Metastatic colorectal cancer cells from patients previously treated with chemotherapy are sensitive to T-cell killing mediated by CEA/CD3-bispecific T-cell-engaging BiTE antibody

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BACKGROUND: Novel technologies to redirect T-cell killing against cancer cells are emerging. We hypothesised that metastatic human colorectal cancer (CRC) previously treated with conventional chemotherapy would be sensitive to T-cell killing mediated by carcinoembryonic antigen (CEA)/CD3-bispecific T-cell-engaging BiTE antibody (MEDI-565).

METHODS: We analysed proliferation and lysis of CEA-positive (CEA+) CRC specimens that had survived previous systemic chemotherapy and biologic therapy to determine whether they could be killed by patient T cells engaged by MEDI-565 in vitro. Results: At low concentrations (0.1–1 ng ml−1), MEDI-565 + T cells caused reduced proliferation and enhanced apoptosis of CEA+ human CRC specimens. High levels of soluble CEA did not impair killing by redirected T cells and there was no increase in resistance to T-cell killing despite multiple rounds of exposure.

CONCLUSIONS: This study shows for the first time that metastatic CRC specimens derived from patients previously treated with conventional chemotherapy can be lysed by patient T cells. Clinical testing of cancer immunotherapies, such as MEDI-565 that result in exposure of tumours to large numbers of T cells, is warranted.

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Antigen-specific cytotoxic T cells (CTLs) have the capacity to kill human cancers, as showed by tumour regression after adoptive transfer of ex vivo-expanded tumour-infiltrating lymphocytes (TILs) and T-cell receptor gene-transfected T cells to melanoma patients (Leen et al., 2007). However, generating antigen-specific T cells in vitro for adoptive transfer is complicated, time-consuming and at best, is only successful in 70% of TIL cultures (Dudley et al., 2003). An alternative is to redirect large numbers of T cells through the use of bispecific antibodies, which target CD3 on T cells and a surface antigen on tumour cells, including bispecific single-chain antibodies of the BiTE class (Wolf et al., 2005). These recombinant constructs transiently link resting T cells to tumour cells, leading to T-cell activation and serial lysis of tumour cells (Hoffmann et al., 2005; Brandl et al., 2007; Brischwein et al., 2007). Mouse models have shown high levels of activity of BiTE antibodies targeted against EphA2 and CD19 (Dreier et al., 2003; Schlereth et al., 2006; Brischwein et al., 2006; Offner et al., 2006; Hammond et al., 2007). Witthauer et al. (2008) reported that a BiTE targeting EpCAM could lead to T-cell-mediated killing of breast cancer cells in pleural effusions. Recently, Bargou et al. (2008) reported clinical activity and a safety profile suitable for continued development of the BiTE antibody blinatumomab (also called MT103/MEDI-538) with dual specificity for CD19 and CD3 in a study of non-Hodgkin’s B-cell lymphoma patients who had experienced relapse after standard therapies.

MEDI-565, also known as MT111, is composed of a human single-chain antibody recognising carcinoembryonic antigen (CEA, CD66e and CEACAM5), which is frequently expressed in carcinomas of the lung, pancreas, stomach, ovary, uterus, breast, colon and rectum (Hammarstrom, 1999), and a de-immunised single-chain antibody specific for CD3, which is connected by a short flexible linker sequence (Lutterbuese et al., 2009). Conventional CEA-specific antibodies can bind with high affinity and selectivity to CEA-expressing (CEA+) tumours in vivo but they do...
not recognise CEA expressed on the luminal side of several normal epithelial tissues, thus limiting their potential toxicity (Mayer et al., 2000). The bispecific BiTE antibodies, CEA/CD3, were recently shown to prevent subcutaneous tumour growth and formation of lung metastases in preclinical models (Lutterbuese et al., 2009).

The purpose of our study was to determine whether T cells from normal donors or cancer patients could be redirected by MEDI-565 to kill human colorectal cancer specimens that had survived previous chemotherapy treatment. We aimed to confirm the function of MEDI-565 with the T cell of cancer patients and against human colorectal cancers. Furthermore, we aimed to confirm that the mechanism of killing by MEDI-565 was similar to that of other forms of T-cell-mediated killing. Finally, we wanted to determine whether tumours remained sensitive to repeated rounds of exposure to MEDI-565. We found that tumour cells, from patients previously treated with chemotherapy, had their growth inhibited and underwent apoptotic cell death when exposed to combinations of T cells with MEDI-565.

