Clinical Studies

Cancer Research

Immunomonitoring Results of a Phase II/III Study of Malignant Ascites Patients Treated with the Trifunctional Antibody Catumaxomab (Anti-EpCAM × Anti-CD3)

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Abstract

Patients with malignant ascites secondary to primary carcinomas benefit from intraperitoneal therapy with the trifunctional antibody catumaxomab (anti-EpCAM × anti-CD3). Here, we report the analysis of peritoneal fluid samples from 258 patients with malignant ascites randomized to catumaxomab or control groups to investigate the molecular effects of catumaxomab treatment. In the catumaxomab group, tumor cell numbers and peritoneal levels of VEGF decreased, whereas the activation status of $CD4^+$ and $CD8^+$ T-cell populations increased more than two-fold after treatment. Notably, $CD133^+/EpCAM^+$ cancer stem cells vanished from the catumaxomab samples but not from the control samples. *In vitro* investigations indicated that catumaxomab eliminated tumor cells in a manner associated with release of proinflammatory Th1 cytokines. Together, our findings show that catumaxomab therapy activates peritoneal T cells and eliminates $EpCAM^+$ tumor cells, establishing a molecular and cellular basis to understand *in vivo* efficacy within the immunosuppressed malignant ascites tissue microenvironment. *Cancer Res; 72(1); 24–32. ©2011 AACR.*

Introduction

Malignant ascites is defined as the abnormal accumulation of peritoneal fluid caused by the dissemination of epithelial cancer cells. Thus, various types of abdominal tumors including those of the ovaries, endometrium, colon, stomach, pancreas, and others can be accompanied by this indication (1, 2). It emerges whether the equilibrium between fluid production and its drainage from the peritoneal cavity is disturbed by parameters such as by vessel obstruction due to high tumor mass, impaired lymphatic drainage, or fluid overproduction often caused by an increased secretion of VEGF by peritoneal tumor cells (3–5).

The trifunctional antibody catumaxomab effectively attaches to tumor and T cells via its epithelial cell adhesion molecule (EpCAM) and CD3-specific–binding site, respectively. In addition, catumaxomab binds to and activates Fcγ receptor $I/IIa/III^+$ accessory cells via its specific Fc region (6, 7). As shown experimentally, catumaxomab supplementation of peripheral blood mononuclear lymphocytes (PBMC)/$EpCAM^+$ tumor cell cocultures mediates a concerted attack of various immune cells on the tumor cells. The former executes a whole array of different killing mechanisms such as T-cell–mediated cytotoxicity, phagocytosis and perforin—mediated cell lysis which ultimately resulted in a significant reduction of tumor cells (8). Because EpCAM is expressed by approximately 87% to 100% of the main ascites-causing carcinomas (9), treatment of such indications, including malignant ascites, with catumaxomab appears very promising. In a clinical pilot and a phase I/II study (10, 11), it has been shown that intraperitoneal administration of catumaxomab effectively eliminated the tumor cells from the peritoneal cavity with the result of a sustained reduction of excessive ascites fluid.

On the basis of these encouraging results, an open-label, multicenter, randomized phase II/III study of patients with malignant ascites was initiated to compare the effect of catumaxomab versus mere paracentesis (control) on peritoneal tumor cell development (12). Patients with malignant ascites because of $EpCAM^+$ malignancies received catumaxomab intraperitoneally on days 0, 3, 7, and 10 at doses of 10, 20, 50, and 150 μg, respectively. Puncture-free survival, the primary investigational parameter of that study, was significantly higher in the catumaxomab group (median, 46 days) than in the control group (median, 11 days; $P < 0.0001$; HR, 0.254). Also the secondary endpoint, time to next paracentesis, showed a significant ($P < 0.0001$) prolongation from 13 days in the control group to 77 days in the catumaxomab group (HR, 0.169). Overall survival (OS) in the intent-to-treat population (full analysis set, $n = 258$) showed a positive trend

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in the pooled population and was statistically significant in the subgroup of patients with gastric cancer ($n = 66$; median, 71 vs. 44 days; $P = 0.0313$), although the study was neither designed nor powered for OS analysis. Noteworthy, OS analysis in the safety set (consisted of patients who received at least one catumaxomab infusion and of all patients randomized to the control group, $n = 245$) revealed that the OS rate at 6 months increased by factor 4 and the OS rate at 1 year tripled for catumaxomab-treated patients versus control patients (13). Here, we report the immunomonitoring results of a phase II/III study using the trifunctional antibody catumaxomab in cancer patients with malignant ascites due to diverse cancer types. Different immunologic parameters of cells in patient samples of peritoneal fluids were investigated. Our results contribute to the understanding of the *in vivo* effects catumaxomab executes, even within the prevailing immunosuppressed environment of malignant ascites (14) and its significance for treatment.

