

A human cytokine/single-chain antibody fusion protein for simultaneous delivery of GM-CSF and IL-2 to Ep-CAM overexpressing tumor cells

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Pro-inflammatory cytokines regulate the growth, differentiation, and activation of immune cells and can play a role in antitumor responses. GM-CSF and IL-2 induce tumor rejection in animal models when expressed by tumor cells, and IL-2 is used for the treatment of melanoma and renal cell cancer. However, high doses of GM-CSF and IL-2 are associated with severe side effects in cancer patients. We generated a dual cytokine fusion protein for simultaneous targeted delivery of human GM-CSF and IL-2 to human tumors. The fusion protein is based on a heterodimeric core structure formed by human CH1 and C kappa domains (heterominibody) with C-terminally fused human cytokines and N-terminally fused human single-chain Ab fragments (scFv) specific for the tumor-associated surface antigen epithelial cell adhesion molecule (Ep-CAM). The dual cytokine heterominibody (DCH) was well expressed and secreted by CHO cells, preserved the specific proliferative activities of the two cytokines, and showed Ep-CAM-specific binding to tumor cells. DCH induced potent tumor cell lysis *in vitro* by two distinct mechanisms. One was activating PBMCs to lyse tumor cells, which was superior to cytotoxicity induced by equimolar ratios of free recombinant human IL-2 and GM-CSF. The other mechanism was redirected lysis, as seen with isolated human T cells, which was solely dependent on the IL-2 fusion part. The therapeutic principle of dual cytokine targeting may warrant *in vivo* testing of murine-specific analogues in appropriate mouse models and further preclinical development of the less immunogenic, human cytokine- and human Ep-CAM-specific DCH molecule described here.

Keywords: human, cytokines, recombinant antibodies, cultured tumor cells, cytotoxicity tests, immunotherapy

Introduction

Many cytokines secreted by the cells of the immune system stimulate the proliferation, differentiation, and activation of immune cells after binding to specific membrane receptors. The systemic and local administration of various cytokines, as produced by recombinant DNA technology, stimulates the immune system and induces potent antitumor effects in cancer patients. For instance, systemic administration of IL-2 is used as immunotherapy for human malignancies, and it induced objective clinical responses in approximately 15% of patients with metastatic melanoma and renal cell carcinoma (1). IL-2 can mediate the regression of even bulky, invasive tumors in selected patients (2). The cytokine stimulates the proliferation and

activation of various immune effector cells such as T cells, NK cells, B cells, and macrophages (3). The side effects of systemic IL-2 administration led researchers to develop targeted IL-2 delivery to the tumor microenvironment by way of Ab-cytokine fusion proteins. Two humanized Ab-IL2 fusion proteins directed against Ep-CAM and the GD2 disialoganglioside are in early clinical trials for the treatment of prostate carcinoma and melanoma (4, 5). Both fusion proteins elicited potent antitumor activity mediated by T cells or NK cells in syngeneic murine tumor models and were superior to the combination of Ab and free IL-2 (6, 7).

GM-CSF regulates the growth and differentiation of hematopoietic cells and stimulates the functional activities of neutrophils and macrophages by upregulation of reactive oxygen intermediates in cytotoxic granules, increased phagocytic activity, and the release of pro-inflammatory cytokines (8). GM-CSF also mediates the upregulation of MHC class II molecules, costimulatory molecules, and adhesion molecules on macrophages and DCs, leading to increased antigen presentation (9). The cytokine has been described as the principal mediator of proliferation and maturation of DCs, which play a major role in the induction of T cell-mediated immune responses. Several vaccines consisting of irradiated tumor cells genetically engineered to secrete GM-CSF are currently in late-stage clinical development for various types of cancer and reportedly induce objective clinical responses in cancer patients (10, 11). Ab-GM-CSF fusion proteins may provide an alternative to vaccination strategies with GM-CSF-secreting tumor cells designed to stimulate an immune response at the site of the tumor. The antitumor activity of Ab-GM-CSF fusion proteins targeting the Her2/neu and transferrin receptor has been demonstrated in immune competent mice (12, 13).

