Therapeutic Window of MuS110, a Single-Chain Antibody Construct Bispecific for Murine EpCAM and Murine CD3

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Abstract

EpCAM (CD326) is one of the most frequently and highly expressed tumor-associated antigens known and recently has also been found on cancer stem cells derived from human breast, colon, prostate, and pancreas tumors. However, like many other tumor-associated antigens used for antibody-based immunotherapeutic approaches, EpCAM is expressed on normal tissues including epithelia of pancreas, colon, lung, bile ducts, and breast. To assess the therapeutic window of an EpCAM/CD3-bispecific single-chain antibody construct of the bispecific T-cell engager (BiTE) class, we constructed murine surrogate of MT110 (muS110) from single-chain antibodies specific for murine EpCAM and CD3 antigens. Immunohistochemical analysis showed that, with minor differences, the expression of EpCAM protein on a large variety of tissues from man and mouse was similar with respect to distribution and level. MuS110 exhibited significant antitumor activity at as low as 5 μg/kg in both syngeneic 4T1 orthotopic breast cancer and CT-26 lung cancer mouse models. Dosing of muS110 for several weeks up to 400 μg/kg by intraaimal dose escalation was still tolerated, indicating existence of a significant therapeutic window for an EpCAM-specific BiTE antibody in mice. MuS110 was found to have similar in vitro characteristics and in vivo antitumor activity as MT110, a human EpCAM/human CD3-bispecific BiTE antibody that currently is in formal preclinical development. [Cancer Res 2008;68(1):143–51]

Introduction

A highly promising concept of treating malignant diseases is the recruitment of cytotoxic T cells for tumor cell elimination using bispecific antibody constructs (1, 2). Many different formats of bispecific T-cell-recruiting antibodies have been developed throughout the past two decades. One class of bispecific antibodies, called “bispecific T-cell engager” (BiTE), is composed of two covalently linked single-chain antibodies (scFv). Biological and structural features of BiTE constructs have been defined in detail (3). BiTE antibodies recognize with one single-chain antibody, a subunit of the CD3 complex present on all T cells, and with the second single-chain antibody, a tumor-associated cell surface antigen. By simultaneously binding CD3-positive effector and target antigen-positive tumor cells, BiTE antibodies redirect cytotoxic T cells to very efficiently induce killing of target-positive tumor cells in vitro and in various animal models (4–8). BiTE mode of action involves formation of a regular cytolytic synapse (9), serial killing of target cells by BiTE-activated, polyclonal effector T cells (10), and a highly conditional T-cell activation leading to T-cell proliferation, release of cytotoxic granule content, and cytokine production (4, 11, 12).

One representative of the BiTE class is called MT110 (4), which targets epithelial cell adhesion molecule (EpCAM; CD326). EpCAM is widely expressed on most human adenocarcinoma and several squamous cell carcinoma (13–15) and is present on cancer stem cells of various carcinomas (16–18). MT110 and related antibodies have been shown to eradicate established tumors in immunodeficient mice at low microgram doses and to reactivate tumor-resident T cells within human metastatic tissue leading to tumor rejection (4, 7, 19). MT110, which is in formal preclinical development, is specific for human CD3 and human EpCAM, but with the exception of chimpanzee, does not crossreact with its orthologs in other species. Because the chimpanzee is no longer considered an appropriate species for in vivo studies, we constructed for the present study a biosimilar antibody called murine surrogate of MT110 (muS110) with specificity for murine EpCAM and murine CD3 using rat and hamster monoclonal antibodies, respectively. MuS110 is used to principally assess safety and therapeutic window of an EpCAM-specific BiTE antibody in a relevant species.

Expression of EpCAM and CD3 in tissues from humans and mice was found to be similar. MuS110 and MT110 had comparable biological activities, and muS110 exhibited a significant therapeutic window in mice. The murine EpCAM–specific BiTE antibody muS110 therefore seems to distinguish between EpCAM expressed on tumor and normal tissue, leading to a well-tolerated inhibition of EpCAM-expressing tumors.

