A Monoclonal Antibody-Cobra Venom Factor Conjugate Increases the Tumor-specific Uptake of a $^{99m}$Tc-labeled Anti-Carcinoembryonic Antigen Antibody by a Two-Step Approach

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Abstract

Monoclonal antibodies (mabs) have considerable potential for specific cancer therapy. However, due to the antigen heterogeneity and especially the low uptake in solid tumors, mabs have not been used successfully in most clinical trials to date. This study investigates the effects of a mab-cobra venom factor (CVF) conjugate in vitro and in vivo in an orthotopic pancreatic cancer model using nude rats. CVF, a nontoxic glycoprotein from cobra venom, permanently activates the alternative pathway of complement. Coupled to a mab with tumor-binding properties, the complement activation can be targeted to the tumor tissue. We studied the activity of a mab CA19-9-CVF conjugate with the human pancreatic cancer cell line PancTu I.

PancTu I cells express the complement resistance factors CD 46, CD 55, and CD 59, as we demonstrated by immunostaining, an observation that may explain the lack of cytotoxicity of the CA19-9-CVF conjugate. However, using ELISA, Western blot, and immunostaining, we showed that CA19-9-CVF activates the complement cascade, including the release of the anaphylatoxin C3a, a mediator of an inflammatory reaction. The in vivo studies of CA19-9-CVF-treated nude rats showed an increased tumor infiltration by natural killer cells and macrophages. The tumor uptake of a $^{99m}$Tc-labeled anti-carcinoembryonic antigen antibody was increased approximately 2-fold in rats pretreated with 70 μg of CA19-9-CVF, compared to animals that received an equimolar mixture of noncoupled mab and CVF.

This study indicates the value of mab-CVF conjugates in adjuvant immunotherapy. mab-CVF conjugates might be useful in pretargeting approaches by increasing the uptake of a therapeutic mab.

Introduction

mabs with tumor-binding properties offer an opportunity for specific tumor cell destruction. Within the last 15 years, various mab therapy approaches have been developed for the treatment of cancer. Unfortunately, despite very promising in vitro results, the use of mabs has not been successful in clinical studies treating patients with solid tumors. Phase I and II trials in colorectal cancer (3) and pancreatic cancer (4, 5) yielded no effect at all. Radioimmunotherapy, as well as immunotoxins, also failed in most patients (6–8).

The main reasons for the disappointing clinical results are the antigen heterogeneity of solid tumors and the low tumor uptake of injected mabs. Whereas the problem of antigen heterogeneity of solid cancers can be overcome by the application of an antibody cocktail containing mabs with different target antigens (7), the limiting factor of mab therapy is the low tumor uptake in solid cancers. Due to high interstitial pressure and the large size of mabs, only 0.1% of the injected dose binds to tumor tissue (8).

In this study, we investigated a mab-CVF conjugate and studied its use to increase the tumor uptake of mabs by the induction of a local inflammation. CVF is a nontoxic glycoprotein (Mr 144,000) that can be isolated from cobra venom. By binding factor B, CVF activates, in analogy to human C3, the alternative pathway of complement and forms a C3/C5 convertase. In contrast to human C3, CVF leads to a permanent activation of the complement cascade because the inhibitors H and I do not react with CVF (9). The complement activation includes the release of C3a and C5a, both of which mediate an inflammatory reaction and attract immunocytes such as NK cells and macrophages to the tumor site. Additionally, CVF leads to the formation of the membrane attack complex, which might cause cell lysis.

CVF was isolated from the mab CA19-9, which binds a carbohydrate antigen frequently expressed on mucins of pancreatic cancer (10). The experiments were performed using the human pancreatic cancer cell line PancTu I. In vitro studies showed that PancTu I cells express complement resistance factors CD 46, CD 55, and CD 59. Complement-mediated cell lysis could not be achieved by applying a CA19-9-CVF. However, we were able to show that the complement cascade was activated by CA19-9-CVF, causing the release of C3a.

The in vivo experiments were performed using an orthotopic pancreatic cancer model in nude rats. Nude rats were used because rat serum reacts with CVF analogously to human serum, whereas mouse serum shows no cytotoxic complement activity with mab-CVF conjugates (11). The tumors of CA19-9-CVF-treated rats were markedly infiltrated by NK cells and macrophages, and the uptake of an anti-CEA antibody was enhanced 2-fold.

