New Gene-Immunotherapy Combining TRAIL-Lymphocytes and EpCAMxCD3 Bispecific Antibody for Tumor Targeting

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Abstract

**Purpose:** To enhance T-cell responsiveness toward cancer cells, we overexpressed TRAIL in lymphocytes, as this death ligand induces tumor-specific apoptosis. To increase contact time of lymphocytes with tumor cells and thereby of TRAIL with its death receptors, lymphocytes were linked to the CD3 arm of bispecific antibody EpCAMxCD3, to guide the lymphocytes to tumor cells positive for the cancer stem cell marker EpCAM/ESA.

**Experimental Design:** Lymphocytes were transduced with TRAIL lentivirus and the antitumor effect in presence and absence of EpCAMxCD3 was evaluated in vitro and in xenograft studies using epithelial cell adhesion molecule (EpCAM)-positive pancreatic and prostate cancer cells.

**Results:** Compared with control lymphocytes, TRAIL-lymphocytes increased cytotoxicity and further induced expression of several apoptosis-related molecules. Cotransplantation of TRAIL-lymphocytes and tumor cells in mice or peritumoral injection of TRAIL-lymphocytes in larger xenografts retarded growth and induced apoptosis. Combination of TRAIL-lymphocytes with EpCAMxCD3 potentiated tumor eradication by enhancing antiapoptotic and antiproliferative signaling and by decreasing tumor vasculature. Intratumoral cyst formation was involved and associated with enhanced chemokine secretion and infiltration of mouse macrophages, suggesting contribution of an inflammatory host response. Most importantly, tumorigenicity of pancreatic cancer cells with cancer stem cell features resistant to conventional chemotherapy was strongly reduced.

**Conclusions:** This gene-immunotherapeutic approach may be a new tool to support endogenous immune responses toward cancer even in its advanced stages. *Clin Cancer Res; 18(4); 1–11. ©2012 AACR.*

Introduction

Pancreatic adenocarcinoma and advanced prostate cancer are aggressive malignancies with poor prognosis (1). Nontoxic new approaches are needed to improve overall and progression-free survival. A promising strategy is the use of TRAIL, which is a member of the TNF superfamily of death receptor ligands. TRAIL is a transmembrane protein that is cleaved into soluble TRAIL (sTRAIL). Preclinical data show that sTRAIL is a cancer cell-specific molecule exerting antitumor activity in vitro and in mice (2, 3). Phase I/II clinical trials have shown a favorable toxicity profile for sTRAIL but limited evidence of antitumor activity. This is probably due to short exposure of tumor cells to low TRAIL concentrations and to short half-life of sTRAIL (4, 5). Strategies to enhance the therapeutic activity of TRAIL include cell-based transportation of the full-length, membrane-bound mTRAIL (6–8). Two examples of cell-based approaches are the retransfusion of mTRAIL-transfected tumor-infiltrating T cells of patients with renal cell carcinoma (9) and of tumor-infiltrating T cells preincubated with recombinant TRAIL-fusion proteins anti-CD3:TRAIL and K12:TRAIL (10). However, the delivery of mTRAIL to tumors using human primary lymphocytes or other immune effector cells from peripheral blood has not been explored so far.

Another novel immunotherapeutic strategy uses bispecific antibodies (bsAb), which are artificial molecules with a
CD44 is a relevant marker for pancreatic cancer stem cells. As shown by Li and colleagues, the ESA cancer stem cell marker (21–23), present in several tumor entities including pancreatic (23, 24) and prostate (25) cancer. Remarkably, EpCAM, also known as ESA, is suggested as a cancer stem cell marker expression as we described recently (26).

In the present study, we stably overexpressed mTRAIL in primary human lymphocytes by lentiviral transduction and found an increased antitumor effect involving reduction of tumorigenicity of pancreatic cancer cells with stem cell character, which was potentiated by combination with EpCAMxCD3.

Translational Relevance

Our proof of concept study is the first step for further development of a systemic gene-immunotherapeutic approach. Autologous patient lymphocytes will be transduced ex vivo to TRAIL-overexpressing lymphocytes, which are in turn guided by bispecific antibody EpCAMxCD3 to the tumor microenvironment. Both components induce cell death by different signaling pathways, which enhances tumor cell death even in cells with cancer stem cell characteristics. In addition, this approach may sensitize for conventional cytotoxic therapy as both EpCAMxCD3 and TRAIL-lymphocytes induce an inflammatory response. The clinical feasibility of such a gene-immunotherapy may be understood as an initial aid to support endogenous immune responses and the effect of cytotoxic tumor therapy.

dual specificity for antigens. Many bsAb constructs directed against the CD3 molecules on lymphocytes and against distinct tumor-associated antigens are currently in preclinical and clinical testing. Her2/neu, epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), EpH2A, CD33, CD19, and epithelial cell adhesion molecule (EpCAM/ESA/CD326) are frequently chosen targets for such bsAb (11). A prominent example for an EpCAM-directed reagent represents the trifunctional antibody catumaxomab (12), which enhances the antitumor activity by redirecting T cells via CD3 and Fcg receptor I/III-positive accessory cells to the tumor. Results of a prospective randomized phase II/III trial indicate prevention of malignant ascites in patients with epithelial cancer resistant to conventional chemotherapy (13). Recently, we showed the effectiveness of our bsAb EpCAMxCD3 (HEA125xOKT3), which redirected T cells to EpCAM-positive tumor cells, increased the duration of contacts between T cells and tumor cells and reduced malignant ascites in patients with advanced ovarian cancer (14, 15).