Materials and Methods

Cell lines and culture. Dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cells, 4T1 cells, and B16F10 cells were purchased from the American Type Cell Culture Collection. DHFR-deficient CHO cells and B16F10 cells were transfected with an expression plasmid coding for murine or human full-length EpCAM. CHO/muEpCAM and CHO/huEpCAM cells were grown in suspension with HyQ medium (HyClone), supplemented with 20 and 500 nmol/L methotrexate, respectively. 4T1 cells and B16F10/muEpCAM cells were grown in RPMI (Invitrogen) supplemented with 10% FCS (Invitrogen). All cells were cultivated at 37°C in a 5% CO2 humidified incubator. Subconfluent cultures were detached by incubation with trypsin and checked for vitality by eosin exclusion.

Construction and production of MT110 and muS110. MT110 was engineered by recombinant DNA technology using an antibody specific for human EpCAM and the antibody diL2K specific for human CD3 (4). To generate muS110, the antibody mCD3-1 specific for murine CD3 and the
antibody G8.8 specific for murine EpCAM were used. The sequences encoding the variable domains of the respective antibodies were amplified and modified as described (20). The coding sequences of the inserts were verified by sequencing according to standard protocols (21). Expression plasmids were transfected into DHFR-deficient CHO cells. Eukaryotic protein expression in DHFR-deficient CHO cells was performed according to Kaufman et al. (22). Transfected cells were expanded in tissue culture and the constructs purified from cell culture supernatant of CHO cells as described earlier (23–25).

**Antibodies.** A hybridoma producing rat anti-mouse EpCAM monoclonal antibody G8.8 was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Resources, and maintained by the Department of Biological Sciences (University of Iowa, Iowa City, IA). For scFv anti-EpCAM in MT110, murine variable domains recognizing human EpCAM were obtained by phage display. Variable domains of the mouse antihuman CD3 monoclonal antibody L2K were subjected to a procedure removing human T helper cells epipetides by point mutation of MHC class II anchor amino acids (26). This resulted in deimmunized L2K (diL2K), which was used to construct MT110. Nonspecific rat IgG2a or mouse IgG1 isotype control antibodies were obtained from SeroTech and Sigma Aldrich, respectively.

**Saturation binding analysis.** Saturation binding assays were performed with CHO cells transfected with either human or murine EpCAM antigen or CD3 enriched human or murine T cells [from peripheral blood mononuclear cells (PBMC) or spleen, respectively]. One hundred thousand cells per well were incubated for 1 h at room temperature with the respective antibody at various concentrations. Staining was detected using a hexahistidine tag-specific secondary antibody (Dianova) and an antimouse IgG–specific labeled detection antibody (Sigma). Cells were fixed in 3.7% formaldehyde solution (Merck). Binding was analyzed by flow cytometry on a FACS Calibur instrument and CellQuest software (BD Biosciences). Dissociation constants (K_D) values were determined using the nonlinear regression function for binding curves integrated in Prism (GraphPad Software, Inc.).

**Cytotoxicity assay.** Redirected T-cell cytotoxicity was analyzed by an assay using human PBMC or murine splenocytes as effector cells (4). The standard assay monitored uptake of propidium iodide by lysed cells using flow cytometry with a FACS Calibur device and CellQuest software (BD Biosciences). Cytotoxicity was calculated according to the formula: [1 − live target cells (sample)/live target cells (control)] × 100. Sigmoidal dose response curves typically had R^2 values of >0.9 as determined by Prism Software (GraphPad Software).

