Targeting EpCAM (CD326) for immunotherapy in hepatoblastoma

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Abbreviations: ADCC, antibody dependent cell cytotoxicity; CDDP, cisplatin; EpCAM, epithelial cell adhesion molecule; E:T, effector-to-target; HB, hepatoblastoma; IL-2, interleukin 2; PE, phycoerythrin; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte

Introduction

Hepatoblastoma (HB) is the most common liver cancer in children. Recurrence of HB after chemotherapy and surgery is frequent among high-risk patients and is associated with chemoresistance. Immunotherapy may improve poor treatment outcomes in HB patients. Cytotoxic leukocytes of the innate and adaptive immune system including different populations of cytotoxic T cells play a major role in fighting developing tumors. In this setting, monoclonal antibodies may be employed to specifically direct immune responses toward tumor cells. We addressed this issue by using humanized antibodies that recognize the cell surface molecule EpCAM (CD326, overexpressed in hepatic tumor cells) to enhance immune responses against HB. EpCAM was constantly expressed on HB cells and its expression was independent of previous therapy based on the DNA-damaging agent cisplatin. Co-culture assays performed with two well-described HB cell lines and tumor tissue cultures demonstrated that tumor cell lysis by γδ T cells can be dramatically augmented by applying EpCAM-specific monoclonal antibodies. These data emphasize the value of antitumor immune responses and encourage adapting immunotherapeutic regimens to improve the outcome of high risk HB.

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molecules. \textsuperscript{21,22} \(\gamma\delta\) T cells are known for their capacity of lysing various tumor cells in vitro. \textsuperscript{23,24}

The present study aimed at analyzing the value of EpCAM as a target for the immunotherapy of HB. An immunotherapeutic approach involving antibodies and \(\gamma\delta\) T cells was investigated in HB cells previously exposed to CDDP.

**Results**

**EpCAM expression by HB cells in vitro and in vivo.** To assess whether immunotherapy could be a promising tool for treating HB we screened for tumor infiltrating lymphocytes (TILs) in vivo. CD45 is a tyrosine-protein phosphatase expressed on leukocytes and is overexpressed in up to 50% of HB cases, as revealed by a gene expression analysis (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-1851, data not shown). Slices from tumors explanted from a patient with epithelial HB were stained for the detection of CD45\(^+\) cells by DAB-based immunohistochemistry. Shown are two regions of the same tissue.

**Figure 1.** Staining for tumor-infiltrating leukocytes. Slices of a hepatoblastoma tissue sample revealed infiltrating CD45\(^+\) cells by DAB-based immunohistochemistry. Shown are two regions of the same tissue.

**Figure 2.** Expression of EpCAM on hepatoblastoma cells treated with cisplatin. (A and B) HepT1 (A) and HuH6 (B) hepatoblastoma (HB) cells were incubated with the indicated concentrations of cisplatin (CDDP) for 48 h. Flow cytometry with CD326-specific antibodies revealed constant expression of EpCAM on live HB cells regardless on previous treatment.

Since CD326 is highly expressed by HB, it represents a possible target for immunotherapeutic applications. \textsuperscript{8,25} As demonstrated by flow cytometry, HuH6 and HepT1 HB cells express EpCAM on the cell surface (Fig. 2). There was no difference in the expression of this tumor marker regardless of the previous treatment with concentrations of CDDP ranging from 1 \(\mu\)g/mL to 4 \(\mu\)g/mL (for 48 h). As these results were obtained from monolayer cultures, we intended to mimic the in vivo setting more realistically by generating tumor spheroids. For this purpose, HB cell lines were cultured on ultra-low attachment surface plates at a starting cell density of 100,000 cells per well over a period of
Discussion

New successful regimens to treat HB have been developed over the past 40 y. Despite all such efforts, survival rates of patients suffering from high-risk HB remain poor (3-y survival = 69%).

New strategies against advanced HB are needed, since the low overall response to chemotherapy and the high incidence of metastasis impair disease outcome. We chose EpCAM as a target for immunotherapy because it is a well-known tumor antigen. Moreover, a reduction of EpCAM expression due to chemotherapy is not expected. We indeed demonstrated that EpCAM is constantly expressed on cultured HB cells irrespective of chemotherapeutic treatment. Gene array analysis performed with hepatic tumor tissue from HB patients after several rounds of cytostatic drug application confirm our results in vivo (http://www.ebi.ac.uk/arrayexpress/experiments/E-EXP-1851).