MATERIALS AND METHODS

Reagents

Fluorescein isothiocyanate anti-lineage cocktail 1 mAb, PerCP-anti-CD4 mAb, APC-anti-CD8 mAb, PE-anti-CD69 mAb, PE-anti-CD25 mAb, FITC-anti-granzyme B mAb, PE-anti-Fas ligand mAb and streptavidin-APC were purchased from BD Bioscience (San Jose, CA, USA). Fluorescein isothiocyanate labelled and PE labelled anti-CEA mAbs were from Sanquin (Amsterdam, The Netherlands), and 7-AAD and Annexin V-biotin kit were purchased from Immunotech (Marseille, France, cat. no. PN IM3422). Ethylene glycol tetraacetic acid (EGTA) was obtained from Sigma (St Louis, MO, USA), and purified human CEA protein was purchased from TriChem (West Chester, PA, USA).

Construction and production of bispecific antibodies

Standard DNA technologies were used to construct MEDI-565, also known as MT111, and the control MEC14 BiTE, as described (Lutterbuese et al., 2009). The selection of MEDI-565 was from a set of bispecific single-chain antibodies (Lutterbuese et al., 2009) and possessed a set of characteristics identified as important for development of a biological drug. It is composed of a humanised anti-CEA single-chain antibody (Chester et al., 1994) and a ‘de-immunised’ human CD3ε-specific single-chain antibody derived from the mouse monoclonal antibody L2K (Brischwein et al., 2006). MEC14 BiTE is composed of a murine anti-Mecoprop (an herbicide) single-chain antibody linked to the same anti-CD3ε single-chain antibody used to construct MEDI-565. The expression vector pEF-DHFR containing the coding sequences of MEDI-565 or MEC14 BiTE (Cont BiTE) was transfected into DHFR-deficient CHO cells. Each antibody was purified from CHO cell culture supernatants using immobilised metal affinity chromatography and gel filtration essentially as described (Kufer et al., 2001). Antibody preparations containing primarily the monomeric form (>97%) of each bispecific antibody was used in all experiments.

Tumour cell lines

Ls174T (ATCC CCL-188), a CEA highly expressing colon carcinoma cell line, AsPC-1 (ATCC CRL-1682), a CEA + pancreatic adenocarcinoma cell line and SW480 (ATCC CCL-228) and HCT116 (ATCC CCL-247), the CEA-negative colorectal carcinoma cell lines, were purchased from ATCC (Manassas, VA, USA). The cell lines were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA).

Mice

NOD.CB17-PrkdcscidIl2rγcÎ±å (NOD/SCID) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in the Duke Comprehensive Cancer Center Isolation Facility. All work was performed in accordance with the approved protocol of the Duke IACUC.

Tumour cell isolation from patients’ colorectal cancer specimens and establishment of explants in NOD/SCID mice

Patients undergoing resection of colorectal cancer metastatic to the liver, which were refractory to standard chemotherapy (including fluorouracil, oxaliplatin and bevazicumab), provided signed informed consent approved by the Duke University Medical Center Institutional Review Board before surgery. After the collection of the colorectal cancer specimen, the tissue was minced with a blade into pieces smaller than 2 mm cube and digested overnight with triple enzyme buffer containing collagenase IV (1 mg ml−1, Sigma, no. C-5138), hyaluronidase (100 µg ml−1, Sigma, no. H-6254) and deoxyribonuclease (20 U ml−1, Sigma, no. D-5025) in RPMI1640 medium. The cells were spun down, washed with PBS thrice, resuspended in Hank’s balanced salt solution and mixed with Matrigel (BD Biosciences) in a 1:1 ratio. Cells (half of available cells from digestion procedure, typically 1 × 10⁶ cells) were injected into the back of NOD/SCID mice. After 2- to 4-month growth in vivo, when tumours reached approximately 1 cm in diameter, the mice were killed and the tumours were excised, minced and put into in vitro culture. Some of the minced cells were injected into the flank of NOD/SCID mice, and serial in vivo passages were performed. Colorectal cancer (CRC) cells growing in vitro were used as target cells of the assays. These cells (CRC007, CRC010 and CRC039) were analysed for their HLA class I expression and CEA expression, and were proven to be positive for both molecules.

Flow-based cytotoxicity assay

T cells were negatively isolated from the PBMCs of the normal donors or patients using a T-cell isolation kit (Invitrogen Dynal AS, Oslo, Norway, cat no. 113.11D). In all experiments, purity of CD3 + cells exceeded 95% of the CD45 + leucocyte population after isolation procedures. For the cytotoxicity assays, 1 × 10⁵ tumour cells and 5 × 10⁴ negatively isolated T cells were put into 96-well U-bottom plates with MEDI-565 or Cont BiTE at concentrations ranging from 0.01 to 10,000 ng ml⁻¹. Alternatively, in some experiments using 12-well plates, 5 × 10⁵ tumour cells and 2.5 × 10⁶ T cells were added to each well with MEDI-565 or Cont BiTE. After 1–7 days of incubation, all cells were harvested with 0.05% trypsin/EDTA and spun down by centrifugation. Cells were then stained with anti-CEA-PE, 7-AAD and propidium iodide, and CEA + cells were analysed for their viability after acquisition using a FACSCalibur flow cytometer (BD Biosciences). Alternatively, cells were labelled with biotin-conjugated Annexin V, and then stained with anti-CEA-PE, 7-AAD and Streptavidin-APC. The CEA + cells were analysed for expression of Annexin V as a marker of apoptosis. To test whether cytotoxicity was dependent on exocytosis of cytotoxic granules, the assay was performed in the presence of 4 µM EGTA, a chelator of extracellular calcium required for exocytosis (Lowin et al., 1996; Voskoboinik et al., 2005, 2006).