**EpCAM protein expression in murine and human tissues.** Immunohistochemical studies were contracted to Charles River Laboratories. Sections of freshly frozen mouse and human tissues were fixed in acetone for ~10 min at room temperature and air dried. After blockade of endogenous peroxidase and biotin, the primary antibody (i.e., G8.8, mCD3-1, anti-human EpCAM, diL2K or respective rat IgG2a, and mouse IgG1 control antibodies) was applied to the sections at 1.0 μg/mL or 5.0 μg/mL for ~25 min. Bound G8.8 or mCD3-1 were detected with a biotinylated rabbit anti-rat IgG secondary antibody and bound anti-human EpCAM or diL2K with a biotinylated rabbit anti-mouse IgG secondary antibody. Bound primary/secondary antibody complexes were visualized with a streptavidin–biotin–horseradish peroxidase conjugate and a diaminobenzidine chromagen substrate. The slides were counterstained in hematoxylin, dehydrated with alcohol to xylene, and overslipped.

**Animal studies.** In vivo experiments were performed in 6- to 10-week-old immunocompetent BALB/c mice (Charles River Laboratories) or immunodeficient nonobese diabetes/severe combined immunodeficient (NOD/SCID) mice. The NOD/SCID mice were tested for leakage of immunoodeficiency and only mice with IgG levels below 100 ng/mL were used. All experiments were performed according to the German Animal Protection Law with permission from the responsible local authorities. In compliance with the Animal Protection Law, mice had to be euthanized when tumor volumes exceeded 10% of their body weight. Statistical analysis of the mean tumor volume of the corresponding treatment groups versus the vehicle control group was performed using the Student’s t test.

For the SW480 human colon xenograft model, 5 × 10^6 human PBMC were mixed with 5 × 10^8 SW480 colon carcinoma cells (~500,000 EpCAM binding sites/cell) in a final volume of 0.2 mL PBS, and the effector/target cell mixture (E:T; 1:1) was s.c. injected into the right flank of NOD/SCID mice. Six animals per group were i.v. treated with different doses (5 and 50 μg/kg) of MT110 or PBS control vehicle starting 1 h after SW480/PBMC inoculation, and treatment was repeated for 4 consecutive days.

For the orthotopic 4T1 murine breast carcinoma model, a mixture of 5 × 10^6 murine T cells and 2 × 10^8 4T1 tumor cells was inoculated in a total volume of 50 μL without FCS into the mammary fat pad of 6 BALB/c mice per group and animals were s.c. treated daily with either PBS or 15, 5, 1.5, or 0.5 μg/kg muS110. Tumors were measured on the indicated days with a caliper in two perpendicular dimensions, and tumor volumes were calculated according to the formula: tumor volume = [(width^2 × length)/2].

For the CT-26 lung cancer model, 1 × 10^6 CT-26/muEpCAM tumor cells were i.v. injected into the lateral tail vein of 8 to 12 BALB/c mice/group. CT-26/muEpCAM tumor cells are trapped in the small pulmonary venules around the alveoles of the lung where they form small tumor colonies on the lung surface. From day 3 to day 17 after tumor cell inoculation, animals were daily i.v. treated with PBS control buffer or different doses of muS110 (0.5, 2, 5, and 12.5 μg/injection) or increasing doses from 12.5 to 400 μg/injection, in which the original dose of 12.5 μg/injection was doubled every second day after the fourth injection). Thereafter, animals were sacrificed and the number of CT-26/muEpCAM tumor cell colonies on the lung surface was determined macroscopically. The observation period was limited to 17 days for ethical reasons because of the severe effects of high lung tumor burden on respiration.

**Pharmacokinetic analyses.** C57BL/6 mice were i.v. injected with 10 μg/mouse of either MT110 or muS110 and allocated to three or four different groups of five mice each. Different groups were alternately bled at different time points after MT110 administration (predose, 5, 15, 30 min and 1, 2, 4, 6, 8, 12, and 24 h) and muS110 injection (predose, 5, 30 min and 1, 2, 3, 4, 5, 6, 8, 10, 12, and 24 h) and plasma concentrations quantified by specific ELISAs.