Ep-CAM (also known as KSA, 17-1A, GA733-2, EGP-2, or ESA) is a 40-kDa transmembrane glycoprotein that is expressed on the basolateral surface of most epithelia and is overexpressed on the majority of human adenocarcinomas (14, 15). Ep-CAM has been the target of numerous immunotherapies, many of them in clinical trials (16, 17, 18). Recent studies have shown that Ep-CAM overexpression correlates with reduced overall survival among breast cancer patients (19), and that specific inhibition of Ep-CAM expression by small inhibitory RNA reduces the proliferation, migration, and invasiveness of breast cancer cells (20). The intracellular domain of Ep-CAM provides a robust proliferation signal to transfected cells by way of c-myc expression (21). Based on these observations, tumor cells appear to gain an advantage from the overexpression of Ep-CAM,

which can reach from 100- to 1000-fold higher levels on tumor cells than on the corresponding normal epithelial cells (20, 22).

In the present study, we constructed, expressed, and characterized *in vitro* a novel fusion protein designed to combine in one molecule the pro-inflammatory cytokines IL-2 and GM-CSF, as well as two single-chain antibodies with specificity for Ep-CAM. All protein components of the DCH were of human origin and of human specificity. The rationale for constructing DCH was to combine the distinct antitumor activities of IL-2 and GM-CSF in one molecule that could be targeted to most human adenocarcinomas, obviating the need to develop two separate immunotherapies. DCH and various control constructs could be expressed in CHO cells. They retained the specific activities of the cytokines, as well as the capacity to bind to the Ep-CAM antigen on tumor cells. The combination of GM-CSF and IL-2 in DCH fusion proteins induced potent tumor cell lysis in the presence of PBMCs and showed increased cytotoxic activity compared to equivalent molar amounts of free GM-CSF and IL-2. By way of its IL-2 portion, DCH induced Ep-CAM-dependent redirected tumor cell lysis by isolated T cells. *In vivo*, simultaneous targeting of IL-2 and GM-CSF to tumor cells may elicit synergistic cytokine effects in the tumor microenvironment, leading to a strong immune response against tumor cells.

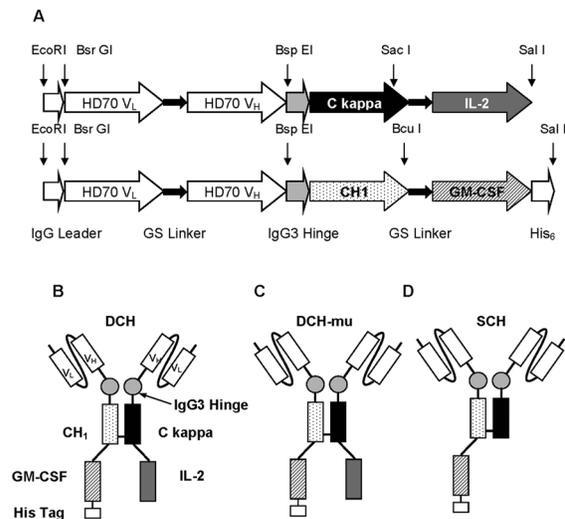
Results

Construction and expression of DCH and variants

A recombinant cytokine fusion protein was constructed that allows simultaneous targeting of GM-CSF and IL-2 to the tumor-associated antigen Ep-CAM by way of single-chain antibodies. The core of the DCH fusion protein and variants is a heterominibody that had previously been used to design constructs for targeting costimulatory molecules such as B7-1, B7-2, and LFA-3 by Ep-CAM-specific single-chain antibodies to tumor cells (23). The cDNA structures of constructs used in this study are shown in Figure 1A, and the domain structures of DCH and variants are shown in Figure 1, panels B-D.

The heterominibody core in DCH is stabilized by a disulfide bond between the heterodimer of the constant Ig domains C kappa and CH1 of human IgG1 (Figure 1B). Two scFvs derived from the human anti-Ep-CAM Ab HD70 (24) were fused to C kappa and CH1 domains by the hinge region of human IgG3. Human GM-CSF and IL-2 were joined by a G_4S linker peptide to the C-terminus of the CH1 and C kappa domains, respectively. A C-terminal polyhistidine tag was added to GM-CSF to allow for highly efficient affinity purification by immobilized metal ion adsorption chromatography. A mutant (mu) fusion protein called DCH-mu was designed that had amino acid mutations in the hypervariable regions of both HD70 V_L and V_H domains, abrogating Ep-CAM binding (Figure 1C). DCH-mu had Ser/Trp and Pro/Glu substitutions in the complementary-determining region (CDR) 3 of the HD70 V_L domain, and Trp/Glu and Tyr/Glu substitutions in the CDR3 of the HD70 V_H domain. A second control protein was designed that contained only GM-CSF and was referred to as single cytokine heterominibody (SCH) (Figure 1D). In addition, the parental scFv HD70 heterominibody, designated HMB, was constructed without IL-2 and GM-CSF, and purified. A fourth control protein containing only IL-2 was also constructed, but it was not analyzed further due to its poor expression in CHO cells.