MTT assay

The tumour cells, AsPC-1, were cultured with T cells in a 1:5 ratio for 7 days in the presence of MEDI-565 or Cont BiTE (100 ng ml⁻¹). On day 7, floating cells were discarded and only
adherent AsPC-1 cells were harvested with 0.05% trypsin/EDTA and washed with PBS thrice. 1 × 10⁶ AsPC-1 tumour cells were added to each well of 96-well flat-bottom plates in 200 μl of complete RPMI1640 medium. The cells were allowed to adhere to the plates overnight at 37 °C (day 0) and were further incubated for 1, 2, 4 or 7 days. A total of 20 μl of 10 × 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg ml⁻¹) solution was added to each well, and incubated at 37 °C for 2 h. The adherent cells were lysed with 150 μl of dimethylsulphoxide (DMSO) and the optical density (OD) at 550 nm was measured. Sensitivity of colorectal cancer cells to oxaliplatin was also determined using MTT assay. In brief, 1 × 10⁴ CRC cells were added to each well of 96-well flat-bottom plates and, after overnight incubation, various concentrations of oxaliplatin were added to the wells. The MTT assay was performed after 2-h incubation with oxaliplatin and the IC₅₀ was reported as the concentration at which 50% reduction in cell proliferation occurred.

Trypan blue dye exclusion assay
To assess MEDI-565 effects on tumour cell proliferation, tumour cells (0.5 × 10⁵ per well) were cultured with or without T cells (2.5 × 10⁵ per well) for 5 or 7 days in 12-well plates in the presence of MEDI-565 or Cont BiTE (each at 100 ng ml⁻¹). Photographs were taken at the magnifications of ×40, ×100 and ×200, and the numbers of tumour cell/T-cell clusters, tumour cell attached by at least three lymphocytes, were counted in three random high-power fields (×100). Cells were harvested with 0.05% trypsin/EDTA and Trypan blue dye was added after which the sum of viable tumour cells (Trypan blue negative) and dead cells (Trypan blue staining) was determined. Smaller cells (morphologically T cells) were eliminated from counting.

Cell-cycle analysis
The tumour cells, AsPC-1, were cultured with normal donor’s T cells in a 1:5 ratio for 7 days in the presence of MEDI-565 or Cont BiTE (100 ng ml⁻¹). On day 7, floating cells were discarded and only adherent AsPC-1 cells were harvested from flasks with 0.05% trypsin/EDTA, washed with PBS and resuspended in 0.5 ml of 0.1% glucose/PBS, and cells were then fixed by gently adding 5 ml of cold 70% ethanol. Cells were kept at 4 °C overnight, and then washed with PBS twice. Propidium iodide was added to the cell suspension, and incubated at 37 °C for 30 min. Cells were acquired using FACS Calibur flow cytometer (BD Biosciences), and DNA content was analysed for tumour cells. Tumour cells were gated based on the size and granularity in forward scatter and side scatter histogram.

Intracellular cytokine staining
2.5 × 10⁶ T cells and 5 × 10⁵ AsPC-1 cells were incubated in the presence of MEDI-565 or Cont BiTE (each at 100 ng ml⁻¹) for 1, 2 or 4 days as described above. For the last 5 h of the incubation period, Brefeldin A (1 μg ml⁻¹) was added to the medium. Then, EDTA (final concentration, 2 μM) was added to the medium to dissociate cells and after 10 min the cells were harvested, washed with PBS twice and fixed with 1% formaldehyde in PBS. Cells were permeabilised with permeabilising solution (BD Biosciences) for 20 min, and stained with anti-CD4-PerCP, anti-CD8-APC and FITC-labelled anti-granzyme B or PE-labelled anti-Fas ligand. Forward Scatterlow Side Scatterlow CD4⁺ and CD8⁺ T cells were analysed for their granzyme B or Fas ligand expression (Supplementary Figures).

Enzyme-linked immunosorbent assay
2.5 × 10⁶ T cells and 5 × 10⁵ AsPC-1 cells were incubated in the presence of MEDI-565 or Cont BiTE (each at 100 ng ml⁻¹) for 1, 3 or 5 days and supernatants were harvested and kept frozen at −80 °C until used. Supernatants were analysed using ELISA for the presence of granzyme B and perforin (Mabtech Inc., Cincinnati, OH, USA) according to the manufacturer’s instructions. Colour was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) stabilised substrate (Promega, Madison, WI, USA), stopped by adding 1 N H₂SO₄, and OD at 450 nm was measured using ELISA plate reader (Bio-Rad, Model 680, Hercules, CA, USA; Supplementary data).