For MT110 quantification, human EpCAM–coated ELISA plates (NUNC) were incubated first with sheep anti-MT110 antibody (Micromet) followed by rabbit anti-sheep and goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma), each for 60 min at 25°C. For muS110 quantification, murine EpCAM–coated ELISA plates were incubated first with biotin-conjugated mouse antipentahistidine antibody (1:1,000; Igen) followed by streptavidin conjugated with horse radish peroxidase (1:5,000; Dako) each for 60 min at 25°C. Finally, plates were incubated with 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate (Sigma) for 60 min at 25°C. The reaction was stopped by the addition of 50 μL 2N H_2SO_4 (Merck). For both ELISAs, absorbance (405 nm) was read on Power WaveX select (Bio-Tek instruments). Standard curves were fitted by four-variable analysis.

The corresponding absorbance value of one dilution that was within the linear part of the standard curve was used to calculate the concentration of MT110 and muS110, respectively. The limit of quantification of the method was determined with ~2 and 20 ng/mL for MT110 and muS110, respectively.

Pharmacokinetic calculations of MT110 and muS110 were performed by the pharmacokinetic software package WinNonlin Professional 4.1 (Pharsight Corporation). Variables were determined by noncompartmental analysis. The noncompartmental analysis was based on model 201 (i.e., bolus injection). The distribution half-life (t_1/2 α) was calculated using a log-linear regression of the first four and three sampling time points, whereas the terminal elimination half-life (t_1/2 β) was calculated by the last five or six sampling time points with a measurable concentration of MT110 and muS110.

**Results**

**Construction of the murine EpCAM/murine CD3-reactive BiTE antibody MuS110.** MuS110 was constructed from two single-chain antibodies reactive with murine CD3 [hamster monoclonal
antibody (mAb) mCD3-1] and murine EpCAM (rat mAb G8.8). Both antibodies showed no crossreactivity with other rodent orthologs tested (data not shown). MuS110 had its four variable domains aligned in the same orientation and by using linkers of the same length and composition as MT110 (4). MuS110 was purified from cell culture media of a CHO cell clone by the same procedure as used for MT110 (4). Both constructs have in their COOH terminus a hexahistidine tag for purification and antibody detection. Monomeric forms of muS110 and MT110 were isolated and used for further characterization.

**MT110 and muS110 have similar EpCAM but distinct CD3 binding affinities.** Saturation binding curves and equilibrium binding constants for MT110 and muS110 for their respective antigens were determined using human and murine EpCAM cDNA–transfected CHO cell lines and purified human and murine T cells, respectively (Fig. 1). CHO cell lines expressing human and murine EpCAM had approximately the same surface antigen density, $1.0 \times 10^6$ binding sites per cell for CHO-huEpCAM and $1.2 \times 10^6$ binding sites per cell for CHO-muEpCAM. Computational analysis revealed $K_D$ for EpCAM binding of muS110 and MT110 of 2.1 $\pm$ 0.26 x $10^{-8}$ mol/L ($n = 7$) and 1.3 $\pm$ 0.25 x $10^{-8}$ mol/L ($n = 4$), respectively. $K_D$ values for CD3 binding to purified T cells were 2.9 $\pm$ 0.4 x $10^{-7}$ mol/L for muS110 ($n = 5$) and 1.0 $\pm$ 0.15 x $10^{-7}$ mol/L ($n = 11$) for MT110. Although EpCAM binding affinity of muS110 and MT110 was very similar, the affinity of muS110 for mouse CD3 was 35-fold higher than that of MT110 for human CD3.

**MT110 and muS110 show highly specific redirected lysis of respective target cells.** Dose-dependent redirected lysis of murine EpCAM–expressing CHO cells was only observed when murine T cells and the murine-specific BiTE muS110 were combined in the cell culture assay (Fig. 2A). MuS110 was completely ineffective with human T cells, and MT110 could not lyse murine EpCAM–expressing CHO cells in the presence of human T cells. This high specificity was confirmed in the reciprocal experiment using CHO cells expressing human EpCAM (Fig. 2B). The half-maximum activity of redirected lysis of MT110 [50% growth inhibition (EC$_{50}$) value] in the human system was at a concentration of 0.65 $\pm$ 0.5 ng/mL MT110 ($n = 5$). The EC$_{50}$ value of muS110 in the murine system was at 2.51 $\pm$ 1.2 ng/mL muS110 ($n = 10$).