Importantly, EpCAM is overexpressed by the majority of epithelial tumors and derived metastases but not by normal cells (16). EpCAM-positive tumor entities include pancreatic and prostate adenocarcinomas (17, 18). Increased EpCAM expression indicates a poor prognosis as shown, for example, in breast and gallbladder carcinomas (19, 20). Remarkably, EpCAM, also known as ESA, is suggested as a cancer stem cell marker (21–23), present in several tumor entities including pancreatic (23, 24) and prostate (25) cancer. As shown by Li and colleagues, the ESA+/CD44+/CD24+/ESA- population from human resected pancreatic tumor tissue formed tumors in mice to a much higher frequency than ESA+ cells (23). The same group showed that the marker combination CD44+/CD24+/ESA+ was most potent in tumor formation in mice compared with CD44+/CD24+/ESA-. Therefore, EpCAM (ESA) alone or in combination with CD24 and CD44 is a relevant marker for pancreatic cancer stem cells.

Analysis of apoptosis in vitro

To detect apoptosis induction by TRAIL-lymphocytes, BxPc-3 cells were grown on chamber slides. TRAIL- or MOCK-lymphocytes were added to 70% confluent BxPc-3 cells. Twenty-four hours later, lymphocytes were removed and attached BxPc-3 cells were fixed in acetone. Staining for active caspase-3 was carried out with rabbit anti-human pAb (R&D Systems). The signal was enhanced by a Vectastain ABC Elite kit. 3,3’-diaminobenzidine (DAB) was used as a chromogen.

Xenograft tumors

In the first experiment, a combination of BxPc-3 or PC-3 tumor cells (5 × 10⁶) and TRAIL-lymphocytes (5 × 10⁶) was most potent in tumor formation in mice compared with CD44+/CD24+/ESA- cells. Therefore, EpCAM (ESA) alone or in combination with CD24 and CD44 is a relevant marker for pancreatic cancer stem cells.

Materials and Methods

Primary and established cell lines

Human peripheral blood mononuclear cells (PBMC) and skin fibroblasts were obtained from healthy donors following approval by the ethical committee of the University of Heidelberg, Heidelberg, Germany. Established human cell lines BxPc-3 (pancreatic adenocarcinoma), PC-3 (prostate adenocarcinoma). HEK 293T (kidney epithelial) were from American Type Culture Collection (ATCC) and authenticated throughout the culture by the typical morphology.

GEM-BxPc-3 were obtained by continuous incubation of BxPc-3 cells during several months in increasing concentrations of gemcitabine up to 100 nmol/L leading to selection of gemcitabine-resistant cells with enhanced cancer stem cell marker expression as we described recently (26).

Antibodies and reagents

EpCAMxCD3 (HEA125xOKT3) and CD19xCD3 (HD37xOKT3) bsAb were produced by hybrid hybridoma technique as described (27). Parental anti-human EpCAM hybridoma (HEA125; IgG1) was raised in our laboratory. Hybridoma OKT3 (IgG2A) directed against the e-chain of the CD3 molecule was purchased from ATCC. Gemcitabine was from Fresenius Kabi.

Transduction of lymphocytes

PBMCs (3 × 10⁶ cells per mL) were activated as described (15) and transduced at multiplicity of infection (MOI) 10 in 24-well plates precoated with retronectin (TaKaRa), followed by incubation at 37°C for 24 hours. This procedure was repeated after 24 hours. After transduction, lymphocytes were incubated in complete cell culture medium supplemented with interleukin (IL)-2 (100 U/mL). Lymphocytes were used for experiments at day 5 after transduction.

Analysis of apoptosis in vitro

To detect apoptosis induction by TRAIL-lymphocytes, BxPc-3 cells were grown on chamber slides. TRAIL- or MOCK-lymphocytes were added to 70% confluent BxPc-3 cells. Twenty-four hours later, lymphocytes were removed and attached BxPc-3 cells were fixed in acetone. Staining for active caspase-3 was carried out with rabbit anti-human pAb (R&D Systems). The signal was enhanced by a Vectastain ABC Elite kit. 3,3’-diaminobenzidine (DAB) was used as a chromogen.