Materials and Methods

Study design and patient samples

The present investigation is a two-arm, randomized, open-label, phase II/III study with patients suffering from malignant ascites secondary to epithelial cancers requiring symptomatic therapeutic paracentesis. The study (EudraCT number: 2004-000723-15; ClinicalTrials.gov identifier: NCT00836654) was approved by an independent ethics committee at each of the involved study centers, and all patients gave written informed consent prior to their trial participation. Briefly, 258 patients with EpCAM-positive cancer were included in the study described by Heiss and colleagues (12). The distribution was 129 ovarian carcinomas and 129 non-ovarian carcinomas. The main cancer types in the non-ovarian stratum were gastric ($n = 66$; 51%), breast ($n = 13$; 10%), pancreas ($n = 9$; 7%), colon ($n = 8$; 6%), and endometrial ($n = 6$; 5%). It was conducted in compliance with Good Clinical Practice guidelines and the Declaration of Helsinki. Ascites samples were analyzed before (screening), during (day 3, before second catumaxomab infusion), and after therapy (day 11, 1 day after the fourth catumaxomab infusion) for different pharmacodynamic parameters, for example, detection of $EpCAM^+$ tumor cells, VEGF levels, and T-cell activation. Because of stability reasons, fresh ascites samples had to be shipped within 24 to 72 hours to the study laboratory. If spontaneous ascites fluid could not be obtained, a peritoneal lavage with a sterile sodium chloride solution was conducted. Such samples were treated in the same way as the ascites samples. The patient population was randomized 2:1 into catumaxomab treatment and control groups.

Tumor cell detection and monitoring of tumor cell numbers

An immunocytochemistry protocol for the detection of $EpCAM^+$ tumor cells in malignant ascites samples was established to evaluate if the individual patient samples would meet the inclusion criteria described below. Ascites cells taken at screening (immediately prior to the study) were harvested by standard sample centrifugation or Ficoll density centrifugation if erythrocytes were present to eliminate the latter. Subsequently, cytospins of 2.5×10^5 ascites cells were prepared by centrifuging them onto microscope slides. After drying 4 individual slides per sample, resembling 10^6 cells in total, slides were assessed for $EpCAM^+$ tumor cells via staining using the anti-EpCAM antibody HO-3 (TRION Research) directly labeled with Alexa Fluor 594 Texas Red fluorescence dye (Molecular Probes). HO-3 (15) is the parental antibody of the anti-EpCAM–binding arm of catumaxomab. The samples were categorized into the following criteria:

- EpCAM-negative ($EpCAM^-$) if no $EpCAM^+$ cells were detectable in 10^6 analyzed ascites cells
- EpCAM < 400 if less than 400 cells were found in 10^6 analyzed ascites cells
- $EpCAM^+$ if $\geq$ 400 $EpCAM^+$ cells were detectable in 10^6 analyzed ascites cells

For patient inclusion, the latter criterion had to be fulfilled.

$EpCAM^-$ samples were additionally counterstained with anti-cytokeratin 8, 18, 19 antibodies A45B-B3 (Micromet) and its corresponding secondary antibody anti-mouse IgG1 tagged with Alexa Fluor 488 (Molecular Probes). This immunocytochemistry staining technique was also used to determine the number of $EpCAM^+$ tumor cells from ascites fluid samples throughout the therapy. To prevent possible competitive inhibition of the staining antibody by catumaxomab residuals in the ascites samples collected during and after therapy, follow-up samples were stained with the Alexa Fluor 594 Texas Red–labeled anti-EpCAM antibody VU1D9 (TRION Research) which binds to a different EpCAM epitope than HO-3/catumaxomab does (11, 15). All cytospins were analyzed by a computerized image analysis system (MDS, Applied Imaging) counting Texas Red–labeled cells.

Determination of peritoneal T-cell activation markers

Fresh ascites cells [5×10^5 per fluorescence-activated cell-sorted (FACS) sample] were centrifuged, washed, and stained for FACS analysis with the antibody combinations anti-$CD45^+$ FITC/anti-$CD4^+$ PE/anti-$CD69^+$ APC or anti-$CD45^+$ FITC/anti-$CD8^+$ PE/anti-$CD69^+$ APC (Becton Dickinson). After 30 minutes of incubation at 2°C to 8°C, samples were washed and propidium iodide was added to exclude dead cells. Samples were analyzed in a FACSCalibur using the CellQuest Pro Software (Becton Dickinson).

Measurement of VEGF and total protein concentrations in ascites fluid samples

Relative VEGF levels (ratio of VEGF concentration to total protein concentration) were determined in ascites fluid samples derived from the catumaxomab patient group l as for the corresponding control group and were statistically analyzed using repeated measures ANOVA. VEGF levels were determined by the Quantikine human VEGF Immunoassay ELISA (R&D Systems GmbH) and total protein concentrations using a BCA Protein Assay kit (Thermo Fisher Scientific) following manufacturers' instructions.