Experiments using the human mammary carcinoma cell line MDA-MB-453 (expressing human EpCAM) in combination with human PBMC and MT110, and the murine mammary carcinoma cell line 4T1 (expressing murine EpCAM) in combination with muS110 and murine T cells gave similar EC$_{50}$ values compared with EpCAM-transfected CHO cells as target cells (data not shown).

**Similar EpCAM protein expression in murine and human tissues and cells.** Human and murine tissues were analyzed for expression of EpCAM and CD3 by immunohistochemical staining with the parental monoclonal antibodies used to construct muS110 and MT110. Staining specificity was controlled by respective isotype control antibodies. The anti-CD3 mAbs predominantly stained murine and human lymphoid tissues, respectively. Occasional other tissue staining could be explained by the presence of infiltrated T lymphocytes. The expression pattern of EpCAM in multiple murine and human tissues was largely overlapping and primarily seen on epithelial cells (Table 1). Figure 3 shows examples for the EpCAM staining of human and murine pancreas and prostate tissues. In both species, EpCAM staining patterns were largely confined to the cell surface, showed no difference with respect to polarized expression on epithelial cells, and stained a similar proportion of cells in tissues (data not shown). Brain and endothelium of murine and human origin did not show any
concentrations during a 48-h assay period. In both experiments, effector and B cells, in vitro, were stimulated with increasing BiTE concentrations during a 48-h assay period. Furthermore, murine EpCAM was tested with murine CD3 cell–enriched splenocytes in a Hu T cell cytotoxicity assay as described in Materials and Methods.

Figure 2. In vitro efficacy of muS110 and MT110 in redirected target cell lysis. A, in vitro efficacy of muS110. Redirected lysis of CHO cells transfected with murine EpCAM was tested with murine CD3 cell–enriched splenocytes in the presence of increasing BiTE concentrations during a 48-h assay period. B, in vitro efficacy of MT110. Redirected lysis of CHO cells transfected with human EpCAM was tested with human PBMC in the presence of increasing BiTE concentrations during a 48-h assay period. In both experiments, effector and target cells were mixed at an effector-to-target ratio of 10:1. Points, mean; bars, SE of triplicate measurements. Cell lysis was assessed via a FACS-based cytotoxicity assay as described in Materials and Methods. Mu T cells, murine T cells; Hu T cells, human T cells.

EpCAM expression. Human epithelia of breast, bladder, colon, intestine, kidney, bile ducts, lung, pancreas, pituitary, prostate, thyroid, and uterus all expressed EpCAM and so did all respective murine tissues (Table 1). Likewise, certain tissues were not stained with either human or murine EpCAM–specific mAbs, including bone marrow, cerebellum, cerebral cortex, endolymphatic, heart, ovary, spinal chord, spleen, and striated muscle. The only differences in EpCAM expression between man and mouse were noted for adrenal gland and testis tissues, which were stained in human but apparently not in murine tissue samples. When the analysis was extended to peripheral blood cells using fluorescence-activated cell sorting (FACS) analysis, a fraction of murine B and T cells was found to express EpCAM, whereas no expression of EpCAM was detectable on human peripheral lymphocytes (data not shown).

Pharmacokinetics of MT110 and muS110. Pharmacokinetic variables were determined in C57BL/6 mice after i.v. administration of 10 μg MT110 or 10 μg muS110. Serum concentrations of MT110 and muS110 were quantified by specific ELISAs. Peak plasma and serum concentrations of MT110 and muS110 were detected 5 min after bolus injection and reached the lower limit of quantification of 2 and 20 ng/mL, respectively, within ~8 to 10 h. t1/2 α were 0.34 and 0.17 h, and t1/2 β were 1.88 and 5.13 h for MT110 and muS110, respectively.