Xenograft tumors

In the first experiment, a combination of BxPc-3 or PC-3 tumor cells (5 × 10⁶) and TRAIL-lymphocytes (5 × 10⁶)
in PBS were transplanted subcutaneously to 5-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice in a volume of 100 μL. Three days later, animals were randomized to 5 groups (n = 5 per group). EpCAMxCD3 (0.01 or 0.1 mg/kg), irrelevant bsAb CD19xCD3, parental bivalent mAb anti-human CD3 (OKT3), anti-human EpCAM (HEA125) mAb (1 mg/kg), or PBS were injected intraperitoneally. All mice were given 1 mg/mL of doxycycline (Sigma) and 5% glucose in the drinking water. Five injections of EpCAMxCD3 or control mAbs were administered intraperitoneally every third day. In a second experiment, BxPc-3 cells (5 × 10^6) were engrafted and 12 days later, when the tumors reached a volume of 30 to 40 mm^3, TRAIL-lymphocytes (5 × 10^6) were preloaded with EpCAMxCD3 (1 mg/mL) for 1 hour and then transplanted subcutaneously around the tumor. Animals were randomized to 3 groups (n = 5 per group) and received intraperitoneal injections of EpCAMxCD3 at a dose of 0.01 mg/kg, 0.1 mg/kg, or PBS. Controls received EpCAMxCD3 at a dose of 0.1 mg/kg or PBS only. In a third experiment, GEM-BxPc-3 cells were left untreated or were incubated with TRAIL-lymphocytes or with TRAIL-lymphocytes preloaded with EpCAMxCD3 (1 mg/mL) for 2 hours at an effector:target (E:T) ratio of 10:1. Lymphocytes were removed by washing and 100 μL viable gemcitabine-resistant BxPc-3 cells (6 × 10^3) were transplanted to each of 4 NMRI Fox nu/nu mice/treatment group in 50% Matrigel/PBS followed by monitoring of tumor engraftment (tumor take). Tumor size was measured and analyzed as described (26). The animal experiments were approved by the Ethical Committee.

Analysis of xenograft tissue

Frozen 6-μm xenograft tissue sections were examined by histochemistry and immunohistochemistry using standard avidin-biotin technique with a Vectastain ABC Elite kit (Vector).

Real-time reverse transcriptase PCR

mRNA was isolated from frozen tumor samples, converted to cDNA by standard techniques and PCR was carried out with the LightCycler FastStart DNA Sybr GreenI kit (RAS) according to the protocol provided in the parameter specific kits. The transcript number was calculated from a standard curve, obtained by plotting known input concentrations of 4 different plasmids at log dilutions to the PCR-cycle number (CP) at which the detected fluorescence intensity reaches a fixed value. The calculated copy numbers were normalized according to the expression of cyclophilin B.

Viability, colony formation, and ALDH1 activity

These features were measured as described in our recent publication (26).

Statistical analysis

Data are presented as the mean ± SD. The Student t test and χ² test were used to evaluate the difference between groups. Significance of growth kinetics over time was evaluated using variance analysis with repeated measurement and Post hoc test for comparison of groups after Dunnett. P < 0.05 was considered statistically significant.

Results

TRAIL-lymphocytes induce apoptosis in tumor cells

Whereas resting lymphocytes express only minimal amounts of TRAIL, expression is enhanced to a median level upon lymphocyte stimulation (28). To further increase expression to therapeutic active levels, we cloned membrane TRAIL into a lentiviral vector. Upon lentiviral transduction of lymphocytes and activation, TRAIL expression was measured by fluorescence-activated cell-sorting (FACS) analysis. While TRAIL expression in activated lymphocytes transduced with control vector was 7%, the level was further increased to 57% by the lentiviral TRAIL construct.
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Our recent study showed that this bsAb retards growth of BxPc-3 xenografts and induces cell death by mediating enhanced contact time between lymphocytes and tumor cells associated with the release of granzyme B, perforin, IFN-γ, TNF-α, IL-10, and TGF-β1 (Supplementary Table S2; ref. 15). To investigate the effect of EpCAMxCD3 to cytotoxicity of TRAIL-lymphocytes in vivo, immunodeficient mice were transplanted subcutaneously with EpCAM-positive BxPc-3 or PC-3 tumor cells in presence or absence of nontransduced or TRAIL-lymphocytes. Three days later, EpCAMxCD3, or parental antibody and irrelevant bsAb control antibodies were intraperitoneally injected 5 times in 3 day intervals. Compared with nontransduced lymphocytes, TRAIL-lymphocytes induced tumor growth retardation in BxPc-3 tumors, whereas the effect in PC-3 xenografts was not significant (Fig. 2A). The TRAIL effect was potentiated in both tumor entities by combination with EpCAMxCD3, which was administered 3 days after transplantation in low doses of 0.1 and 0.01 mg/kg. Whereas in PC-3 cells, both doses reduced tumor sizes below the levels obtained by TRAIL-lymphocytes alone, only the higher dose