Detection of putative CD133$^+$/EpCAM$^+$ cancer stem cells in malignant ascites fluid samples

Samples from a subset of catumaxomab and control group patients were analyzed for the presence of putative CD133$^+$/EpCAM$^+$ cancer stem cells (CSC) using double staining procedures on cytospin preparations. An anti-EpCAM antibody VU1D9 (TRION Research) tagged with Alexa Fluor 594 Texas Red fluorescence dye (Molecular Probes) and an anti-C133 antibody (Miltenyi Biotec) with its corresponding Alexa Fluor 488 FITC–tagged antibody were used. Malignant ascites fluid samples harvested at screening, before the second catumaxomab administration as well as 1 day after the last administration were analyzed. A total of 5×10^5 cells in total were evaluated per visit with a computerized image analysis system (MDS, Applied Imaging).

In vitro tumor cell elimination in ascites fluid samples (long-term clonogenic assay)

For long-term clonogenic assays, cells harvested from samples at screening were seeded in 24-well plates (10^6 cells per well). Catumaxomab was supplemented at day 0 (10 ng/mL) and day 3 (100 ng/mL); control samples were incubated without catumaxomab. After 11 days of incubation, all cells were harvested from the wells and cytospins were prepared from these. Cytospins were subsequently stained with the Texas Red–tagged anti-EpCAM antibody VU1D9 and the remaining EpCAM$^+$ tumor cells were quantified via an automated imaging analysis device (MDS).

Assessment of cytokines

Catumaxomab-mediated cytokine release was determined by culturing ascites cells harvested at screening for 24 hours in flat-bottomed 24-well plates. Catumaxomab was supplemented at a concentration of 10 ng/mL. A culture well devoid of catumaxomab served as the control. After 24 hours, the supernatants (100 μL) were collected and frozen at −20°C. Interleukin (IL)-2, IL-6, IFN-γ, and TNF-α were assessed via an in-house validated human Th1/Th2 cytometric bead array (CBA) kit (BD Biosciences Pharmingen). Data analysis was conducted using FACSCalibur with CBA software (BD Biosciences).

Statistics

All statistical tests were two-sided, at the 5% significance level without adjustment for multiple testing. Time-to-event parameters (OS, puncture-free survival, and time to first puncture) were compared between the treatment groups using the log-rank test and HRs. Comparison between groups with regard to tumor cell counts was conducted using the Wilcoxon test. Further descriptive statistical analyses such as means, medians, SDs, and ranges were calculated. Statistics was conducted by using the SAS software version 9.1.3.

Results

Patient selection based on EpCAM$^+$ cells

To determine the number of EpCAM$^+$ cells in the ascites fluid samples of study patients, an EpCAM detection test based on cytospin preparations and immunohistochemical staining combined with a computer-based evaluation was established and validated. In total, 433 patient fluid samples were sent to our central testing laboratory. Of these, 51 (12%) could not be evaluated because of logistic problems or other reasons. Three-hundred eighty-two samples were tested for EpCAM expression. Eleven of 382 samples (3%) had neither detectable EpCAM-positive nor cytokeratin 8, 18, 19-positive tumor cells questioning the malignant origin of these ascites samples. In the remaining screening population (371 patients), 55 patients were screening failures (15%) and were regarded to contain EpCAM-negative cells only. Finally, 316 patients (85%) were tested EpCAM-positive, 44 samples (14%) did not contain sufficient numbers of EpCAM-positive cells to pass the inclusion criteria (>400 cells of 10^6 analyzed cells), and 19 patients (6%) whose samples contained EpCAM-positive cells denied consent, suffered from deteriorating health, or died before study termination. Consequently, 253 patients with a positive EpCAM screening and 5 additional patients without having been tested for EpCAM upon patient exception request added up to the 258 patients with ovarian or non-ovarian primary cancer entities (pooled population) that were randomized to the study (Table 1).

Monitoring of EpCAM$^+$ tumor cells in malignant ascites during and after treatment