Th1/Th2 CBA analysis
Culture supernatants were collected after 5 days of T-cell/tumour cell co-incubation with MEDI-565 or Cont BiTE (each at 100 ng ml⁻¹). The concentrations of IL-2, IL-4, IL-5, IL-10, TNF-z and IFN-γ were measured with a BD Cytometric Bead Array Th1/Th2 cytokine kit (BD Biosciences), according to the manufacturer’s instructions, and analysed on a FACSCalibur flow cytometer using BD CBA software (BD Biosciences; Supplementary data).

Statistical analysis
The Student’s t-test was used to analyse differences in tumour cell killing between MEDI-565 and control MECl4 BiTE-treated tumours. Differences at P<0.05 were considered statistically significant.

RESULTS
Human CEA + tumour cells are recognised and undergo apoptosis by normal human donor T cells redirected by MEDI-565
To examine the cytolytic effects of MEDI-565, the CEA + human pancreatic cancer cell line AsPC-1 was incubated with purified, human T cells and MEDI-565 or a control BiTE (which binds the herbicide mecoprop and human CD3; Cont BiTE) for 5 days. As shown in Figure 1, in the presence of MEDI-565, but not Cont BiTE, tumour cells were surrounded by T cells, detached from the bottom of the wells, and showed apoptotic changes in cell morphology. Similar observations were made when using human CEA + tumours (e.g., CRC039) obtained from resection specimens as target cells. We found a markedly greater number of tumour cells with adherent T cells in the MEDI-565 containing co-cultures compared with the Cont BiTE containing cultures (AsPC-1: 23.0 ± 3.6 vs 1.3 ± 0.6 clusters per field, CRC039: 15.7 ± 2.1 vs 1.7 ± 1.5 clusters per field, P<0.005). These data show that MEDI-565 redirects large numbers of T cells to engage tumour cells.

Proliferation of human CEA + tumour cells is inhibited and cytotoxicity is enhanced by T cells redirected by MEDI-565
We analysed the cytotoxicity of tumour cells caused by MEDI-565 using flow cytometric analysis to detect uptake of a dye into killed tumour cells. As shown in Figure 2A, the cytotoxicity of MEDI-565 required the presence of T cells, suggesting that MEDI-565 alone does not cause cell death. We next analysed whether MEDI-565 reduced proliferation of tumour cells in the presence of T cells by counting viable cells with Trypan blue dye exclusion. As shown in Figure 2B, MEDI-565 significantly inhibited the cell proliferation and resulted in only one-eighth of the number of viable tumour cells in culture after a 7-day incubation period (P<0.005). Interestingly, T cells proliferated in MEDI-565 containing co-cultures, but not in Cont BiTE containing co-culture (3.31 ± 0.49 × 10⁶ vs 0.72 ± 0.11 × 10⁶ cells per well), possibly because of activation as evidenced by upregulation of CD69 and CD23 observed only in MEDI-565 cultures. The expression (percent positivity/mean fluorescence intensity (MFI)) of CD69
Normal donor- and cancer patient-derived T cells with MEDI-565 induce apoptotic cell death of a CEA + cancer cell line

To examine the effect of MEDI-565 and T cells on apoptotic cell death of tumour cells, AsPC-1 cells were incubated with T cells and a range of MEDI-565 concentrations. On study days 1 to 4, subpopulations of tumour cells undergoing apoptosis were identified by flow cytometry using annexin V and 7-AAD (Figure 3C). Figure 3C shows the analysis at day 4 with predominantly early apoptosis (annexin V + and 7-AAD−) with some component of late apoptosis/cell death (annexin V + and 7-AAD+) over a wide range of MEDI-565 concentrations (0.1–1000 ng ml⁻¹). Apoptosis was not observed until day 2, but reached a maximum at day 4 (Figure 3D) and did not consistently increase further by day 7 (data not shown).

CEA + human metastatic colorectal tumour explants are recognised and undergo reduced proliferation and apoptotic cell death when exposed to T cells redirected by MEDI-565