Materials and Methods

Materials. CVF was isolated from lyophilized cobra venom (Naja naja kaouthia; Latexan, Rosans, France) as described previously (12). Human pancreatic cancer cell line PancTu I was obtained from Dr. M. v. Bülow (University of Mainz, Mainz, Germany) and maintained in continuous culture as described previously (13). The mab CA19-9 was isolated from the supernatant of hybridoma cultures by protein A affinity chromatography. Normal human serum was stored at −80°C and was used within 2 weeks of preparation. SPDP and Sephadex-G200 Gel were purchased from Pharmacia (Piscataway, NJ), molecular weight markers for SDS PAGE were purchased from Bio-Rad (Richmond, CA), and Na$_2^{15}$CrO$_4$ (1 mCi/ml) and Na$_{125}$I (5 mCi/ml) were purchased from Amersham (Arlington Heights, IL). Peroxidase and alkaline phosphatase labeled F(ab')$_2$ goat antimouse antibodies for immunostaining and ELISA were purchased from Dianova (Hamburg, Germany).

Nude rats (nu/nu) were obtained from the animal breeding colony of the University Hospital Eppendorf (Hamburg, Germany). Animal experiments were performed according to the guidelines of the “Ministerium für Natur und Umwelt des Landes Schleswig Holstein” dated October 7, 1992.

Preparation of CA19-9-CVF Conjugate. The conjugation of mab CA19-9 and CVF was performed as described previously (12). Six mg of CA19-9 antibody were derivatized with SPDP (400 μmol). After purification by size exclusion chromatography (G-25 Sephadex PD10 columns, Pharmacia), the antibody fractions were concentrated by ultrafiltration tubes (Sartorius, Göttingen, Germany) and incubated with 50 mM DTT. After 20 min, DTT

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was removed, and the free sulfhydryl-containing antibodies were immediately added to equimolar amounts of SPDP-derivatized CVF (4 mg). The derivatization with SPDP yielded to 3.5 pyridylthiole residues per CVF and 3 residues per CA19-9 molecule, respectively. After 22 h, the reaction mixtures were subjected to size exclusion chromatography at 4°C on a Sephadex-G200 gel column (2.5 X 100 cm) equilibrated with PBS, pH 7.45 (GIBCO, Paisley, UK). The conjugate fractions were pooled and concentrated by ultrafiltration tubes (Sartorius) to a final concentration of 200 µg/ml CA19-9-CVF conjugate. After filter sterilization, the conjugate was found to be stable for at least several months at 4°C. All calculations are based on a Mw of 155,000 for the antibody and 299,000 for the conjugate with CVF (Mw, 144,000). Determination of the hemolytic activity and antigen-binding capability are based on the assumption of an equimolar CVF-antibody ratio in conjugates.

Protein concentrations were determined spectrophotometrically at 280 nm using extinction coefficient of E1%1cm = 0.99 for CVF (12), E1%1cm = 1.4 for CA19-9 antibody, and E1%1cm = 1.2 for the CA19-9-CVF conjugate.

Analysis of Conjugate Binding to PancTu I Cells. The conjugate binding was determined by ELISA. Briefly, tumor cells (105 cells/well) were plated on microtiter plates until confluent, fixed with 0.05% glutaraldehyde, and blocked at 4°C with 1% BSA in PBS overnight. Plates were washed and CA19-9-CVF, and noncouples proteins were added in varying amounts (100 µl/well) and incubated for 90 min at 20°C. Cells were washed extensively (3 times) and incubated with peroxidase-conjugated goat antimouse IgG (Dianova, Hamburg, Germany) diluted 1:30,000 in PBS, pH 7.45 (100 µl/well), for 1 h at 20°C. After washing, cells were incubated with 100 µl/ well substrate (4 mg o-phenylenediamine in 10 ml 0.1 m citrate-disodiumphosphate buffer (pH 5.0) containing 3.3 µl 30% H2O2) for 20 min at 20°C. The reaction was blocked by adding 8 n H2SO4 (25 µl/well), and absorbance was determined at 492 nm.

Hemolytic Assay for CVF Activity. The complement-activating activity of free and conjugated CVF was determined in a bystander lysis assay of guinea pig erythrocytes (14). Briefly, unsensitized guinea pig erythrocytes (5 X 105 cells/20 µl) were incubated for 30 min at 37°C with 20 µl of guinea pig serum and 20 µl of varying amounts of CA19-9-CVF or unconjugated proteins in PBS (pH 7.45). Hemolysis was determined by spectrophotometric quantitation of released hemoglobin at 412 nm. The same assay was used to determine the complement activity of rat serum after treatment with CVF. Rat serum lyses guinea pig erythrocytes via complement activation. CVF can diminish this effect because it causes a serum decomposition. Twenty µl of serum from rats treated with 30, 3, and 0.3 µg CVF, respectively, were added to guinea pig erythrocytes instead of guinea pig serum. The hemolysis of CVF-treated rat serum was compared with the effect of normal rat serum (not treated with CVF), and maximal lysis was considered to be fully retained complement activity.