Reduced lymphatic drainage from the peritoneal cavity due to vessel obstruction as a consequence of tumor cell growth represents one major cause for malignant ascites formation (1–4). Therefore, we monitored tumor cell counts during and after therapy. The number of EpCAM$^+$ tumor cells per 10^6 ascites cells was analyzed in the catumaxomab and the control group at screening and at puncture visit of each included patient. Additional tumor cell assessments were conducted in the catumaxomab group before the second catumaxomab infusion (day 3) and one day after the last catumaxomab infusion (day 11). In the catumaxomab group, EpCAM-positive tumor cells dropped from a median screening value of 6,510 EpCAM$^+$ cells (165 patients) to a median of 27 cells on day 3 (133 patients) and to 0 cells (115 patients) on day 11 (Table 2). In the control group, the tumor cell number increased from 9,373 EpCAM$^+$ tumor cells at screening (85 patients) to 18,929 EpCAM$^+$ tumor cells (74 patients) at the puncture visit. Puncture visits in the control group were conducted after a median of 13 days after screening. In the catumaxomab group, the number of EpCAM$^+$ tumor cells at the puncture visit (77 days after catumaxomab treatment) were still significantly lower than screening analysis (2,090 vs. 6,510 cells, respectively; $P = 0.0012$, Wilcoxon rank-sum test; Table 2). Furthermore, overall analysis of the catumaxomab-treated pooled population revealed that already before the second infusion (day 3), in 42% of the patients no EpCAM$^+$ tumor cells could be detected in the ascites/lavage samples. This percentage further increased to 83% after the last catumaxomab infusion on day 11. Moreover, 16% of patients with malignant ascites requiring paracentesis after catumaxomab treatment were still free of detectable EpCAM$^+$ tumor cells in the malignant ascites fluid, whereas in all ascites samples of control patients, EpCAM-positive tumor cells were present and even had increased in

Table 1. EpCAM screening results in patients with malignant ascites

Patient status	*n* (%)
Screening samples derived from patients	433
Ascites samples could not be tested because of poor quality or other reasons	51 (12)
Screened patient samples	382
No EpCAM$^+$ or cytokeratin 8, 18, 19-positive tumor cells detectable in the samples after appropriate staining	11 (3%)
Patients screened with malignant ascites	371
Patients tested EpCAM$^-$	55 (15%)
Patients tested EpCAM$^+$	316 (85%)
Patients tested EpCAM$^+$	316
Patients rated as EpCAM$^+$ < 400 (inclusion criteria not fulfilled)	44 (14%)
Patients tested EpCAM$^+$ $\geq$ 400, but not included in the study because of serious adverse events, denying informed consent or death	19 (6%)
Patients randomized with positive EpCAM test	253
Patients randomized without EpCAM test (included as exceptions)	5
Total number of patients randomized for study	258

numbers at puncture visit (Fig. 1). The tumor load results presented are coherent with the clinical data showing that (i) the median time to the next therapeutic puncture was significantly longer ($P < 0.0001$) in the catumaxomab group (77 days) than in the control group (13 days) and (ii) the median puncture-free survival was significantly longer ($P < 0.0001$) in the catumaxomab group (46 days) than in the control group (11 days; ref. 12).

Relative VEGF levels in malignant ascites samples

The angiogenesis factor VEGF enhances vascular permeability and among other factors it is expressed and secreted by tumor cells (16, 17). The release of VEGF has also been identified as one of the main factors promoting intraperitoneal accumulation of fluid and thus the formation of malignant ascites (17, 18). We have compared ascites sample VEGF levels relative to total protein content throughout the study of treatment and control patient groups. The median VEGF concentration compared with the median total protein concentration in screening ascites samples was set to 100% in both the catumaxomab and the control groups. In the control group ($n = 49$), relative VEGF levels increased from screening (100%) to puncture visit (120%), suggesting tumor growth in these patients. In contrast, relative VEGF levels exhibited by the catumaxomab group ($n = 93$) decreased with statistical significance ($P < 0.001$; ANOVA) from 100% at screening to 37% at day 11 (Fig. 2). In summary, the measured VEGF reduction in the catumaxomab group further supported the observed tumor cell elimination affected by catumaxomab.

T-cell activation in malignant ascites samples

Unlike conventional monospecific antibodies, catumaxomab simultaneously binds and activates Fcγ receptor–positive accessory cells and T cells (7, 8, 19). T cells are activated by catumaxomab in a two-step process, including binding of the antibody to CD3 (step 1) and to activating Fcγ-receptors on accessory cells, which in response leads to co-stimulatory signaling events between T cells and accessory cells (step 2). To assess T-cell activation *in vivo*, the expression pattern of T-cell activation marker CD69 was analyzed using CD45$^+$/CD4$^+$ and CD45$^+$/CD8$^+$ T-cell populations from fresh malignant ascites fluid samples. CD69 is involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes (20). Percentage of CD45$^+$CD4$^+$CD69$^+$ cells in malignant ascites fluids increased after catumaxomab therapy from 6.48% at screening ($n = 126$) to 15.32% at day 3 ($n = 121$) and up to 19.46% at day 11 ($n = 79$). Similar results were obtained for the CD45$^+$CD8$^+$ T-cell subpopulation, where the percentage of CD69$^+$ cells increased from a median of 13.03% (at screening; $n = 126$) to 23.73% (day 3; $n = 121$) and to 33.80% (day 11; $n = 79$; Fig. 3A). At the therapeutic puncture visit, the median activation status of both T-cell subpopulations was found to have dropped to initial levels monitored at screening. In contrast, the activation status of T cells from the controls did not vary considerably between screening and puncture visits (Fig. 3B).