Delivery of TRAIL-lymphocytes by EpCAMxCD3 increases retardation of xenografts

To enforce the antitumor activity of TRAIL-lymphocytes, we carried out combination experiments with EpCAM as our recent study showed that this bsAb retards growth of mainlly CD4+ and CD8+ cells were transduced and that lentiviral transduction does not affect lymphocyte function (Supplementary Fig. S1B and S1C). Next, BxPc-3 and PC-3 were used as targets, as these cells express TRAIL receptor 1 and 2 and are sensitive to recombinant sTRAIL (Supplementary Fig. 1E and S1F). In addition, both cell lines harbor tumor cells with cancer stem cell features and express EpCAM (15, 29, 30). To examine the effect of TRAIL-lymphocytes to apoptosis, we cultured BxPc-3 cells alone or together with preactivated TRAIL- or MOCK-lymphocytes. Twenty-four hours later, the typical apoptotic blebbing of dying cells was observed, and caspase-3 activity was measured by immunofluorescence staining followed by counting positive cells. Whereas the basal apoptosis induction was 5%, MOCK-lymphocytes increased it to 60%. This strong apoptosis induction already by control lymphocytes may be due to extensive activation by anti-CD3 mAb and IL-2 prior to coincubation. This most probably led to spontaneous release of cytotoxic substances from granules as perforins and granzyme B, which are responsible for cell death. Apoptosis was further increased by TRAIL-lymphocytes to 80% suggesting additional specific induction of apoptosis by TRAIL (Fig. 1A). These data were confirmed by measurement of apoptosis by propidium iodide staining and FACS analysis 4 hours after coincubation (Fig. 1B). To investigate suitability of TRAIL-lymphocytes in a model reflecting the tumor microenvironment, we established 3-dimensional tumor reconstructs by embedding BxPc-3 cells together with lymphocytes and fibroblasts in collagen matrix. Seventy-two hours later, supernatants and cleared lysates of 3-dimensional gels were collected and subjected to protein array analysis. Compared with control 3-dimensional constructs harboring BxPc-3 cells and MOCK-lymphocytes, expression of apoptosis-related proteins was increased in the presence of TRAIL-lymphocytes (Supplementary Fig. S2A and S2B). Marked upregulation of procaspase-3 and of cleaved caspase-3 occurred, confirming our in vitro results (compare Fig. 1A and data not shown). In addition, enhanced expression of the proapoptotic proteins cytochrome c, FADD, CD95/TNFSF6, HTRA2/Omi, and SMAC/Diablo was strongly activated. Activation of expression of the antiapoptotic proteins catalase, HIF-1α, HO-1, HO-2, Hsp27, Hsp60, and survivin was also observed, although the level of upregulation was rather low in most cases. To exclude the possibility that the effects of TRAIL-lymphocytes could be tumor type or cell line specific, we carried out experiments with PC-3 prostate cancer cells, which confirmed the earlier described results (data not shown). These data suggest that TRAIL-lymphocytes are superior to MOCK-lymphocytes in inducing apoptosis in tumor cells.

Figure 2. EpCAMxCD3 enhances tumor retardation by TRAIL-lymphocytes. A, BxPc-3 or PC-3 cells were subcutaneously grafted in the presence of lymphocytes to NOD/SCID mice (n = 6 per group) and treated 3 days later with intraperitoneal injections of EpCAMxCD3 in doses indicated as described in Materials & Methods. L, nontransduced lymphocytes. Tumor size was measured every third day. Tumor volume is depicted as mean ± SD. B, weight of tumors at the end point of the experiment at day 23. *, P < 0.05.
of 0.1 mg/kg further increased the TRAIL-effect in BxPc-3 cells. Parental or irrelevant control antibodies did not change tumor growth as expected (data not shown). At day 23, tumors were resected and the weight was determined, which confirmed tumor growth kinetics (Fig. 2B, data not shown). Metastases in lung, liver, or spleen were not detectable in either treatment group as examined by macroscopic inspection, FACS analysis, and immunohistochemistry of tissues for expression of human MHC class I antigen and for human cytokeratins 4, 5, 6, 10, 13, and 18. Body weight or general condition of mice was not affected by tumor growth and treatment (data not shown).

**Delivery of TRAIL-lymphocytes by EpCAMxCD3 inhibits proliferation, angiogenesis and induces apoptosis and inflammation**

For evaluation of mechanisms involved in xenograft retardation, we conducted immunohistologic staining for proliferation (Ki-67), vessel density (CD31), and apoptosis [terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), active caspase-3] using frozen tissue sections obtained at day 23 after treatment. Therapeutic effects to these parameters were obvious for combination treatment but were less pronounced for TRAIL-lymphocytes alone (Fig. 3 and data not shown). This was expected and may be due to the rather late time point after treatment at which apoptosis induction by the relatively insensitive TUNEL and active caspase-3 measurements may be not detectable anymore. To overcome these assay-specific limitations, we examined mRNA expression of apoptosis- and inflammation-related genes in tumor tissue by reverse transcriptase PCR (RT-PCR). The most prominent changes induced by TRAIL-lymphocytes were downregulation of the death receptors TRAIL-R2 and CD95, downregulation of the antiapoptotic gene survivin, and upregulation of the antiapoptotic genes Hsp27 and Hsp70, as well as upregulation of the inflammation-related genes HIF-1α, NF-κB, and VEGF (Supplementary Table S1). Combination with EpCAMxCD3 enhanced expression of the proapoptotic genes Bax, TRAIL, p21, TNF-R1 and restored TRAIL-R2 expression. However, combination treatment induced also an antiapoptotic response, as Bcl-2, Bcl-xL, cIAP1, cIAP2, XIAP, survivin, Hsp27, and Hsp70 were upregulated along with HIF-1α, NF-κB, and VEGF. These results suggest that combination of TRAIL-lymphocytes with EpCAMxCD3 induces more pronounced tumor retardation and changes in expression of genes involved in antiapoptotic, proapoptotic, and inflammation signaling.