Figure 1



Schematic representation of DCH and variants. (A) cDNA domains encoding scFv HD70-IL-2 and scFv HD70-GM-CSF polypeptide chains. Structure of (B) the DCH fusion protein; (C) DCH-mu variant with amino acid mutations in the hypervariable regions of the V_L and V_H domains; and (D) SCH variant without an IL-2 fusion at the C kappa C-terminus. Abbreviations: HD70 V_L and V_H , light and heavy chain variable region domains derived from the human anti-Ep-CAM Ab HD70; GS linker, glycine-serine linker peptide (G_4S); IgG3 hinge, upper hinge region of human IgG3; C kappa, constant region domain of human Ig kappa light chain; CH1, first constant region domain of human IgG1 heavy chain; His₆, hexahistidine tag.

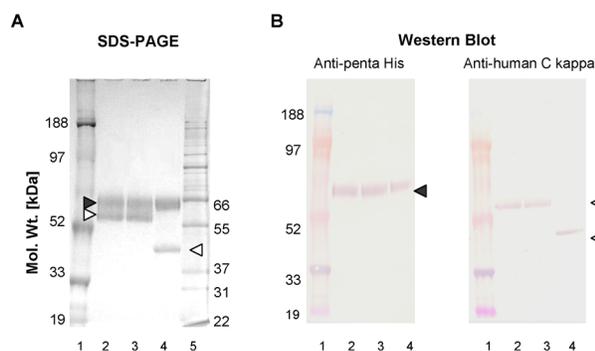
CHO cells deficient in dihydrofolate reductase were double transfected with cDNAs encoding the two polypeptide chains of DCH, DCH-mu, SCH, and HMB on pEF-dhfr and pEF-ada vectors and selected for stable expression. The expression of constructs was increased by gene amplification using the dihydrofolate reductase and adenosine deaminase enzyme inhibitors methotrexate and deoxycoformycin.

Purification and biochemical analysis of DCH and variants

DCH, DCH-mu, and SCH fusion proteins were purified from CHO cell supernatants using a three-step purification procedure of Q-Sepharose anion exchange chromatography, immobilized metal ion affinity chromatography (IMAC), and gel filtration. Average protein yields were between 1.3 and 4 mg purified DCH per liter of cell culture supernatant. The high degree of purity of DCH and variants was confirmed by SDS-PAGE under reducing conditions (Figure 2A). The identity of the more slowly migrating Coomassie blue-stained bands of 65 kDa with HD70-GM-CSF and HD70-mu-GM-CSF polypeptides was verified by Western blotting using an anti-penta-His detection Ab (Figure 2B). The identity of the faster migrating Coomassie blue-stained bands of 60 kDa with HD70-IL-2 and HD70-mu-IL2 polypeptides was confirmed by Western blot analysis with an anti-human kappa light chain Ab (Figure 2B). The HD70-C kappa chain of SCH showed an apparent molecular mass of 43 kDa.

All polypeptide chains showed relatively broad bands upon SDS-PAGE and apparent molecular masses that were greater than the molecular weights calculated. These findings are consistent with glycosylation of GM-CSF and IL-2 in CHO cells,

Figure 2



SDS-PAGE and Western blot analysis of DCH and variants. DCH (lane 2), DCH-mu (lane 3), and SCH (lane 4) were expressed in CHO cells and purified by Q-sepharose anion exchange chromatography, immobilized metal ion affinity chromatography, and gel filtration. (A) Purified proteins were analyzed on 12% SDS-PAGE under reducing conditions. (B) Proteins were blotted onto nitrocellulose membranes. HD70-GM-CSF (solid arrowhead) and HD70-mu-GMCSF (solid arrowhead) polypeptides were detected with an anti-penta-His mouse IgG1 Ab in combination with an alkaline phosphatase-conjugated goat anti-mouse Ab. The HD70-IL-2 (open arrowhead), HD70-mu-IL-2 (open arrowhead), and HD70-C kappa (open arrowhead) polypeptides were detected using an alkaline phosphatase-conjugated anti-human kappa light chain Ab. Molecular weight markers and their molecular weights in kilodaltons are shown in lanes 1 (Multi Mark multicolored standard) and 5 (Mark 12 unstained standard).