Putative CD133$^+$/EpCAM$^+$ CSCs in malignant ascites fluids

Cancer stem cells are defined as "tumor-initiating cells" that have the capacity of self-renewal and to give rise to a variety of differentiated cells found in malignancy. There is increasing evidence that CSCs may play an important role in tumor disease progression (21–25). The CD133 membrane glycoprotein is considered as a valid CSC marker (26–31) that has previously been shown to be capable of identifying a cancer-initiating subpopulation in EpCAM-positive solid tumors. A subset of patients of the pivotal study (a total of 29 patients with malignant ascites: 9 ovarian, 12 gastric, 2 pancreatic, and 2 patients with cancers of unknown origin as well as 1 gall bladder, 1 uterus, 1 lung, and 1 patient with breast cancer) was analyzed for the presence of CSCs in malignant ascites. Cytospin double staining of ascites cells has revealed the presence of CD133$^+$/EpCAM$^+$ cells in 18 of 29 (62%) patients with a mean value of 1,472 per 5×10^5 analyzed cells at screening in the catumaxomab group and in 6 of 6 patients (100%) with a mean of 4,328 per 5×10^5 analyzed cells in the control group. At day 3, CD133$^+$/EpCAM$^+$ cells were detected in 8 of 28 patients (29%; mean value, 530 per 5×10^5 analyzed cells). At day 11, CD133$^+$/EpCAM$^+$ cells have been eliminated

Table 2. EpCAM$^+$ tumor cell count and time to first puncture

Parameter	Visit	Catumaxomab treatment group		Control group		Group comparison
		n	Median	*n*	Median	
Tumor cell number/10^6 analyzed cells	At screening	165	6,510	85	9,373	
	Before second administration (day 3)	133	27	NA	NA	
	After last administration (day 11)	115	0	NA	NA	
	Therapeutic puncture	43	2,090	47	18,929	$P = 0.0012$ (Wilcoxon test)
Time to first puncture		170	77 Days	88	13 Days	$P < 0.0001$ (log-rank test)

Abbreviation: NA, not available.

in all analyzed patients ($n = 21$). In contrast, CD133$^+$/EpCAM$^+$ cells were found in all control group patients ($n = 6$; mean value, 1,450 per 5×10^5 analyzed cells) after paracentesis (Table 3).

In vitro tumor cell elimination and cytokine profile

Malignant ascites fluids provide a convenient cellular environment to investigate catumaxomab-mediated interactions between tumor and immune cells *in vitro* as all of the involved cell types are present in them. Thus, experiments were carried out with fresh ascites cells harvested from screening samples. These experiments were designed to investigate the *in vitro* efficacy of catumaxomab on autologous patient cells and to compare the results with the *in vivo* pharmacodynamic data described in this study. To study tumor cell elimination, an autologous long-term clonogenic assay was conducted with 10^6 cells from malignant ascites screening samples which were seeded in 24-well plates. Catumaxomab was supplemented at days 0 and 3; control samples were incubated without catumaxomab. After 11 days of incubation, the cells were analyzed by EpCAM immunostaining. In summary, control samples contained a median of 2,334 EpCAM$^+$ tumor cells ($n = 170$) and catumaxomab-supplemented samples ($n = 169$) had a median of only 32 cells after the incubation period (Fig. 4A). To determine the *in vitro* stimulated cytokine profile (e.g., IFN-γ, TNF-α, IL-6, and IL-2) released by these autologous ascites cell preparations, the respective supernatants were harvested after 24 hours of incubation. Ascites samples without catumaxomab exhibited median value of 1,978 pg/mL for IL-6 ($n = 185$) with no detectable amounts of IFN-γ ($n = 190$), TNF-α ($n = 190$), nor IL-2 ($n = 190$). In contrast, release of proinflammatory cytokines was markedly increased in the presence of catumaxomab:

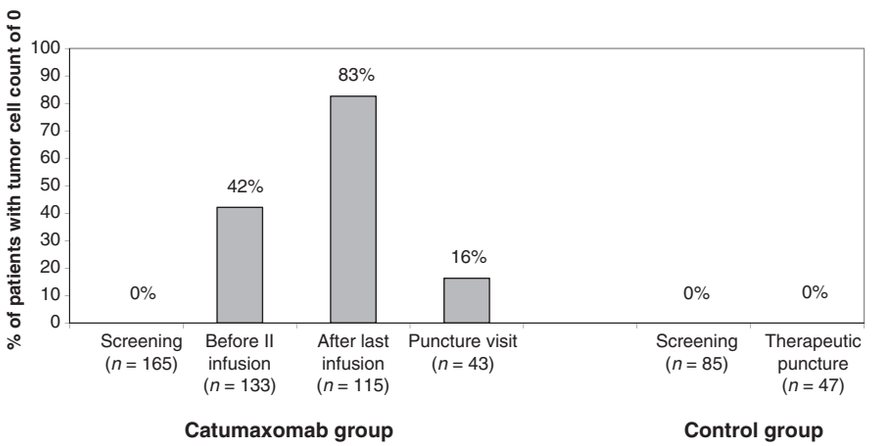

Figure 1. Percentage of randomized patients without detectable EpCAM$^+$ ascites tumor cells in the catumaxomab treatment and control group. *n*, number of patient samples analyzed at the indicated assessment times.

