting experiments, since the relative amounts and the activities of CE2 proteins in cells and supernatants of transfected COS-7 cells were comparable.

Binding of the C28-sCE2 fusion protein to EpCAM was demonstrated by FACS analysis of EpCAM expressing SW1398 colon cancer cells incubated with transfected COS-7 supernatants (Figure 4A), whereas sCE2 did not bind the EpCAM expressing cells. Thus, the C28 moiety of C28-sCE2 mediated EpCAM binding. Furthermore, SW1398 cells were plated and incubated with the transfected COS-7 supernatants for 24 h. Hereafter, cells were stained with anti-myc antibody to detect bound fusion protein. C28-sCE2 was detected at the membrane of SW1398 cells, shown in Figure 4B, whereas cells incubated with sCE2 were not stained.
mainly remained intracellular while sCE2 and C28-sCE2 were secreted. As expected, CE2 proteins migrated with an apparent molecular weight of 75 kDa whereas CE2 was detected with an antibody directed against the C-terminal cellular retention signal. In lanes 1, 3 and 4 cellular lysates (c) and in lanes 2, 5 and 6 supernatants (s) of COS-7 cells transfected with pCE2 (lanes 1,2), psCE2 (lanes 3,4) and pC28-sCE2 (lanes 5,6) respectively are shown. The CE proteins migrated with an apparent molecular weight of 75 kDa whereas the fusion protein had a molecular weight of 100 kDa. As expected, CE2 mainly remained intracellular while sCE2 and C28-sCE2 were secreted by transfected COS-7 cells.

Prodrug activation and antiproliferative effects

Concentrated supernatants of COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2 were analysed for CPT-11 conversion using HPLC. Supernatants were incubated with CPT-11 for 22 h at 37°C. It was found that both sCE2 and C28-sCE2, which were secreted in the culture medium of transfected cells, were able to activate the prodrug CPT-11, since the drug SN-38 was formed (data not shown).

To show the effect of CPT-11 conversion into SN-38 by CE2, sCE2 and C28-sCE2 on the viability of colon cancer cells, the EpCAM-expressing colon carcinoma cell line SW1398 was incubated overnight with the concentrated supernatant of COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2. After incubation, culture medium or a non-toxic concentration (1 μM) of CPT-11 was added. In Figure 5 it is shown that the supernatants of COS-7 cells transfected with sCE2 or C28-sCE2 render SW1398 cells susceptible to CPT-11. Incubation with supernatants of pCE2 transfected COS-7 cells and CPT-11, which do not secrete CE, or incubation with sCE2 or C28-sCE2 supernatant only, did not show augmented toxicity.

DISCUSSION

CPT-11 is a prodrug for the treatment of colon cancer. One enzyme that converts CPT-11 into the toxic drug SN-38 is CE. By increasing the concentration of CE at the site of a tumour via a GDEPT approach, the conversion of CPT-11 to SN-38 will be enhanced at the site of the tumour, leading to tumour specific cytotoxicity. Human liver CE1 and rabbit CE have been employed in a GDEPT approach in combination with CPT-11. Although rabbit CE appeared to convert CPT-11 very effectively (Danks et al, 1999), an enzyme of human origin is preferred for in vivo applications to treat patients. Human CE1 showed a low conversion velocity and a low hydrolysis rate for CPT-11 in comparison with CE2 (Humerickhouse et al, 2000). Therefore, in this study we used the human liver CE2 enzyme to sensitize human tumour cells to CPT-11. Because current gene transfer technology does not allow expression of transgenes in all cells of a targeted tumour in vivo, a bystander effect is required, in order to achieve efficient tumour reduction. Extracellularly produced SN-38 should not only kill the tumour cells in which CE2 is formed, but also neighbouring tumour cells that do not express CE2. To investigate whether extracellular conversion of CPT-11 would lead to a larger bystander effect than intracellular conversion, we constructed a secreted form of CE2 (sCE2). Furthermore, we hypothesised that a fusion protein consisting of sCE2 fused to a tumour specific scFv antibody would be retained in the tumour thereby preventing leakage of the enzyme into the circulation and therefore further reducing
unwanted side effects. An example of a tumour-associated antigen is EpCAM. This molecule is an attractive target for enzyme prodrug therapy, since it is highly expressed on the cell surface of most carcinomas, including colon tumours. Furthermore, EpCAM is highly expressed on distant metastasis (Litvinov et al., 1994). Therefore, we constructed a fully human fusion protein consisting of sCE2 fused to a human scFv antibody directed to EpCAM (C28-sCE2). Intratumoural expression of this protein in cancer patients is expected to be less immunogenic than expression of non-human fusion proteins.

The secreted and the targeted protein were detected in the supernatant of transfected COS-7 cells and the secreted proteins exhibited comparable enzymatic activities as determined by conversion of pNpAc. Comparing the secreted proteins to intracellular wildtype CE2, it was observed that secreting COS-7 cells with pCE2 resulted in a much lower total amount of CE-activity than cells transfected with pCE2 or the fusion protein C28-sCE2. Whether this is due to a greater amount of protein or a higher enzyme activity of sCE2 when compared with CE is not clear. C28-sCE2 showed enzyme activity and specific binding to EpCAM (C28-sCE2). Therefore, we constructed a secreted form of CE2 that was capable to convert the prodrug CPT-11 to SN-38 cells that were incubated with secreted or targeted protein and a non-toxic concentration of CPT-11 showed complete growth inhibition of these cells.

In conclusion, we constructed a secreted form of CE2 that was capable to convert the prodrug CPT-11, leading to enhanced toxicity of CPT-11 to colon cancer cells. This construct holds promise in GDEPT approaches since transduction of tumour cells with pCE2 will most likely result in high concentrations of sCE2 throughout the whole tumour. Therefore, CPT-11 will be converted to SN-38 very efficiently throughout the tumour, resulting in a larger bystander effect than intracellular conversion of CPT-11. The C28-sCE2 fusion protein is as active as sCE2, and therefore this construct is as useful as sCE2 for GDEPT, but the theoretical advantage of C28-sCE2 is that the targeting moiety will prevent leakage of the construct into the circulation. However, from this study it can not be concluded that C28-sCE2 will have this additional advantage as compared to sCE2. To prove this hypothesis, in vivo experiments are necessary in which sCE2 and C28-sCE2 are expressed in colon carcinoma xenografts followed by CPT-11 administration.

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