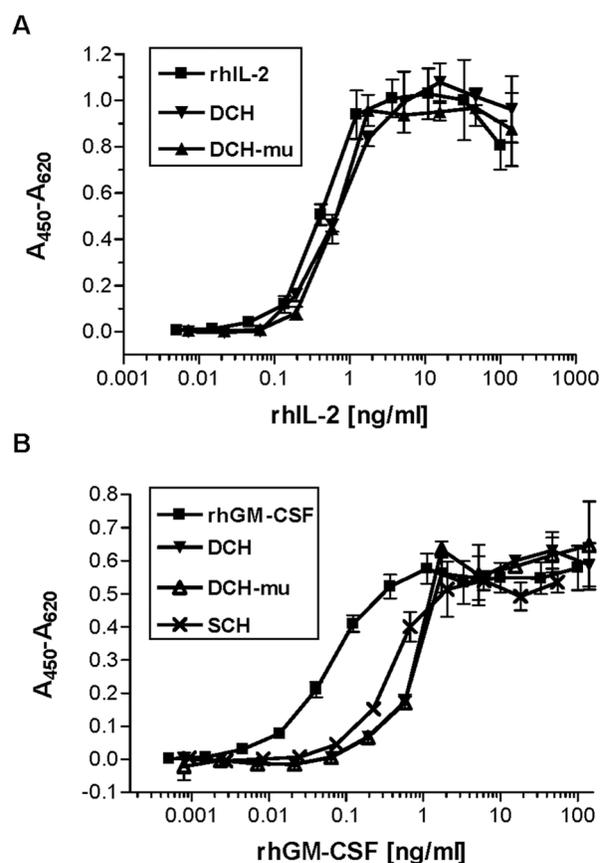
as well as of the single-chain HD70 Ab, which contains an N-glycosylation consensus site. In the absence of reducing agents, DCH and variants showed apparent molecular weights of approximately 125 kDa upon SDS-PAGE (data not shown), confirming the heterodimeric structure of the molecules and formation of a disulfide bridge between cysteine residues of the CH1 and C kappa domains. Under native conditions, DCH, DCH-mu, and SCH eluted as single peaks from gel filtration columns. Parental HMB, in the absence of GM-CSF and IL-2, was purified to homogeneity by cation exchange chromatography, IMAC, and gel filtration (data not shown).

Biological activity of IL-2 and GM-CSF in DCH and variants

IL-2 bioactivity was measured in a proliferation assay with the IL-2-dependent mouse cell line CTLL-2. A recombinant human IL-2 standard from *E. coli* induced half-maximal proliferation of CTLL-2 cells at a concentration of 0.33 ng/ml and maximum proliferation at 1.2 ng/ml IL-2 (Figure 3A). The concentration of DCH and DCH-mu in the proliferation assay was adjusted to obtain molar concentrations of fused IL-2 equivalent to that of recombinant IL-2 standard. The bioactivity of IL-2 fused to the C-termini of DCH and DCH-mu proteins was essentially identical to the activity of free human IL-2 standard (Figure 3A).

GM-CSF bioactivity was tested in a proliferation assay using the GM-CSF-dependent human erythroleukemia cell line TF-1. Purified DCH, DCH-mu, and SCH proteins were all able to stimulate the proliferation of TF-1 cells in a dose-dependent manner (Figure 3B). Maximum TF-1 proliferation was achieved by fusion proteins at normalized GM-CSF concentrations greater than or equal to 1.7 ng/ml, with half-maximal activities between 0.34 and 0.77 ng/ml. When compared to equimolar concentrations of recombinant human GM-CSF produced in *E. coli*, GM-CSF fusion proteins were approximately 6- to 13-fold less active (Figure 3B). The reduced biological activity of