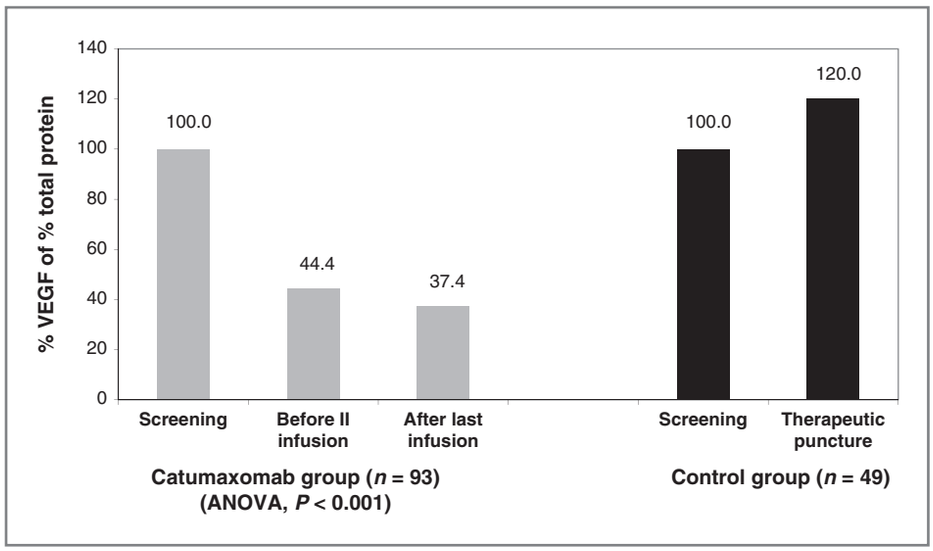

Figure 2. Analysis of VEGF levels in malignant ascites fluid samples of the catumaxomab treatment and control group. VEGF levels assessed are indicated in percentage initially starting with 100% at screening puncture. *n*, number of patient samples analyzed at the indicated assessment times.

Median values of IFN-γ amounted to 2,300 pg/mL ($n = 189$), of TNF-α to 333 pg/mL ($n = 189$), of IL-2 to 1,284 pg/mL ($n = 189$), and of IL-6 to 4,300 pg/mL ($n = 184$; Fig. 4B).

Discussion

The therapeutic benefit of catumaxomab for patients suffering from malignant ascites is well described (10, 19) and has recently been reported on a pivotal clinical trial (12), which led to approval of catumaxomab for the treatment of malignant ascites by the European Medicines Agency (EMA) in 2009. With the present study, we have investigated cellular, molecular, and immunologic parameters of the effects of catumaxomab versus paracentesis alone in patients with malignant ascites to possibly explain the previously observed clinical benefits. We have shown that catumaxomab administration induced the following: (i) a significant decrease of the peritoneal tumor cell burden, (ii) a significant increase of the activated $CD4^+$ and $CD8^+$ T-cell populations, (iii) a significant reduction of relative VEGF levels, (iv) elimination of putative $CD133^+/EpCAM^+$ cancer stem cells, (v) the *in vitro* elimination of $EpCAM^+$ tumor cells, and (vi) a sharp increase of proinflammatory Th1 cytokine release (IL-2, IL-6, TNF-α, and IFN-γ) in an autologous *in vitro* assay setting.

The data indicate that intraperitoneal catumaxomab treatment triggers the activation of different types of immune effector cells within the peritoneal cavity resulting in the depletion of EpCAM-positive tumor cells. Curiel and colleagues (14) have concluded that ovarian cancer–induced malignant ascites is accompanied by immunosuppressive effects as