MuS110 efficacy and toxicity in an orthotopic breast cancer model. Given the restricted species specificity of MT110 and muS110, no direct comparison for efficacy of the two BiTE antibodies was possible in the same mouse model. MT110 requires an immunodeficient model in which human T cells are mixed with tumor cells expressing human EpCAM. MuS110 can be tested in an immunocompetent mouse model and, depending on the syngeneic tumor cell line, no extra expression of murine EpCAM is required.

Consistent with earlier data (4), MT110 showed high antitumor activity in a human colon SW480 xenograft model (Fig. 4A). Although five daily i.v. injections of 5 μg/kg MT110 significantly reduced tumor growth, 50 μg/kg MT110 led to a complete inhibition of tumor growth. At the higher dose, four of six animals were still free of tumor on day 26 after tumor cell inoculation, and the other two showed very small tumors with diameters of 0.06 and 0.01 cm³. As shown elsewhere (7), a nonspecific BiTE or T cells alone showed no antitumor activity in this animal model.

In the 4T1 orthotopic breast cancer model, daily s.c. treatment with 5 or 15 μg/kg muS110 starting at day 0 induced a dose-dependent retardation of tumor growth (Fig. 4B). Although 0.5 and 1.5 μg/kg muS110 were not effective, 5 μg/kg significantly reduced tumor growth. At 15 μg/kg, 68% of tumors were no longer detectable. Although all animals developed tumors, 1 of 6 animals in the 15 μg/kg dose group had tumors with a size below 0.1 cm³ at the end of the study. Side effects in the highest dose groups such as loss of body weight, diarrhea, and mild sedation/excitation were only transient. The side effects in the highest dose groups such as loss of body weight, diarrhea, and mild sedation/excitation were only transient.

Pharmacokinetics of muS110 and MT110 were further assessed in the CT-26 lung tumor model. Daily i.v. treatment with muS110 starting from day 3 after tumor cell inoculation led to a significant and dose-dependent inhibition of lung tumor colony formation (Fig. 5A and B). Although treatment with 0.5 μg/kg muS110 starting from day 3 after tumor cell inoculation led to a significant and dose-dependent inhibition of lung tumor colony formation (Fig. 5A and B). Although treatment with 0.5 μg/kg did only show a 6.7% reduction of lung tumor colonies, treatment with 2 or 5 μg/kg led to a 38.9% and 50.6% reduction, respectively. Treatment with 12.5 μg/kg muS110 reduced the number of lung tumor colonies by 56%. With a reduction of 63%, the strongest effect was seen in the intranasal dose escalation group (12.5–400 μg/kg muS110). Side effects in the highest dose groups such as loss of bodyweight, diarrhea, and mild sedation/excitation were only transient. The loss in body weight was ~5% to 7.5% and only seen after the first two injections with a rapid recovery and normalization thereafter (Fig. 5C). In the lower dose groups, no such treatment-related side effects were observed.
**Table 1. Comparison of EpCAM staining patterns of human and murine tissues**

<table>
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<tr>
<th>Antibody concentration</th>
<th>Mouse tissues</th>
<th>Human tissues</th>
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<tr>
<td></td>
<td>Antimurine EpCAM</td>
<td>Isotype control</td>
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<td>1 μg/mL</td>
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<tr>
<td>Pos. ctrl (CHO/Ep-CAM)</td>
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<td>0</td>
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<tr>
<td>Neg. ctrl [CHO]</td>
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<tr>
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<td>0</td>
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<tr>
<td>Bone marrow</td>
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<td>2</td>
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<tr>
<td>Breast/skin</td>
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<td>1</td>
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<tr>
<td>Colon</td>
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NOTE: Sections of fresh frozen mouse and human tissues were stained as described in Materials and Methods. All slides were evaluated by a certified pathologist to ensure that the quality of staining is sufficient for interpretation. The relative density of positive cells and the intensity of staining was graded on the following scale: 0, no labeled cells; 1+, light staining and/or occasional cells/types of cells ("Minimal"); 2+, light-medium staining and/or small numbers of cells/types of cells ("Mild"); 3+, moderate staining and/or medium numbers of cells/types of cells ("Moderate"); and 4+, dark staining and/or large numbers of cells/types of cells ("Marked"). Mouse and human tissues both expressing EpCAM are highlighted by mint color; unique EpCAM staining is highlighted in pink.