Figure 3



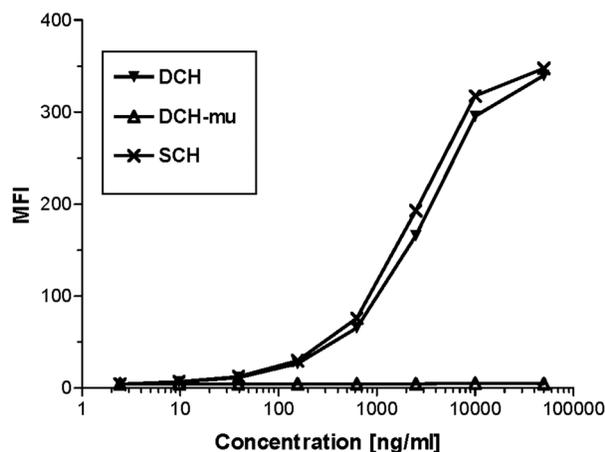
Biological activity of IL-2 and GM-CSF in DCH and variants. (A) The biological activity of IL-2 was measured by cellular proliferation of the IL-2-dependent murine T cell line CTLL-2. CTLL-2 cells were incubated with various concentrations of free recombinant human IL-2 (rhIL-2) and equivalent amounts of IL-2 in purified DCH proteins. (B) The biological activity of GM-CSF was measured by cellular proliferation of the GM-CSF-dependent human erythroleukemia cell line TF-1. TF-1 cells were incubated with various concentrations of free recombinant human GM-CSF (rhGM-CSF) and equivalent amounts of GM-CSF in purified DCH molecules. The number of viable cells was determined in a WST-1 colorimetric assay. All results are expressed as mean absorbance $A_{450}-A_{620}$ of triplicate determinations.

glycosylated GM-CSF in CHO-produced fusion proteins versus nonglycosylated *E. coli*-produced GM-CSF is in agreement with the findings of other research groups, who observed a 20-fold increase in specific biological activity following removal of oligosaccharides from recombinant CHO-produced GM-CSF (25). In summary, cell proliferation assays indicated that IL-2 and GM-CSF cytokines retained their biological activity when fused to the C-termini of heteromimicry constructs.

Binding of DCH and variants to Ep-CAM

Binding of DCH and variants to Ep-CAM-expressing Kato III cells was analyzed by flow cytometry, and fusion proteins were detected using anti-penta-His mouse IgG1 and FITC-conjugated goat anti-mouse F(ab)₂. DCH and SCH fusion proteins showed saturable binding to Kato III cells, whereas virtually no binding of DCH-mu was detected up to a concentration of 50 μg/ml (Figure 4). These data demonstrate

Figure 4



Binding of DCH and variants to Ep-CAM. The human adenocarcinoma cell line Kato III was incubated with various amounts of recombinant fusion proteins. Binding was analyzed by flow cytometry using an anti-penta-His mouse IgG1 and FITC-conjugated goat anti-mouse F(ab)₂ detection system. The mean fluorescence intensity (MFI) of cells from duplicate determinations is plotted against the concentration of DCH and variants.

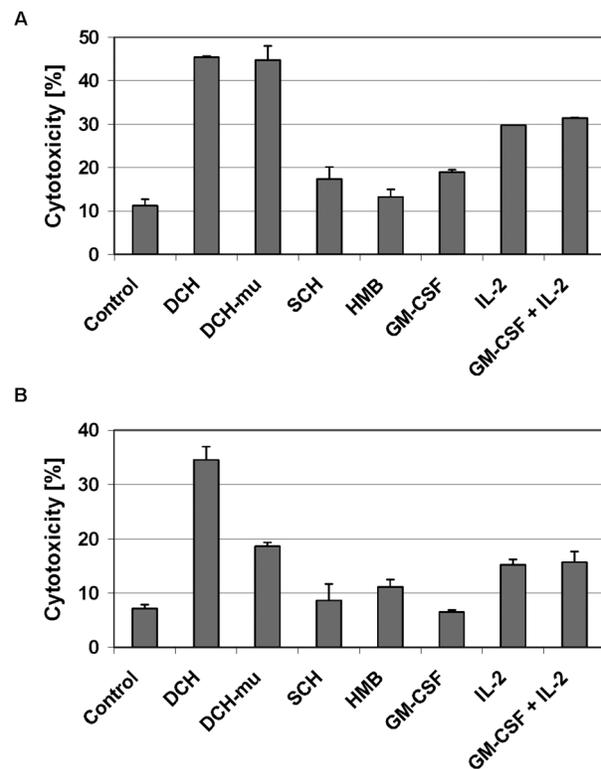
that the HD70 scFv fragments in DCH and SCH fusion proteins retained their ability to bind to Ep-CAM expressed on a human gastric carcinoma-derived cell line, and that the four amino acid alterations in the CDR3 regions of the HD70 V_L and V_H domains abolished Ep-CAM binding.