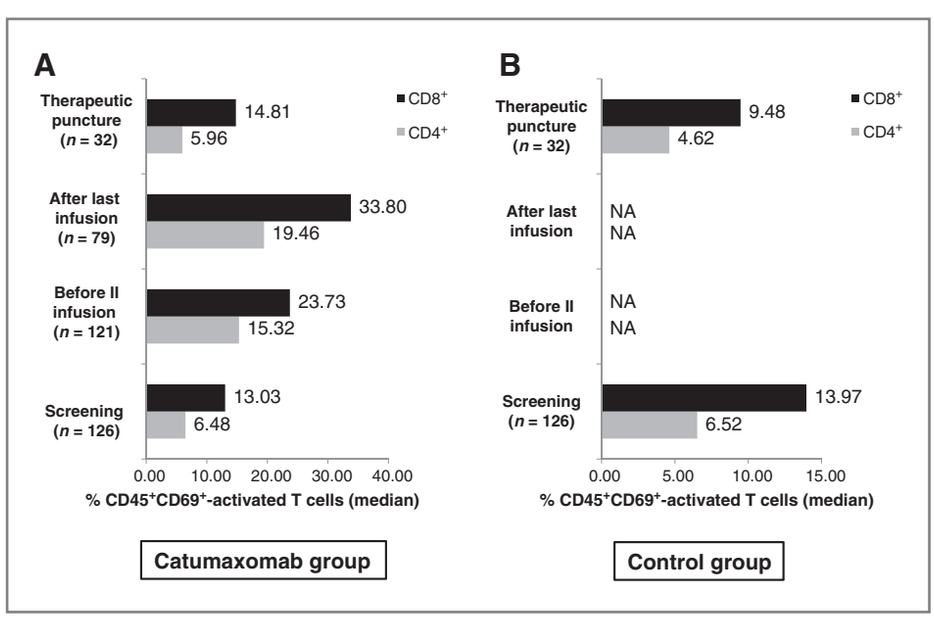

Figure 3. Flow cytometric analysis of *in vivo* T-cell activation ($CD4^+$ or $CD8^+$ T cells) in malignant ascites fluid samples of the catumaxomab treatment and control groups. Catumaxomab group (A) and control group (B). *n*, number of patient samples analyzed at the indicated assessment times; NA, not available.

Table 3. Detection of putative $CD133^+/EpCAM^+$ CSCs in patients with malignant ascites

	Catumaxomab group		Control group	
Assessment time	$CD133^+/EpCAM^+$ CSCs/0.5 × 10^6 analyzed cells (mean value)	Number of patients with detectable $CD133^+/EpCAM^+$ CSCs in malignant ascites	$CD133^+/EpCAM^+$ CSCs/0.5 × 10^6 analyzed cells	Number of patients with detectable $CD133^+/EpCAM^+$ CSCs in malignant ascites
At screening	1,472	18/29 (62%)	4,328	6/6 (100%)
Before second administration (day 3)	530	8/28 (29%)	NA	NA
After last administration (day 11)	0	0/21 (0%)	NA	NA
Therapeutic puncture	NA	NA	1,450	6/6 (100%)

Abbreviation: NA, not available.

HER2/neu-specific T-cell proliferation was inhibited by $CD4^+CD25^+FOXP3^+$ T-regulatory cells. The finding that catumaxomab administration still induces T-cell activation as well as $EpCAM^+$ tumor cell depletion even within this immunosuppressive milieu is an important finding and of pivotal importance for treatment of patients with malignant ascites. Moreover, it should be noted that besides $EpCAM^+$ tumor cells, catumaxomab is also able to kill putative $CD133^+/EpCAM^+$ CSCs also present in malignant ascites, which are believed to be resistant to chemotherapy and may therefore be the main cause of tumor growth and disease progression (21–25, 32–35). It should be stressed that CD133 represents a valid CSC marker for many primary malignant diseases, for instance, ovarian, gastric, prostate, lung, brain, and pancreatic cancers (26–31). Of note, only the article published by Shmelkov and colleagues (36) yet described CD133 expression as not restricted to CSCs. According to the published data, both CD133-positive and CD133-negative metastatic colon CSCs are capable of initiating tumors. In this context, it should be mentioned that all samples of all 29 patients with malignant ascites tested for $EpCAM^+CD133^+$ CSCs did not suffer from colon carcinomas underlining the relevance of CD133 as CSC marker for the respective primary cancer diseases described by this report. However, future *in vitro* investigations and experiments in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice should underline the tumor-initiating capacity of $CD133^+/EpCAM^+$ CSCs isolated from patients with malignant ascites.

The relatively long-lasting clinical improvement of patients observed after a short 10-day catumaxomab treatment period may, at least in part, be explained by the elimination of both $EpCAM^+$ tumor cells and putative $EpCAM^+CD133^+$ double-positive CSCs. The *in vitro* cytotoxicity data obtained from the autologous cell assays are particularly impressive, as immune effector cells from patients with cancer often tend to be less responsive (37).