**EpCAMxCD3 potentiates TRAIL-lymphocyte-induced retardation of larger tumors**

To investigate the effect of TRAIL-lymphocytes to larger tumors, we transplanted BxPc-3 cells and treated the mice after xenograft tumors had developed 12 days later. TRAIL-lymphocytes alone or TRAIL-lymphocytes preloaded with EpCAMxCD3 were injected subcutaneously around the tumor. Injections of EpCAMxCD3 were repeated 3 times in 3-day intervals. At day 23 after treatment, tumor xenografts were resected and evaluated. TRAIL-lymphocytes, EpCAMxCD3, and both together had induced large liquid areas within the tumor tissue due to voluminous cyst

![Figure 3. Reduction of proliferation and blood vessel density and induction of apoptosis in vivo. BxPc-3 tumor xenografts as described in Fig. 2B were harvested at day 23 after treatment. Ki-67 expression indicating proliferation was detected in frozen tissue sections by immunohistochemistry and quantified in 10 vision fields under 400× magnification. Blood vessel density was detected by staining with CD31/PECAM-1 and quantified under 100× magnification. Apoptosis was detected by staining of active caspase-3 and TUNEL assay and quantified in 10 vision fields under 400× magnification. *, P < 0.05. par, parental; irr, irrelevant.](cancerres.aacrjournals.org)
formation (Fig. 4A). These cysts were not due to hemorrhage and did not contain cell debris as determined by macroscopic and microscopic inspection (data not shown). Also, such cysts had already been observed in the former experiment presented in Fig. 3, although less pronounced, which may be due to the earlier treatment. We assume that these cysts are due to induction of inflammation by both, TRAIL-lymphocytes and EpCAMxCD3, as suggested by the results of the RT-PCR experiment, in which induction of expression of inflammation-related genes was found (compare Supplementary Table S1). Although no significant differences in relative tumor size were detectable by staining for active caspase-3 and TUNEL assay according to the results of Fig. 3. Together, the most obvious effect by which TRAIL-lymphocytes alone or combined with EpCAMxCD3 induce tumor retardation is by liquefying the tumor tissue in intratumoral cysts and inhibition of tumor angiogenesis.

**Tumor tissue may be lysed by TRAIL-lymphocyte- and EpCAMxCD3-induced inflammation and associated cyst formation**

To evaluate involvement of infiltrating mouse macrophages to inflammation and cyst formation, we used BxPc-3 tumor tissue obtained from the experiment shown in Fig. 3 and stained it with mouse specific F4/80 and CD68 antibodies. TRAIL-lymphocytes and EpCAMxCD3 alone or in combination strongly promoted infiltration of tumor tissue by TRAIL-lymphocyte- and EpCAMxCD3-induced inflammation and associated cyst formation.

**Figure 4.** Gene-immunotherapy reduces tumor mass of advanced xenografts by cyst formation. BxPc-3 xenografts (n = 5 per group) were engrafted in mice. Twelve days later treatment was initiated by subcutaneous injections around the tumor. PBS (CO), EpCAMxCD3 (bsAb), or (TRAIL-L + bsAb) TRAIL-L preloaded with EpCAMxCD3 1 hour *in vitro* prior to injection as described in Materials and Methods. A, at day 23 after treatment, mice were sacrificed and xenografts were resected. Tumor morphology was evaluated by microscopy following hematoxylin and eosin (H&E) staining of frozen tissue. Representative mouse xenografts were photographed and images are shown at 5× and 100× magnification. B, tumor growth is shown as the increase of tumor size starting from day 12 after tumor cell implantation. C, blood vessel density was analyzed by staining of CD31 and evaluation of CD31-positive structures under 100× magnification (n = 4 per group). *, P < 0.05.
islets with F4/80- and CD68-positive macrophages (Fig. 5). In contrast, macrophages in tumors, which are derived from mice treated with control lymphocytes were localized predominately in collagen-rich stroma surrounding BxPc-3 tumor islets. Macrophages could be detected at the periphery of the islets only occasionally. These results were confirmed by examination of tumor tissue obtained from the experiment shown in Fig. 4 (data not shown). Interestingly, such a cyst formation was not observed by treatment of BxPc-3 xenografts with control lymphocytes and EpCAM alone (15). Thus, these results suggest that TRAIL-lymphocytes induce an inflammatory host response, which is increased by EpCAMxCD3, and inflammation is most likely the reason for cyst formation.