51Cr Release Cytotoxicity Assay. PancTu I cells were labeled with Na251CrO4 to a specific activity of 0.1–0.2 cpm/cell as described (14). The labeled cells (5 X 104) were incubated for 45 min at 37°C with varying amounts of either unconjugated antibody, CA19-9-CVF conjugate, or free CVF in a total volume of 100 µl medium. The cells were washed and resuspended with human serum 1:2 diluted in PBS (pH 7.45), and cell culture cluster microtiter plates (Costar, Cambridge, MA) were coated with 104 cells/well. After 4 h at 37°C, the supernatant was collected using a SCS microelisa auto reader at 492 nm.

Detection of C3a by Western Blot. To further demonstrate the formation of C3a, we performed a Western blot. SDS gel was blotted on nitrocellulose paper overnight, blocked with PBS containing 5% BSA for 3 h, and finally washed with PBS. Seven ml of mouse anti-C3a antibody (5 µg/ml) were added. After 2 h, the nitrocellulose was washed 3 times, and a peroxidase conjugated F(ab')2, goat antimouse IgG antibody was added (1:1,000 diluted in PBS, pH 7.45, containing 5% BSA). After 1 h at 20°C and washing, substrate was added (4 ml of a solution with 150 µg 4-Cl-l-naphthol in 50 ml methanol added to 20 ml 20 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 10 µl 3% H2O2).

Detection of C3a by Immunohistochemistry. PancTu I cells from experiments for CA19-9 ELISA were centrifuged on microscope slides by cytocrufination, fixed with acetone, and incubated with the mouse anti-C3a mab (Quidel). Staining was performed with the APAAP staining system purchased from Dianova.
Antibodies against rat macrophages were purchased from Dianova, and NK cells were detected by the mouse mab 3.2.3., which was kindly provided by Professor W. H. Chambers (Department of Pathology, Pittsburgh Cancer Center, Pittsburgh, PA). Microscopic evaluation of the tumor sections was performed by an investigator who was not aware whether an animal was pretreated. The number of NK cells and macrophages was counted in 10 microscope fields (X400) of 2 microscope slides/tumor. Eight tumors were evaluated (4 pancreatic and 4 s.c. tumors) in the control as well as the CA19-9-CVF-treated groups.

Results

Characterization of the CA19-9-CVF Conjugate. CVF was coupled to CA19-9 antibody using the heterobifunctional cross-linking reagent SPDP. CA19-9-CVF conjugates, free of noncoupled protein species, were pooled and used in this study. The purity of pooled fractions was tested by SDS-gel electrophoresis and showed the presence of mainly dimeric and trimeric conjugates (data not shown).

Binding of the conjugate to PancTu I cells was determined by ELISA techniques and showed virtually the same binding activity of the conjugate compared to noncoupled antibody (Fig. 1a).

The complement-activating activity of CA19-9-CVF antibody was determined in a hemolytic assay using guinea pig erythrocytes. On the basis of the assumption that the conjugates contain antibody and CVF at equimolar ratios, the conjugate exhibited an activity that was approximately 30–40% lower than that of unconjugated CVF (Fig. 1b).

In Vitro Effect of CA19-9-CVF. The complement-mediated cytotoxicity of CA19-9-CVF was measured by a 51Cr release assay. No complement-mediated cell lysis was found for CVF-conjugated and noncoupled antibody (data not shown).

Immunostaining showed that approximately 90% of PancTu I cells strongly express the complement resistance factors CD 46 and CD 55, and about 70% of the tumor cells possess CD 59.

An ELISA was developed to demonstrate a complement activation by CA19-9-CVF. PancTu I cells were incubated with either conjugate, antibody, or CVF. PancTu I cells alone caused a partial release of C3a, but the noncoupled proteins did not increase the amount of C3a in contrast to CA19-9-CVF (25 µg), which increased the C3a release 3–4-fold (Fig. 2). The finding of C3a formation was confirmed by Western blot analysis of the serum samples (data not shown) and additionally by immunostaining of CA19-9-CVF-treated PancTu I cells. The membrane region of CA19-9-CVF-treated cells exhibited a strong reaction for C3a, compared to only weak antibody staining of cells that were not treated or that were incubated only with noncoupled protein species.
Characterization of the Tumor Model. PancTu I cells were injected into the pancreatic head and s.c. in nude rats (n = 60). Sixty-five % of the rats developed a s.c. tumor, and all animals except three developed a s.c. tumor and an orthotopic pancreatic cancer simultaneously. The tumor diameter ranged between 0.5 and 1 cm. Twenty % of the animals showed peritoneal carcinosis in addition to the primary tumor, and 3 rats showed an isolated liver metastasis. Histological evaluation of the tumor revealed a moderate to low differentiated adenocarcinoma (Fig. 3). Tumor tissue was strongly positive for CA19-9 expressed virtually without heterogeneity by almost 100% of the tumor cells, whereas CEA positivity was found in only 10–15% of cancer cells, as determined by immunohistochemistry.