To provide a more clinically relevant assessment of MEDI-565 activity, we used the short-term cultured human metastatic colorectal cancer cells derived from surgical resections of patients previously treated with oxaliplatin and fluorouracil-containing regimens. The colorectal cancer cells had been implanted into NOD/SCID mice, grown, removed and then maintained in vitro. Flow cytometric and immunohistochemical analyses showed that these metastatic tumour explants expressed CEA, and had histological characteristics of metastatic colorectal cancers (data not shown). In addition, these explants (CRC007, CRC010 and CRC039) were tested for their sensitivity to oxaliplatin in vitro and were found to have IC₅₀ of 2.5, 12.0 and 30.5 μM, respectively. Compared with the IC₅₀ in a screen of colorectal cancer cell lines treated with oxaliplatin (LS180, <0.3 μM; Colo205, <0.3 μM; and HCT8, <0.3 μM), these explants were determined to be relatively insensitive to oxaliplatin. These metastatic tumour explants were incubated with T cells isolated from healthy volunteer donors for 5 days (Figure 4 and Supplementary Figure 1). Metastatic tumour explants cultured with MEDI-565, but not Cont BiTE, were surrounded by T cells, detached from the bottom of the well, and were gradually killed (Supplementary Figure 1). We confirmed apoptotic cell death of cells from the metastatic tumour explants incubated with MEDI-565 and T cells by annexin V and 7-AAD staining using flow cytometry at day 5 (Figures 4A and B). The average values of cytotoxicity of these three colorectal cancer explants (CRC007, CRC010 and CRC039) from repeated assays are shown in a bar graph (Figure 4B). MEDI-565 induced significantly higher apoptotic cell death of CEA + colorectal tumour explants when compared with Cont BiTE or without BiTE antibody condition (P < 0.005). These data also indicate that, despite being insensitive to oxaliplatin, human colorectal cancer cells are sensitive to killing mediated by MEDI-565.

To assess the combined effects of reduced cell growth and induction of apoptotic cell death, we also analysed the number of live and dead cells using Trypan blue dye exclusion. Similar reductions in total cells for all three cancer cell cultures were observed (Figure 4C). Viable cancer cells in the presence of MEDI-565 and T cells were reduced by 66–80% relative to Cont BiTE/T cells, or no treatment. Thus, treatment of cultures with MEDI-565
combined with T cells induced both anti-proliferative and/or apoptotic effects on human metastatic tumour cells, resulting in a massive reduction in the total number of viable tumour cells after treatment compared with untreated tumour cells.

**Granzyme B and FasL have a role in MEDI-565/T-cell-induced apoptosis**

It is known that T cells kill tumours by the perforin/granzyme B and the Fas/FasL pathways. Therefore, we aimed to confirm that these pathways were also potentially involved in MEDI-565-induced apoptosis. Indeed, we observed greater percentages of granzyme B-and FasL-expressing CD8+ T cells after co-culture of tumours, T cells and MEDI-565 (Supplementary Figures 2 and 3) when compared with control-BiTE. Interestingly, the MEDI-565 induced gradual secretion of perforin by T cells, which corresponded to the slow induction of apoptosis in target cells. These results indicate that the perforin/granzyme B system has an important role in MEDI-565-induced T-cell cytotoxicity.

We next evaluated the cytokines secreted by T cells responding to and destroying the CEA+ tumour cells in cultures containing MEDI-565. Using beads to capture the cytokines, we showed that IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were all secreted, but IFN-γ was found in the highest amount, suggesting that the T cells

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**Figure 2** MEDI-565/T-cell inhibits proliferation of CEA+ cancer cells. (A) AsPC-1 cells (5 × 10^5 per well) were cultured with or without T cells (2.5 × 10^6 per well) for 7 days in 12-well plates in the presence of MEDI-565 or Cont-BiTE (100 ng/ml⁻¹). MEDI-565-induced T-cell cytotoxicity was assessed by staining tumour cells with PE-labelled anti-CEA mAb and 7-AAD. Carcinoembryonic antigen-positive tumour cells were analysed for their7-AAD positivity. The assay was repeated four times and the average values of cytotoxicity for each condition are shown. *P<0.001 (Student’s t-test). (B) MEDI-565 effect on tumour cell proliferation was assessed by counting cells with Trypan blue dye exclusion method. The assay was performed with duplicated wells for each condition and the sum of viable cells (grey) and dead cells (black) are shown. MEDI-565/T-cell-mediated killing of metastatic colon cancer cells.

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**Table 1**

| Number | 0 | 2000 | 4000 | 6000 | 8000 | 10000 |
|--------|---|------|------|------|------|-------|
| Channel | FL2-A-FL2-Area | FL2-A-FL2-Area | FL2-A-FL2-Area | FL2-A-FL2-Area | FL2-A-FL2-Area | FL2-A-FL2-Area |
| G1     | 54.6% | 56.1% | 56.1% | 56.1% | 56.1% | 56.1% |
| G2/M   | 20.2% | 16.9% | 16.9% | 16.9% | 16.9% | 16.9% |
| S      | 25.3% | 18.6% | 18.6% | 18.6% | 18.6% | 18.6% |