Histologically, EpCAM expression appeared to be distributed heterogeneously within the tumor. Our experiments also showed a differential expression of EpCAM on malignant cells forming 3D spheroids. HepT1 and Huh6 hepatoblastoma (HB) cells were cultured on culture plates (2D) or as spheroids (3D). Flow cytometry revealed a lower expression of EpCAM on a subpopulation of hepatoblastoma (HB) cells cultured as spheroids as compared with a homogeneous staining observed for cells cultured on plates.

Figure 3. Expression of EpCAM on hepatoblastoma cells cultured spheroids. HepT1 and Huh6 hepatoblastoma (HB) cells were cultured on culture plates (2D) or as spheroids (3D). Flow cytometry revealed a lower expression of EpCAM on a subpopulation of hepatoblastoma (HB) cells cultured as spheroids as compared with a homogeneous staining observed for cells cultured on plates.

EpCAM as a target for anti-HB immunotherapy. Since EpCAM expression is higher on HB than on normal liver cells, we sought out whether it could serve as a target for immunotherapy. For this purpose, peripheral blood mononuclear cells (PBMCs) and \( \gamma \delta \) T cells were incubated with HB cells, respectively, in the presence of different concentrations of EpCAM-specific antibodies (Fig. 5). PBMCs at an E:T ratio of 2:1 did not effectively kill HUH6 or HepT1 cells. The addition of the mono-specific antibody MT201 resulted in a slight increase in tumor cell lysis (reduction of tumor cell viability of 25%). Lysis was significantly higher in cultures treated with the bi-specific antibody MT110, which—at odds with MT201—concurrently binds EpCAM and CD3. At the highest concentration tested (50 \( \mu \)g/mL) only 25% of tumor cells remained alive. \( \gamma \delta \) T cells are more efficient at lysing HB cells than PBMCs as at an equal cellular ratio a 25% lower tumor cell viability was measured. With increasing concentration of MT110, a prominent killing of HB cells was achieved. We next analyzed the efficiency of \( \gamma \delta \) T cells at lysing tumor cells within xenograft-derived tissue slices, both in the presence and in the absence of MT110 (Fig. 6). These tumors were derived from HUH6 cells that had previously been transfected with Gaussia luciferase (GLuc). Hence, viable cells in tissue slices secreted GLuc into the culture media. Tumor slices accommodated for 8 h in culture released within the first hour of co-culture with \( \gamma \delta \) T cells an enzymatic activity ranging between 9,000 to 15,000 RLU/sec, depending on the weight of the slice. At the end of co-incubation period, the enzyme activity released in the medium of antibody-free co-cultured increased up to 2-fold. In MT110 containing co-cultures, 50% of the initial GLuc activity was observed. These two different types of co-culture, i.e., cells in suspension and tissue slides, emphasize the positive effect of the bi-specific antibody MT110 on tumor recognition and lysis by \( \gamma \delta \) T cells.

A possible option for HB-directed immunotherapy would be constitute by the adoptive transfer of activated and expanded autologous \( \gamma \delta \) T cells, either as freshly isolated populations or upon cryopreservation. \( \gamma \delta \) T cells expanded in culture in the presence of 400 nM zoledronic acid and 200 IU/mL interleukin-2 (IL-2) were compared with cryopreserved cells from the same isolation in a killing assay with HB cells (Fig. 7). Expanded \( \gamma \delta \) T cells lysed up to 25% HB cells at an E:T ratio of 4:1. The addition of 5\( \mu \)g/mL MT110 significantly enhanced tumor cell lysis, an effect that was similar in freshly expanded and cryopreserved \( \gamma \delta \) T cells. Cryopreservation did not have any negative impact on the capacity of \( \gamma \delta \) T cells to lyse target cells, irrespective of the presence of MT110.
The enormous capacity of this antibody to direct the activity of cytotoxic T cells to different tumor cells in vitro and in vivo has been shown elsewhere. Therefore, BiTE MT110 stands out as a promising tool for treating EpCAM+ tumors like HB. One of the advantages of administering antibodies is the represented by the direct activation of effector cells that infiltrate and surround the tumor. This may allow for the circumvention of inhibitory signals coming from stromal cells. As in this study we analyzed the impact of MT110 in vitro, its efficiency at redirecting cytotoxic effector cells toward EpCAM+ hepatic tumor cells in vivo remains to be determined. It is yet unclear tumor infiltration by antibodies and lymphocytes may be as strong as in vitro, because it depends not only on interstitial pressure but also on liver perfusion.