Effect of CVF on Complement Levels in Rat Serum. To estimate the effect of i.v. injected CVF on the complement levels in the serum, groups of two animals were treated with 30, 3, and 0.3 μg CVF. Two animals served as a control group. Thirty and 3 μg of CVF initiated a depletion of complement activity, as determined by a hemolytic assay using guinea pig erythrocytes, whereas 0.3 μg CVF did not influence the complement activity. The complement activity recovered within 3 (0.3 μg CVF) and 5–6 days (30 μg; Fig. 4).

Fig. 3. H & E staining of an orthotopically grown pancreatic (low to moderately differentiated) human adenocarcinoma in nude rats. T, tumor tissue; D, duodenum; P, normal pancreatic tissue. ×200.

Fig. 4. Time course of serum complement depletion after i.v. injection of 30 (○), 3 (■), and 0.3 (★) μg CVF, in comparison to untreated rats (×). The complement activity was determined in a hemolytic assay.
Inflammatory Reaction in Tumors Treated by CA19-9-CVF.

Four nude rats with a s.c. and an orthotopic pancreatic tumor were treated with 70 μg CA19-9-CVF conjugate i.v. As a control, another 4 rats received a mixture of 35 μg noncoupled CA19-9 antibody and 35 μg CVF. The tumor was investigated 6 days after treatment, and tissue sections were stained with an anti-NK cell mab, as well as an antimacrophage antibody. In both groups, 8 tumors could be evaluated by counting the number of NK cells and macrophages. In the control group, 43 ± 24 NK cells and 39 ± 19 macrophages were found compared to 149 ± 98 NK cells and 143 ± 91 macrophages in the treated group.
mab-CVF CONJUGATE

Table 1 Uptake of a 99mTc-labeled anti-CEA antibody in pancreatic tumor tissue 24 h after pretreatment with CA19-9-CVF conjugate or a mixture of equimolar amounts of uncoupled CA19-9 and CVF (control)

The table shows the mean values of cpm/mg tissue (SD in parentheses), the mean ratio of the anti-CEA mab uptake (tumor:normal tissue), and the increase of tumor uptake (tumor:normal tissue ratio) in percentages.

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<tr>
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<th>Control</th>
<th>CA19-9CVF pretreated</th>
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<tr>
<td></td>
<td>cpm/mg tissue</td>
<td>Tumor:normal tissue</td>
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<td>s.c. tumor</td>
<td>15 (12)</td>
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<td>Muscle tissue</td>
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<tr>
<td>Pancreatic tumor</td>
<td>44 (16)</td>
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<td>Duodenum</td>
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CA19-9-CVF-treated group. No differences in the cell infiltration were seen between s.c. and orthotopic tumors. Figs. 5, a and b, show the microscopic slides of a CA19-9-CVF-treated and a control pancreatic tumor stained for NK cells.

Increase of 99mTc-labeled Anti-CEA Antibody Uptake. Four nude rats with a s.c. and orthotopic pancreatic tumor were given i.v. injections of 70 μg CA19-9-CVF conjugate. A further 4 rats received a mixture of 35 μg unconjugated antibody and 35 μg of free CVF (control group). Five days after conjugate injection, a 99mTc-labeled anti-CEA antibody was applied i.p. One day later, the animals were sacrificed, and the anti-CEA antibody uptake was determined in a γ counter.

The CEA-antibody tumor uptake in CA19-9-CVF-pretreated rats increased by approximately 90% in orthotopic tumors and 80% in s.c. tumors, compared to control animals. A statistical evaluation was not performed because of the low number of cases. Table 1 summarizes the results of this experiment.

Discussion

The effects of a mab-CVF conjugate, binding to a human pancreatic cancer cell line, were investigated in vitro and in an orthotopic tumor model using nude rats. CVF, a glycoprotein isolated from cobra venom, activates the alternative pathway of complement analogously to human C3. In contrast to C3, the activity of CVF cannot be inhibited by complement inhibitor factors H and I, resulting in a permanent activation of the complement cascade (9). It was shown that mab-CVF conjugates induce complement-mediated cancer cell lysis, as well as cytotoxic mab, which activates the classical pathway of complement (11). The properties of mab-CVF conjugates have been shown by the complement-mediated killing of leukemia cells (15), melanoma cells (14), and neuroblastoma (11). By conjugation with CVF, any nontoxic mab can access complement-activating activity. The creation of mab-CVF cocktails binding to different tumor antigens can improve mab therapy, thus overcoming the problem of antigen heterogeneity in solid tumors (16).