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**Figure 3**

(A) AsPC-1 cells (5 × 10^5 per well) were cultured with or without T cells (2.5 × 10^6 per well) for 7 days in 150 cm² flask. Floating cells were discarded and adherent cells were harvested with 0.05% trypsin/C2. (B) AsPC-1 cells (6 × 10^6 per flask) were incubated with T cells (30 × 10^6 per flask) for 7 days in 150 cm² flask. Floating cells were discarded and adherent cells were harvested with 0.05% trypan/EDTA. Cells were washed with PBS, resuspended and then fixed with cold 70% ethanol. Cells were stained with propidium iodide and samples were acquired using a FACSCalibur flow cytometer and DNA content of tumour cells were analysed. The percentages of G0/G1, G2/M and S phases are shown (A). Harvested AsPC-1 cells (1 × 10^6 per well) were put into 96-well flat-bottom plates on day −1, and incubated overnight. On days 0, 1, 2, 4 and 7, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed. In brief, 20 μl of 10 × MTT (5 mg/ml⁻¹) solution was added to each well, and incubated at 37 °C for 2 h. Adherent cells were lysed with 150 μl of dimethylsulphoxide (DMSO) at 37 °C for 5 min, and OD 550 nm was measured using an ELISA plate reader. Value of day 0 was used as a baseline data, and the ratios of OD550 nm values of each time point were plotted (D). *P<0.001 (Student’s t-test).
do become activated and secrete cytokines when activated by MEDI-565 bound to CEA on tumour cells; both Tc1 (Th1) and Tc2 (Th2) type cytokines were detected (Supplementary Figure 4).

**Soluble CEA protein does not affect apoptosis induced by T cells redirected by MEDI-565**

As many colorectal cancer patients have large amounts of soluble circulating CEA protein in their serum, the MEDI-565 antibody might be competitively inhibited by binding soluble CEA before it can bind membrane-bound CEA on tumour cells. We analysed whether soluble CEA protein in clinically relevant concentrations could affect MEDI-565-induced T-cell cytotoxicity in our assay system. To the cell culture, CEA protein was added at concentrations from 2.5 to 1000 ng ml⁻¹, and AsPC-1 cells and CRC010 colorectal cancer cell were used as target cells. Apoptosis at each condition was compared with the apoptosis in MEDI-565/T-cell culture without CEA protein (0 ng ml⁻¹) and shown as a

![Graph](image)

**Figure 3** MEDI-565/T-cell-induced apoptotic cell death of a CEA + tumour cell line. (A) MEDI-565 induced cytotoxic capacity of T cells derived from colorectal cancer patients compared with normal healthy donor T cells. T cells were isolated from the PBMCs derived from normal healthy donors (left panel) or colorectal cancer patients (right panel) using T-cell-negative isolation kit. Isolated T cells were co-cultured with AsPC-1 tumour cells in a 5:1 effector-to-target ratio in the presence of various concentrations of MEDI-565 (0.01–10 000 ng ml⁻¹) in 96-well U-bottom plates as described in Materials and Methods. Cells were incubated for 2 days, and the cytotoxicity was analysed using flow cytometry with 7-AAD staining. No significant difference (P>0.05 for Student’s t-test, see text) in MEDI-565-induced T-cell cytotoxicity was observed between T cells from cancer patients and T cells from normal donors when compared at each concentration of MEDI-565. (B) Carcinoembryonic antigen (CEA) specificity of MEDI-565/T-cell mediated killing of tumour cells. The CEA+ tumour cell lines (Ls174T and AsPC-1) and CEA-negative tumour cell lines (SW480 and HCT116) were incubated in a 96-well U-bottom plate with T cells negatively isolated from the PBMCs of normal donors (triangle symbols) or cancer patients (square symbols). MEDI-565 or Cont BiTE was put into the culture at the indicated concentrations. After 2 days of incubation, cells were harvested from the plates with 0.05% trypsin/EDTA and stained with FITC-labelled anti-lineage marker and 7-AAD. The 7-AAD-positive cells in lineage marker negative cells are analysed and shown in each line graph. (C, D) Effects of time and concentration of MEDI-565 on T-cell-induced apoptosis of CEA+ tumour cells. Both the AsPC-1 cells (1×10⁵ cells per well) and normal donor’s T cells (5×10⁵ cells per well) were put into a 96-well U-bottom plate with indicated concentrations of MEDI-565 or Cont BiTE. After 4 days of incubation, cells were harvested with 0.05% trypsin/EDTA and stained with FITC-labelled anti-lineage marker and 7-AAD. The 7-AAD-positive cells in lineage marker negative cells are analysed and shown in each line graph. (C) The AsPC-1 cells were incubated with T cells in the presence of MEDI-565 or Cont BiTE (100 ng ml⁻¹) for 1, 2 or 4 days and stained as described in (B). Percentages of annexin V-positive cells (including 7-AAD negative and 7-AAD positive) in CEA+ tumour cells are shown in each line graph.
The purpose of this study was to determine whether CEA + colorectal cancer cell lines remain sensitive to T-cell-mediated killing despite repeated exposure to T cells and MEDI-565.