Inhibition of tumor take of cancer stem-like cells by TRAIL-lymphocytes and EpCAMxCD3 is associated with inhibition of proliferation and induction of apoptosis

Finally, we analyzed whether TRAIL-lymphocytes and EpCAMxCD3 may have the potential to eliminate tumorigenicity of highly therapy resistant cancer cells with cancer stem cell characteristics. We used gemcitabine-resistant BxPc-3 cells (GEM-BxPc-3), which we recently selected by continuous treatment with increasing concentrations of gemcitabine for several weeks. This resulted in enhanced expression of the cancer stem cell markers CD44/CD24 (26). While GEM-BxPc-3 compared with parental BxPc-3 cells are resistant toward gemcitabine, they are still sensitive for TRAIL-induced cytotoxicity as measured by MTT assay (Fig. 6A). In support of the cancer stem cell character of GEM-BxPc-3 cells, we found that these cells possess higher colony-forming capacity and ALDH1 activity than parental cells (Supplementary Fig. S3A and S3B). Correspondingly, coincubation of GEM-BxPc-3 for 24 hours with TRAIL-lymphocytes at an effector:target ratio of 10:1 led to specific activation of caspase-3 as determined by immunofluorescence microscopy (Supplementary Fig. S3C). Likewise, the number of viable cells after treatment with TRAIL-lymphocytes was reduced compared with cells treated with MOCK-lymphocytes, as found by detection of morphology by light microscopy. For evaluation of tumorigenicity, we treated GEM-BxPc-3 cells with TRAIL-lymphocytes alone or preloaded with EpCAMxCD3. Two hours later, viable cells were counted and $6 \times 10^3$ viable cells were subcutaneously transplanted to mice ($n = 4$ per group). C, tumor engraftment (tumor take) was monitored for 35 days. D, frozen tissue sections of grown GEM-BxPc-3 tumors were analyzed for proliferation by immunohistochemistry. Ki-67-positive tumor cells were quantified in 10 vision fields under 400× magnification. Apoptosis was detected by staining for active caspase-3 and positive tumor cells were quantified in 10 vision fields under 400× magnification.

* $P < 0.05$.  

Figure 6. Inhibition of tumor take, proliferation, and apoptosis resistance of tumor cells with cancer stem features by TRAIL-lymphocytes and EpCAMxCD3. A, viability was measured in BxPc-3 and gemcitabine-resistant GEM-BxPc-3 cells 72 hours after treatment with 50 nmol/L gemcitabine (GEM) or recombinant sTRAIL at concentrations indicated. B, for in vivo experiments, GEM-BxPc-3 cells were left untreated (CO) or were coincubated with TRAIL-L alone or TRAIL-L preloaded with EpCAMxCD3. Two hours later, surviving cells were counted and $6 \times 10^3$ viable cells were subcutaneously transplanted to mice ($n = 4$ per group).
positive cells was higher in tumors derived from TRAIL-lymphocyte-pretreated cells whereas combination with EpCAMxCD3 further increased these effects (Fig. 6D). These results suggest a long-term effect of TRAIL-lymphocytes and EpCAMxCD3 to tumorigenic cells, which might not have been eliminated immediately but over time. We did not detect changes in the cancer stem cell marker CD24/CD44 expression profile in tumors derived from TRAIL-lymphocyte-treated cells and were not able to measure it in EpCAMxCD3 combination treatment due to incompatibility of secondary antibodies with EpCAMxCD3 antibody (data not shown). Nevertheless, reduced tumor take along with reduced proliferation and enhanced apoptosis strongly suggest that tumorigenic properties are targeted by TRAIL-lymphocytes with enhanced effect after combination with EpCAMxCD3.

Discussion

The present study shows that overexpression of mTRAIL in primary human lymphocytes increases T-cell responsiveness toward cancer cells of pancreas and prostate and combination with EpCAMxCD3 potentiates the effect as analyzed in vitro and in vivo. From our results, we conclude that EpCAMxCD3 guided TRAIL-lymphocytes to the tumor microenvironment and increased exposure time of TRAIL overexpressed by lymphocytes to its receptors expressed by tumor cells. Although we did not directly detect that EpCAMxCD3 increases the exposure time of TRAIL and its receptors by linking tumor cells and TRAIL-lymphocytes in vivo, there are strong indirect hints that this was indeed the case. First, we know that EpCAMxCD3 increases duration of the contact between lymphocytes and carcinoma cells without affecting lymphocyte migration in vitro (15). Second, we found that intraperitoneally injected EpCAMxCD3 increased cytotoxicity of TRAIL-lymphocytes, which were cotransplanted with tumor cells in mice. In contrast, parental antibody and irrelevant bsAb control antibodies did not increase the antitumor effect of TRAIL-lymphocytes. Importantly, we did not observe toxic side effects of this gene-immunotherapeutic approach, such as a decrease of body weight, liver toxicity, or metastasis in mice.