Abbreviations: Pos. ctrl, positive control; neg. ctrl, negative control; GI tract, gastrointestinal tract.

**Discussion**

EpCAM (CD326) is expressed at high level and with high frequency on differentiated cancer cells of most human adenocarcinoma and certain squamous cell carcinoma (13, 14, 27–29). A number of studies have recently used anti-EpCAM antibodies for isolation of so-called cancer stem cells from human breast, colon, pancreas, and prostate tumor tissues (16–18, 30), indicating that EpCAM is also expressed on cancer stem cells. This may relate to the findings that overall survival prognosis of several human cancers negatively correlates with expression of EpCAM (29, 31–33), and that overexpressed EpCAM behaves like an oncogene inducing in quiescent cells proliferation, growth factor independence, colony formation in soft agar, and up-regulation of c-myc and cyclins (34). Likewise, blockade of EpCAM expression in human breast cancer cell lines by specific siRNA inhibited proliferation, migration, and invasiveness of cells (35). These recent findings suggest that EpCAM plays an important role in human cancers, supporting its use as immunotherapeutic target for the eradication of both differentiated and pluripotent cancer cells.

Like most other tumor-associated antigens used for targeted therapies, including HER-2, epidermal growth factor receptor, carcinoembryonic antigen, CD20, or CD52, EpCAM is also expressed on normal cells and tissues. Staining of many normal epithelia by EpCAM antibodies raised early on concerns about a therapeutic window (36), which seemed only based on an overexpression of EpCAM on tumor relative to normal tissue. Overexpression of EpCAM relative to parental tissue is indeed the
case for breast (35) and ovarian cancers (37) but, for instance, is not evident for colon cancer. Acute pancreas toxicity was induced by two monoclonal antibodies tested in clinical phase I studies (38, 39), suggesting accessibility of EpCAM on normal pancreas tissue by the antibodies. On the other hand, two other anti-EpCAM antibodies showed good tolerability in man, suggesting differences in EpCAM antigen recognition by the various antibodies (40, 41). For development of a T-cell–recruiting anti-EpCAM BiTE antibody, it was therefore important to establish an animal model in which both efficacy and toxicology of this novel therapeutic approach can be principally assessed.

In the present study, we have observed that a BiTE antibody with specificity for murine CD3 and murine EpCAM had a robust therapeutic window in mice, leading to potent control of tumor growth with no significant or irreversible effect on clinical variables of animals. A detailed toxicology study is ongoing in which muS110 BiTE doses up to 500 μg/kg are being tested that are by far exceeding the efficacious doses in the range of 2.5 to 10 μg/kg identified in the present study. Preliminary analysis of immunologic and histopathologic variables suggests that dose-limiting toxicities of muS110 in mice at doses of 100 μg/kg and above are related to cytokine release rather than damage to EpCAM-expressing organs. What could be the basis for a differential recognition by a bispecific antibody of EpCAM on tumor cells versus the EpCAM abundantly expressed on normal tissues?