Many anticancer drugs will induce outgrowth of drug-resistant cancer cells, and repeated treatments may become ineffective. To test whether we could repeatedly treat tumour cells with MEDI-565 and T cells, we created an analogous in vitro model in which AsPC-1 cells were exposed to three cycles of killing by T cells in the presence of MEDI-565 or Cont BiTE. Harvested cells were analysed using flow cytometry for their susceptibility to MEDI-565/T-cell-mediated apoptosis and levels of CEA expression each cycle. As shown in Figure 6, AsPC-1 cells repeatedly exposed to MEDI-565/T-cell maintained their susceptibility to MEDI-565/T-cell-mediated apoptosis at a similar level to non-treated AsPC-1 cells. It should be noted that the CEA expression levels on the AsPC-1 cells remained constant throughout the assay period (data not shown). On the basis of this result, MEDI-565/T cells may not induce escape mechanisms in cancer cells, and thus might be suitable for long-term and repeated treatment.

DISCUSSION

The results of our study are noteworthy for several reasons. First, we showed that metastatic colorectal cancers, which had survived previous conventional systemic therapy, could be killed by a T-cell-mediated therapy. In addition, we analysed the sensitivity of these tumour cells to oxaliplatin in vitro and found that the IC_{50} (2.5 12.0 and 30.5 μM) for inhibition of proliferation was higher than the majority of 25 colorectal cancer cell lines we had previously tested for sensitivity to oxaliplatin (such as LS180, Colo205 and HCT8, which are considered sensitive with IC_{50} <0.3 μM). Therefore, we believe that the colorectal cancer explants are relatively oxaliplatin-insensitive tumour cells.

Second, the low concentrations of MEDI-565 required for activity suggest a potent anti-tumour capacity (Figure 3). This low concentration has been reported for other BiTE antibodies, Bargou et al (2008) showed the safety of a BiTE antibody, blinatumomab (a BiTE with dual specificity for CD19 and CD3), in their clinical trial, and confirmed that responses to blinatumomab as a single agent occurred in B-cell lymphoma patients at a serum level of 0.6 ng ml^{-1}. This is about five orders of magnitude below serum levels reported for the monoclonal antibody rituximab at standard doses, which similarly elicits objective responses as a single agent in this disease (Hainsworth et al, 2003; Ghielmini et al, 2005). The enormous potency difference between the BiTE blinatumomab and a conventional antibody could be explained by a greater number of T cells attacking an individual tumour cell (both due to more T cells that are able to recognise tumour and explants. We confirmed that T-cell-mediated tumour cell death occurred at very low concentrations of MEDI-565 (1 ng ml^{-1}) and without the addition of costimulatory agents. Similar levels of MEDI-565-redirected T-cell lysis were observed when T cells from colorectal cancer patients or from normal healthy donors were used. Importantly, MEDI-565/T-cell treatment could efficiently induce cell death of colorectal cancer cells derived from different patients. We found that anti-proliferative activity required MEDI-565, T cells and target cells expressing CEA. T cells alone or T cells with Control BiTE did not show these effects on CEA-expressing cancer cells. The enormous potency difference between the BiTE blinatumomab and a conventional antibody could be explained by a greater number of T cells attacking an individual tumour cell (both due to more T cells that are able to recognise tumour and explants. We confirmed that T-cell-mediated tumour cell death occurred at very low concentrations of MEDI-565 (1 ng ml^{-1}) and without the addition of costimulatory agents. Similar levels of MEDI-565-redirected T-cell lysis were observed when T cells from colorectal cancer patients or from normal healthy donors were used. Importantly, MEDI-565/T-cell treatment could efficiently induce cell death of colorectal cancer cells derived from different patients. We found that anti-proliferative activity required MEDI-565, T cells and target cells expressing CEA. T cells alone or T cells with Control BiTE did not show these effects on CEA-expressing cancer cells. The enormous potency difference between the BiTE blinatumomab and a conventional antibody could be explained by a greater number of T cells attacking an individual tumour cell (both due to more T cells that are able to recognise tumour and explants. We confirmed that T-cell-mediated tumour cell death occurred at very low concentrations of MEDI-565 (1 ng ml^{-1}) and without the addition of costimulatory agents. Similar levels of MEDI-565-redirected T-cell lysis were observed when T cells from colorectal cancer patients or from normal healthy donors were used. Importantly, MEDI-565/T-cell treatment could efficiently induce cell death of colorectal cancer cells derived from different patients. We found that anti-proliferative activity required MEDI-565, T cells and target cells expressing CEA. T cells alone or T cells with Control BiTE did not show these effects on CEA-expressing cancer cells. The enormous potency difference between the BiTE blinatumomab and a conventional antibody could be explained by a greater number of T cells attacking an individual tumour cell (both due to more T cells that are able to recognise tumour and explants. We confirmed that T-cell-mediated tumour cell death occurred at very low concentrations of MEDI-565 (1 ng ml^{-1}) and without the addition of costimulatory agents. Similar levels of MEDI-565-redirected T-cell lysis were observed when T cells from colorectal cancer patients or from normal healthy donors were used. Importantly, MEDI-565/T-cell treatment could efficiently induce cell death of colorectal cancer cells derived from different patients. We found that anti-proliferative activity required MEDI-565, T cells and target cells expressing CEA. T cells alone or T cells with Control BiTE did not show these effects on CEA-expressing cancer cells.
proliferation of the engaged T cells) or more efficient lysis of tumour cells by the engaged T cells (due to activation of T-cell-mediated killing by engagement of only very few CD3 receptor subunits). Interestingly, we observed the activation of both CD4 and CD8 T cells against tumour cells in the presence of MEDI-565 (Supplementary Figures 2 and 3). Consistent with our findings, Kischel et al (2009) showed that both CD4 and CD8 T cell subset can contribute to redirected target cell killing with EpCAM/CD3-BiTE antibody. Thus, the high potency of BiTE molecule may derive from a broader spectrum and a greater number of effector T cells engaged to kill target cells. We also observed that the number of T cells in the MEDI-565 co-cultures was greater than the number placed into the cultures and was also greater than that in the control BiTE cultures after 5 days, suggesting that there was proliferation of the effector T cells. Finally, there was upregulation of CD69 on these T cells, suggesting that they were activated.