The mechanisms by which the 2 independent stimuli, TRAIL-lymphocytes and EpCAMxCD3, each for its own induce cell death are already known and published. TRAIL induces apoptosis in sensitive tumor cells expressing TRAIL receptors by death receptor-mediated apoptosis signaling (4). EpCAMxCD3 brings T cells and tumor cells in close contact by redirecting T cells to kill carcinoma cells (27). By binding to CD3 molecules on lymphocytes with one arm and to EpCAM antigen on tumor cells with the other arm, EpCAMxCD3 activates lymphocytes in an antigen-dependent manner. This lymphocyte activation leads to the production of effector cytokines and cytolytic substances such as IFN-γ, TNF-α, granzyme B, and perforin (15). Furthermore, Wimberger and colleagues showed that tumor resident T cells following treatment with EpCAMxCD3 efficiently lysed malignant cells ex vivo. Therefore, in the present study it may be assumed that TRAIL-lymphocytes combined with EpCAMxCD3 eliminated tumor cells by a combination of TRAIL receptor-induced apoptosis and activation of lymphocytes, which in turn produced effector cytokines and cytolytic substances and eliminated the cells by the granzyme B and perforin death system. Another advantage of our strategy is that delivering of mTRAIL by lymphocytes may increase the half-life of TRAIL in vivo and thus potentiate the cytotoxic effect, as fast dilution and loss of efficacy has been described for directly delivered sTRAIL (32). Our data are confirmed by a recent study in which cell-mediated mTRAIL delivery has been successfully investigated for neural and mesenchymal stem cells in solid tumors (7, 33). In related publications describing targeting of lymphocytes by bsAb in vitro, tumor cells were eradicated by pore-forming, proapoptotic components of cytotoxic T cells involving perforin and granzyme B (34, 35). These mechanisms of general T-cell cytotoxicity may also be involved in the antitumor effects observed in our experiments, due to EpCAMxCD3-induced expression of perforin and granzyme B (15). Importantly, the combined cytotoxicity of TRAIL-lymphocytes and EpCAMxCD3 even reduced the tumor-initiating potential of advanced pancreatic cancer cells, which exhibit resistance toward gemcitabine chemotherapy. This result is underlined by a recent report (33), where delivery of TRAIL by mesenchymal stem cells was able to target tumor-initiating cells of squamous and lung cancer. Quite similarly, Herrmann and colleagues showed that EpCAMxCD3-mediated engaging of human T cells eliminates colorectal tumor-initiating cells.

We also observed inhibition of tumor vessel density in xenograft tumors with a most significant effect for combination treatment. These results are in line with recent publications showing that TRAIL induces apoptosis in cultured endothelial cells (37, 38). Antiangiogenic effects of TRAIL were also observed in xenografts of glioblastoma, multiple myeloma, and pancreatic cancer transplanted to immunodeficient mice (26, 39). More recently, intravenous injection of mTRAIL-expressing CD34+ cells exerted a potent antitumor activity in NOD/SCID mice bearing systemic multiple myeloma xenografts, which was characterized by early vascular disruption, resulting in hemorrhagic necrosis and tumor destruction (8). In the present study, EpCAMxCD3-induced release of IFN-γ and TNF-α from activated lymphocytes may have contributed to the observed antiangiogenic effect, as these molecules induce expression of chemokines CXCL-9 (MIG), CXCL-10 (IP-10), and CXCL-1 (I-TAC), which inhibit chemotaxis of endothelial cells (40). On the other side, we detected enhanced VEGF mRNA expression in xenograft tissue upon treatment with TRAIL-lymphocytes alone or in combination with EpCAMxCD3. This observation is in line with data from Secchiero and colleagues, who report that soluble recombinant TRAIL acts proangiogenic by inducing endothelial cell migration and vessel tube formation (41). Besides VEGF expression, TRAIL-lymphocytes alone and to a stronger extend in combination with EpCAMxCD3 led to enhanced mRNA expression of NF-κB, a factor associated with inflammation and...
survival. Survival signaling in parallel to apoptosis signaling is a well known matter upon treatment with TRAIL (42). This was reflected in our experiments by induction of for example FADD and SMAC/DIABLO by TRAIL-lymphocytes in 3-dimensional constructs suggesting upregulated apoptosis signaling. At the same time enhanced HIF1α and later on also enhanced VEGF occurred and reflect antiapoptotic signaling. However, because we did not detect enhanced CD31⁺ vessels but reduction after combined treatment with TRAIL-lymphocytes and bsAb, induction of hypoxia and enhanced angiogenesis by the observed expression of HIF1α and VEGF may be excluded. We suggest that the cellular balance of signaling pathways upon treatment with TRAIL-lymphocytes is shifted toward cell death, as membrane TRAIL may have stronger effects than soluble TRAIL and lymphocytes enhance TRAIL-induced cytotoxicity, especially when EpCAMxCD3 comes into play, which acts as an additional enhancer.