Unfortunately, most malignant tumor cells express resistance factors, preventing them from complement-mediated killing (17, 18). But in addition to the formation of the membrane attack complex, CVF causes the release of C3a and C5a, which induce an inflammatory reaction, including the attraction of macrophages, increase of blood flow, hypervascularization, and formation of vascular leaks (19). To our knowledge, these effects of mab-CVF conjugates have not been studied extensively in vivo.

In this study, we investigated a mab-CVF conjugate using the mab CA19-9, which binds very efficiently to the human pancreatic cancer cell line PancTu I. Immunostaining showed that PancTu I cells strongly express the complement resistance factors CD 46, CD 55, and CD 59. This finding is in accordance with the observation that CA19-9-CVF could not mediate a complement-dependent cell killing. However, using an ELISA system, Western blot, and immunostaining, we demonstrated that CA19-9-CVF conjugates activate the complement cascade after binding to PancTu I cells and induce the release of the anaphylatoxin C3a.

To investigate the in vivo effects of CA19-9-CVF, an orthotopic cancer model was developed using nude rats, which harbor a complement source equivalent to the human serum (11). Recent studies reported that orthotopic implantation results in a tumor growth that resembles human cancers, showing invasive growth and metastases (20). Very few studies have been performed with nude rats. This is most likely attributable to the observation that the number of T cells increases at the age of 3–4 months, which can result in tumor rejection (21). In addition to the short follow-up period of a maximum of 6 weeks, the recovery of T cells could explain the low rate of metastases in our model, compared to mice studies in which metastases occur frequently 8–12 weeks after tumor cell injection (22).

The human pancreatic tumors were examined after pretreatment with CA19-9-CVF conjugate to assess the infiltration of NK cells and macrophages (indicator for inflammatory reactions) and the tumor uptake of a 99mTc-labeled anti-CEA antibody. The infiltration of NK cells and macrophages increased in rats that were treated for 6 days with 70 μg CA19-9-CVF. Within the short observation period, we did not observe a therapeutic effect of the conjugates. One might speculate that this inflammatory reaction can attribute an immune response in the presence of an intact immune system, leading to tumor remission. Further studies have to be performed to investigate this effect in a syngenic animal model.

Apart from their immunotherapeutic potential, CA19-9-CVF conjugates appear to be promising compounds in pretargeting approaches. Although the anti-CEA antibody that was used to investigate the effects of CA19-9-CVF binds only 10–15% of the cancer cells, we observed an increase of the radiolabeled target antibody of approximately 2-fold. Even higher uptake rates might be achieved if a radiolabeled mab is applied that binds more efficiently to the cancer tissue. Pretargeting of tumors with varying vascular active drugs such as histamine, tumor necrosis factor, and interleukin-2 coupled to a mab resulted in a 1.5, 2-, and 3-fold increase of a F(ab')2 antibody fragment, respectively (23). The drawback with these drugs are in contrast to the nontoxic CVF) the severe side effects (e.g., lung edema), whereas CVF induces only a temporary deactivation.

Our data strongly indicate a value of mab-CVF conjugates in adjuvant as well as in pretargeting therapy. Further studies are being currently performed to derive more information about pharmacokinetics and the optimal treatment protocol for pretargeting concepts. A disadvantage of this approach is the large size of mab-CVF conjugates that have a Mr of approximately 300,000–500,000 and thus diffuse only to a very limited degree into tumor tissue. The effect of these conjugates might be increased if the mab targets tumor endothelial cells. Recently, mabs were developed that bind to proliferating endothelial cells found predominantly in tumors and healing wounds (24). These mabs should be quite specific for tumor vessels and might improve pretargeting therapy approaches.

Another problem of mab-CVF conjugates is their immunogenicity.
The cloning of human C3 and CVF should enable the construction of a human C3 that possesses the capacity of permanent complement activation. This molecularly engineered C3 could be used in combination with humanized mabs and will help to solve the problem of immunogenic conjugates. Furthermore, it might be possible to construct a fusion protein of a mab and the small active moiety of CVF or its genetically engineered C3 analogue. Such fusion proteins with reduced molecular weight will diffuse more readily in tumor tissue.

Acknowledgments

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References


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