Third, we showed that resistance to T-cell-mediated killing may not occur. We created an in vitro model of repeated MEDI-565/T cell exposure, by repeating three cycles of MEDI-565/T-cell attack and rest with the CEA-expressing cell line AsPC-1. We observed that these cells maintained their susceptibility to MEDI-565/T-cell attack after prolonged treatment in vitro (Figure 6). Interestingly, CEA expression of tumour cells was retained despite prolonged treatment, suggesting that MEDI-565/T-cell treatment may not easily induce escape mechanisms in cancer cells. Hypothetically, re-treatment with MEDI-565 can be still effective for progressive disease that was once treated with MEDI-565 successfully at its earlier stages.

Fourth, we were concerned that the high circulating levels of CEA could impair MEDI-565 function through competitive inhibition of binding to surface CEA. As many colorectal cancer patients have high levels of soluble CEA protein in their serum,.....
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Figure 5 Soluble CEA protein does not affect MEDI-565/T-cell-mediated apoptosis of CEA-+ cell line and colorectal cancer cells. AsPC-1 cells or CRC010 colorectal cancer cells (5 x 10⁶ cells per well) were incubated with T cells (2.5 x 10⁶ cells per well) in a 12-well plate in the presence of MEDI-565 (100 ng ml⁻¹; 1.8 nM) with the indicated concentration of soluble CEA antigen in the medium (2.5 to 1000 ng ml⁻¹; 0.01 to 5 nM). After 5 days of incubation, tumour cells were harvested and stained as described in the Figure 4 legend. Annexin V-positive cells in lineage marker-negative, CEA+ tumour cells were evaluated. The cytotoxicity value of the culture with MEDI-565 without soluble CEA protein was set as 100%, and each cytotoxicity value is shown as a percentage relative to this MEDI-565-positive, CEA protein-negative culture. The assay was repeated twice for both AsPC-1 cells and CRC010 cancer cells (filled symbols indicate AsPC-1 cells and open symbols indicate CRC010 tumour cells; each symbol represents an individual T-cell donor). The bars show the average cytotoxicities of each condition.

Figure 6 Repeated treatment with MEDI-565 and T cells does not abolish tumour cell susceptibility to MEDI-565/T-cell-mediated apoptosis. AsPC-1 tumour cells (6 x 10⁵) were incubated with T cells (3 x 10⁶) of 150 cm² T flasks with MEDI-565 or Cont BiTE (100 ng ml⁻¹). Additional BiTE molecules were added every 3 days, and after 10-day incubation, floating cells were washed out and cells were allowed to expand for 7 days (one cycle). Three cycles of attack/rest were repeated for each condition over 50 days. Adherent AsPC-1 cells were harvested and used as target cells for cytotoxicity assay. After 5 days of incubation with T cells, tumour cells were stained as described in the Figure 4 legend. Annexin V-positive cells in lineage marker-negative, CEA+ tumour cells were evaluated. The cytotoxicity value of the incubation with AsPC-1 target cells from long-term culture without BiTE molecule was set as 100%, and each cytotoxicity value is shown as a percentage relative to this condition. The assay was repeated twice with similar results.
T cells, and showed that perforin/granzyme B had a key role in cytotoxicity. As CEA is expressed by many different kinds of cancers, MEDI-565 may be applicable to a wide range of adenocarcinoma as a novel treatment with the potential for high efficacy and less toxicity than conventional therapies. The next studies will be in vivo models to confirm activity against implanted colorectal cancers and if the results continue to show anti-tumour activity, these data would support the initiation of clinical trials with this therapy.

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