In the present study, we observed cyst formation in xenografts of mice, which were injected with TRAIL-lymphocytes alone or in combination with EpCAMxCD3. Because we did not observe cyst formation in xenografts of mice injected with EpCAMxCD3 alone (15), TRAIL-lymphocytes may be the reason for cyst formation. The reason for cysts is not entirely clear, however, as tumor cells die, for example, upon induction of apoptosis by TRAIL-lymphocytes, they release several factors that attract phagocytes, in particular macrophages (43) as observed in our study. EpCAMxCD3 may have contributed by induction of secretion of multiple chemokines in lymphocytes (15). Combined with TRAIL-lymphocytes this may have induced pronounced inflammation sufficient for induction of liquid containing cysts within the tumor tissue. This hypothesis is supported by our observation of enhanced NF-κB mRNA expression upon TRAIL-lymphocytes alone or combined with EpCAMxCD3, as NF-κB is a key player of inflammation. In line with our results, pancreatic cyst formation is frequently observed in inflamed pancreas of patients (44). Examinations of the fluid inside the cysts of patients suggest that it contains liquefied pancreatic tissue and cells of inflammation. In addition to inflammation, antiangiogenic effects mediated by our gene-immunotherapeutic approach may have increased cyst formation by inducing leaky blood vessels. Accordingly, Lavazza and colleagues showed that CD34⁺ cells engineered to express nTRAIL induced hemorrhagic necrosis in multiple myeloma xenografts (8). Thus cyst formation by TRAIL-lymphocytes and combination with EpCAMxCD3 may be a result of inflammation, induction of host response, and vasculature disruption together.

In our cell lines, we see that 50% of the population are TRAIL resistant and do not die upon TRAIL treatment. This matches with the observation that TRAIL resistance is frequently observed in around 50% of tumor cell lines. Upon treatment with TRAIL-lymphocytes, only a small cell population of the gemcitabine-resistant cancer stem-like BxPc-3 cells has active caspase-3 and showed less tumorigenicity in mice. This suggests that TRAIL-lymphocytes alone do not completely target cancer stem-like cells. However, combination with EpCAMxCD3 strongly increased therapeutic efficacy as shown by treatment of mice with tumor xenografts, by tumor take experiments in mice transplanted with pretreated cells and by in vitro experiments of caspase-3 analysis. We also carried out 3-dimensional experiments but saw no additional effect upon combination of TRAIL-lymphocytes with bsAb compared with TRAIL lymphocytes alone. This may be due to the fact that bsAb does only weakly induce apoptosis signaling but rather acts by the perforin/granzyme system (15).

Regarding translational relevance of local injection of TRAIL-lymphocytes and EpCAMxCD3, our model does not clearly mimic human T-cell-based adoptive cancer immunotherapy, where tumor-reactive/specific T lymphocytes are usually systemically administered to patients. We used a direct injection model as systemic administration of lymphocytes results in high organ distribution, that is, in lungs and liver and removal from circulation. In addition, preactivated lymphocytes have a short half-life due to activation-induced cell death. Our model rather reflects a proof-of-concept study similar to experimental approaches carried out by Schlereth and colleagues (45) or Brischwein and colleagues (46). Even in a more patient-related metastatic model with systemic injection, we assume that TRAIL-lymphocytes combined with EpCAMxCD3 may affect tumor cells stronger than EpCAMxCD3 or agonistic TRAIL receptor antibodies, which are already in clinical use. The advantage of our approach is that 2 different cell death systems act together, which potentiates the effect. Even highly therapy-resistant cells with cancer stem cell characteristics may be targeted as EpCAMxCD3 sensitizes tumor cells for TRAIL-induced apoptosis and vice versa. Regarding recent concerns about the safety of older retroviral vectors in patients, previous work has focused attention on self-inactivating (SIN) lentiviral vectors as potential alternative delivery platforms. In our study, we used these improved lentiviral vectors for TRAIL transfer to lymphocytes. Lentiviral vectors limit the risk of viral long terminal repeat (LTR) enhancer mutagenesis and permit the use of lineage-specific expression cassettes (47). However, we are aware that before our gene-immunotherapy may be applicable to treatment in patients, further experimental studies in a model closely resembling the situation in patients are necessary.

In conclusion, we show that TRAIL-lymphocytes combined with EpCAMxCD3 induce a strong antitumor response in our experimental system. This therapeutic approach was even effective in tumorigenic cells with cancer stem cell characteristics, which do not respond to conventional chemotherapy. An important positive effect of our method may be the "opening" of the tumor microenvironment for conventional cytotoxic therapy due to induction of an advanced inflammatory response, which may sensitize tumor cells for cytotoxic therapy or innate immune responses. The clinical feasibility of such a systemic gene-immunotherapy may be understood as an initial aid to
support innate immune responses and to enhance induction of cell death by cytotoxic tumor therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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References


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New Gene-Immunotherapy Combining TRAIL-Lymphocytes and EpCAMxCD3 Bispecific Antibody for Tumor Targeting

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