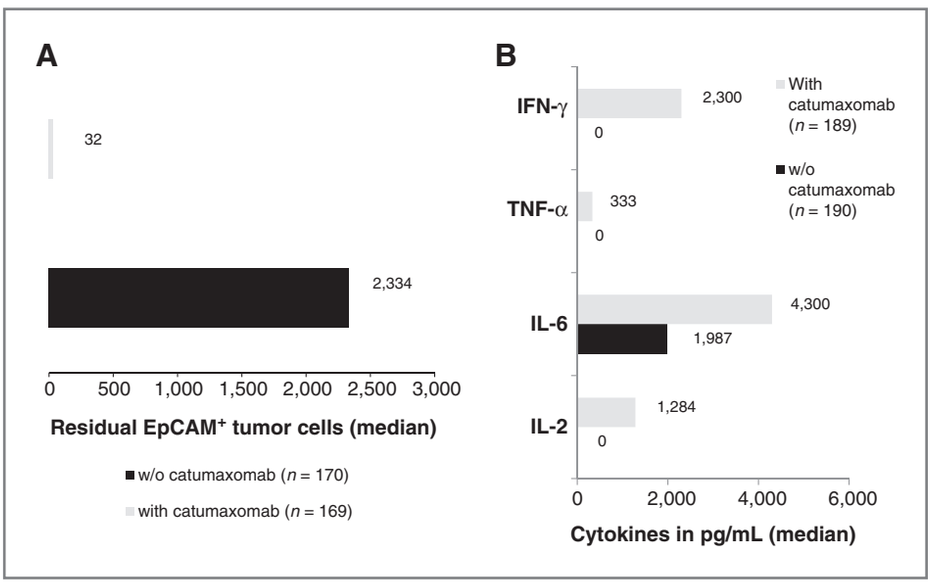

Figure 4. *In vitro* experiments in an autologous setting. A, number of surviving $EpCAM^+$ ascites tumor cells with and without catumaxomab supplementation. B, cytokines profiles exhibited by malignant ascites fluid samples after 24 hours of incubation with or without catumaxomab. Values were converted to WHO standards by the following factors IL-2 (1.52), IL-6 (0.86), IFN-γ (0.46), and TNF-α (0.76). *n*, number of patient samples analyzed; w/o, without.

It has generally been considered that factors, which are produced by tumor cells and which increase vascular permeability and induce angiogenesis, are present in malignant ascites fluid and contribute to tumorigenesis. The etiology of malignant ascites has been attributed to insufficient lymph drainage caused by the tumor spread into draining lymph vessels and/or increased capillary permeability which could be both supported by angiogenic factors such as VEGF (18, 38, 39). Increased VEGF expression has been observed across a range of tumor types—including ovarian, colon, lung, breast, renal, glioblastoma, prostate, and other cancers—and was widely correlated with tumor development and/or poor prognosis.

Zebrowski and colleagues (17) reported on significantly increased VEGF levels in malignant ascites in contrast to those assessed in nonmalignant cirrhotic ascites. Furthermore, malignant ascites from patients with colon and gastric cancer caused an increase in permeability in human umbilical vein endothelial cells (HUVEC) in all cases. Upon neutralization of VEGF activity in colon cancer, ascites decreased *in vitro* HUVEC permeability. Taken together, these data clearly support the notion that VEGF is produced by tumor cells and that VEGF appears to be central for the development of malignant ascites. Therefore, our primary working hypothesis that catumaxomab treatment of patients with malignant ascites may have an impact on the peritoneal VEGF levels has been confirmed by our investigations.

The *in vivo* data are further confirmed by *in vitro* experiments (Fig. 4). First, the catumaxomab-induced *in vitro* elimination of $EpCAM^+$ tumor cells from screening ascites samples confirms the clinical data obtained *in vivo*. Noteworthy, catumaxomab was indeed capable of destroying $EpCAM^+$ tumor cells in an autologous and highly immunosuppressive environment. Moreover, the observed release of IFN-γ, IL-6, TNF-α, and IL-2 induced by catumaxomab in an autologous *in vitro* killing assay indicates immune cell activation and self-supporting T-cell proliferation even under the assumed immunosuppressive influence of T-regulatory cells (14).

In conclusion, all immunologic, cellular, and molecular antitumor effector mechanisms assessed through our study appeared to correlate with the therapeutic benefit that patients with malignant ascites experienced with the catumaxomab treatment. As $EpCAM^+$ tumor ascites cells from primary carcinomas were targeted and eliminated by that antibody, the immunomonitoring data are especially significant in view of novel drug development strategies toward solid cancers.

Disclosure of Potential Conflicts of Interest

H. Lindhofer: employment and ownership interest, TRION Pharma GmbH. P. Wimberger: commercial research grant in translational research project, honoraria, expert, consultant/advisory board member, leader of clinical trial phase III study (CASAPTS trial). B. Schmalfeldt: technical consultant/advisory board member. M. Ströhlein: commercial research grant, TRION Pharma and Fresenius Biotech; honoraria from Speakers' Bureau of Fresenius Biotech; consultant/advisory board member, TRION Pharma. M.M. Heiss: commercial research grant, Fresenius Biotech and TRION Pharma GmbH; consultant/advisory board member, Fresenius Biotech and TRION Pharma GmbH. The other authors disclosed no potential conflicts of interest.

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