A number of recent studies have shown that EpCAM on normal epithelial cells is in complex with other proteins in the plasma membrane within so-called tetraspanin webs. Protein partners of EpCAM include tetraspanins CD9 and 14.1, claudin 7, and CD44 (42–44). These protein-protein interactions may reduce the accessibility of EpCAM on the surface of normal tissue and leave only few epitopes for recognition by antibodies. Upon neoplastic transformation, EpCAM can be overexpressed on certain tumor cells, which may titrate out protein partners. Likewise, free EpCAM may arise from loss of protein partners during transformation, as has been reported for CD9 (45). Accessibility of EpCAM-expressing cells to both antibodies and immune effector cells may further increase upon degradation of extracellular matrix by tumor cell–derived proteases and by loss of tight junctions in tumor tissue. In support for a sequestration of EpCAM in normal epithelia, immunohistochemical studies showed that EpCAM is concentrated within intercellular boundaries, whereas it is evenly distributed on the surface of tumor cells (46). There may in fact be three

Figure 3. EpCAM expression on selected human and murine tissues. Sections of fresh frozen EpCAM-positive (pancreas and prostate) and EpCAM-negative (brain and endothelium) mouse and human tissues were stained as described in Materials and Methods. The mouse and human EpCAM–specific antibodies as well as the corresponding isotype matched control antibodies were used at a concentration of 10 μg/mL, and EpCAM expression was evaluated by immunohistochemical staining of bound antibodies. Isotype ctrl, isotype control.
levels contributing to a differential recognition of EpCAM: (a) a molecular level, where accessibility of certain EpCAM epitopes is controlled by protein partners; (b) a cellular level, where EpCAM is sequestered at least in part by its subcellular distribution within highly structured normal epithelium; and (c) a tissue level, where access of immune effector cells to epithelium is diminished by intact extracellular matrix. Future research is required to investigate these various possibilities and determine their contribution to a therapeutic window as seen with some but not all EpCAM-directed therapeutic approaches.

How transferable are safety results obtained with muS110 in mice to the human EpCAM/CD3-bispecific BiTE antibody MT110 in humans? Transferability relies on both the comparability of muS110 and MT110 in terms of biological activity and of EpCAM expression in man and mouse. To the extent possible, muS110 was constructed to yield structural, biochemical, and biological characteristics similar to those of drug candidate MT110. After all, distinct antibodies had to be used for generation of muS110 and MT110, and the comparison of the two BiTE antibodies is
hampered by the use of different effector cells and target cells, and a pronounced species difference.

The affinity of muS110 and MT110 for EpCAM was almost identical, but muS110 did bind more avidly to CD3 than MT110. Although this may increase the risk of nonconditional T-cell activation by muS110, it apparently did not positively affect the potency of redirected lysis by muS110. If anything, MT110 was more potent with human T cells than muS110 with murine splenocytes in redirected lysis of human or mouse EpCAM-expressing CHO target cells, respectively. Control of tumor growth in respective mouse models was achieved by muS110 and MT110 at similar doses, suggesting that the two BiTE antibodies had rather comparable biological activities in vivo. At present, we do not know whether muS110 and MT110 recognize the same domain on their respective EpCAM orthologs and whether possible differences matter for a therapeutic window.

With respect to EpCAM expression, a significant overlap was found between mouse and human tissues. The parental anti-EpCAM antibodies used for muS110 and MT110 stained most critical organs with similar intensity. In particular, pancreas, a critical organ for toxicity of anti-EpCAM antibody, showed comparable EpCAM expression in man and mouse. A key difference was EpCAM expression on mouse lymphocytes, which was not seen on human lymphocytes. As reported for the B-lymphocyte-specific BiTE antibody MT103 in chimpanzee, the immediate encounter of T cells and B target cells in blood can cause transient cytokine release by MT103 (47). This may be less pronounced with a BiTE targeting localized EpCAM target in tumor tissue. The 33-fold higher affinity of muS110 for the murine CD3 ortholog and the species-specific expression of EpCAM on murine lymphocytes may rather enhance the toxicity profile of muS110 in relation to MT110. However, only a careful dose-escalation clinical phase I study will ultimately determine the tolerability of MT110 in humans. The present data with muS110 are nevertheless encouraging and suggest that a therapeutic window may also exist for EpCAM-directed BiTE antibodies administered to man.

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