Titration experiments revealed that 5 μg/mL of the antibody were sufficient to initiate efficient cell killing in vitro by γδ T cells, as cell lysis could not be significantly enhanced by higher antibody concentrations. Mouse experiments demonstrated that increasing concentrations of MT110 can either efficiently induce tumor elimination or even prevent tumor formation. Combining the adoptive transfer of γδ T cells and the administration of MT101 antibody in orthotopic HB xenografts in mice...
may provide proof-of-principle for this therapeutic concept in vivo.

The impressive antitumor activity of activated γδ T cells, especially in combination with the bispecific antibody MT110, led to a nearly complete elimination of tumor cells in vitro. Since γδ T cells can be amplified and retain their cytotoxic activity also after prolonged cryopreservation periods, this unconventional, minor T-cell subset could be especially suitable for adoptive T-cell transfer against HB. The use of the T-cell engaging antibody MT110 after liver transplantation or autologous adoptive T-cell transfer, for instance against metastases or to prevent recurrence, also appears as a therapeutic option. So far the clinical testing of EpCAM-antibodies like catumaxomab has not been extended to situations of pathological liver. However, side effects can be expected, as transient higher expression of EpCAM was observed during cirrhosis or in liver regenerating post-hepatectomy. Mild toxicity was observed despite EpCAM expression in normal liver cells including biliary duct and hepatic stem cells (http://clinicaltrials.gov/). We will therefore consider an antibody therapy targeting EpCAM for subjects at a very high risk of relapse upon hematopoetic stem cell transplantation following liver transplantation. This concept has already been proposed in the treatment of particular cases of HB.35

Materials and Methods

Cell cultures. HUH6 and HepT1 HB cells were derived from a mixed HB and from a multifocal embryonal HB, respectively.36,37 Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) L-glutamine and 1% penicillin/streptomycin (all from Biochrom AG) on plastic culture dishes (Greiner-Bio One GmbH). Cells were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C and were proved to be Mycoplasma negative. Spheroid cultures were grown in 200 μL culture medium on ultra-low attachment surface plates (Corning Inc.–Life Sciences) over a period of 7 d.38 Some cultures were incubated with increasing concentrations of cisplatin (CDDP, Neocorp AG)—ranging from 1 μg/mL to 4 μg/mL—for 48 h.

Isolation of PBMCs and γδ T cells. Blood samples were obtained as buffy coats from the Centre for Transfusion
Humanized antibodies (BiTE antibody MT110 and MT201/adecatumumab, from Amgen) were administered up to a final concentration of 50 μg/mL. Cell viability was assessed by 24 h lasting 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity assays (Sigma). Twenty-five μL MTT solution (5 mg/mL dissolved in PBS) were added to each well 6 h prior to the end of the assay. To stop the reaction, 100 μL/well lysis solution (10% SDS in 0.1 N HCl) were added and plates were further incubated in the dark at room temperature overnight. Cell viability was assessed by measuring the absorption at 570 nm using Milenia Kinetic Analyzer (DPC Bierman). All assays were performed in triplicates. Relative cell viability was calculated upon normalization based on tumor cell cultured in the absence of effector cells and background of cultures containing no cells (only culture medium) or effector cells only, as controls.

Flow cytometry. Flow cytometry analyses were performed according to established protocols, as described elsewhere. Briefly, 1 million cells were stained with anti-CD326-PE (Miltenyi Biotech). γδ T cells were subsequently isolated by negative MACS isolation according to the manufacturer’s instructions (TCRγδ+ isolation kit human, Miltenyi Biotec GmbH). The purity of γδ T cells after negative MACS isolation, as estimated by flow cytometry using an anti-TCRγδ-PE antibody (Miltenyi Biotech), was > 99%.

Figure 7. Lysis capacity of freshly isolated γδ T cells vs. cryopreserved γδ T cells. (A-D) HepT1 (A and B) and HUH6 (C and D) hepatoblastoma (HB) cells were incubated with increasing numbers of freshly prepared (A and C) or cryopreserved (B and D) γδ T cells in the presence or absence of 5 μg/mL MT110 and MT201 antibodies. Cell viability was measured by MTT assay after 24 h. All data points represent mean values of triplicates.
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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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