Cellular cytotoxicity of DCH and variants

A FACS[®]-based assay was established to analyze cellular cytotoxicity mediated by DCH and variants. Kato III cells prelabeled with the membrane dye PKH-26 were mixed with PBMCs at an E/T ratio of 20:1 and incubated with 10 µg/ml DCH, variant proteins, parental heterominibody (designated HMB), or cell culture medium (control) for 16 h. Recombinant human GM-CSF and IL-2, as well as the combination of both cytokines, were tested at equimolar concentrations of 1.4 µg/ml. PKH-26 is a red-orange fluorescent dye (emission wavelength 540-620 nm, maximum 567 nm) with long aliphatic tails that stably incorporates into the lipid regions of the cell membrane and allows one to distinguish fluorescent tumor cells from unlabeled effector cells (FL2, 585 nm). At the end of the incubation period, the red fluorescent DNA-binding dye PI (emission wavelength 560-670 nm, maximum 600 nm) was added in order to identify nonviable tumor cells (FL3, 660 nm). Cells with damaged membranes allow the entrance of PI, which undergoes a fluorescence enhancement upon binding to nucleic acids, promoting a red fluorescence in dead cells. Two-color FACS[®] analysis (FL2/FL3) was used to discriminate dead (PKH26/PI double positive) and live (PKH26 single positive) tumor cells in the presence of unlabeled effector cells.

Both DCH and DCH-mu induced potent lysis of tumor cells in the presence of PBMCs as compared with a reaction using the parental HMB without cytokines (Figure 5A). The SCH fusion protein and free GM-CSF, at equimolar concentrations, showed only weak activation of PBMCs to lyse tumor cells under the chosen experimental conditions (Figure 5A). An equivalent molar amount of free recombinant human IL-2 was efficient in activating PBMCs to induce lysis of Kato III cells over the 16-h

Figure 5



PBMC- and T cell-dependent cytotoxic activity of DCH and variant proteins.

A two-color FACS[®]-based assay was performed. Kato III target cells prelabeled with the orange fluorescent dye PKH26 were incubated in a 16-h assay with immune effector cells (PBMCs or isolated human T cells) and DCH, variant proteins, or free cytokines at 37°C and 5% CO₂. A control reaction without fusion protein was included. The DNA-binding, red fluorescent dye PI was added at the end of the incubation, and cells were analyzed by flow cytometry using 585 nm (orange) and 660 nm (red) band pass filters. (A) PBMC-dependent cytotoxicity of 10 µg/ml DCH, variant proteins, and parental HMB without cytokines at an E/T ratio of 20:1. Recombinant human GM-CSF and IL-2, as well as the combination of both cytokines, were included at equimolar concentrations of 1.4 µg/ml. (B) T-cell dependent cytotoxicity of 10 µg/ml DCH, variant proteins, and parental HMB without cytokines at an E/T ratio of 10:1. Recombinant human GM-CSF and IL-2, as well as the combination of both cytokines, were included at equimolar concentrations of 1.4 µg/ml. Percentage of cytotoxicity (CT) was calculated as CT = [number of dead target cells (orange/red events) in sample / total number of target cells (orange events) in control] × 100. Error bars indicate standard deviations from duplicate determinations.

incubation period (Figure 5A), presumably by generating LAK cells from PBMCs. The addition of GM-CSF to IL-2 did not further increase cytotoxic activity (Figure 5A). In contrast, the combination of GM-CSF and IL-2 in the DCH fusion proteins led to increased cytotoxic activity over equimolar amounts of IL-2 or the combination of GM-CSF and IL-2 (Figure 5A). A dose-dependent lysis of Kato III cells was observed with both DCH and DCH-mu fusion proteins in the presence of PBMCs, and a half-maximal efficacy of tumor cell lysis (EC₅₀) occurred at 743 and 233 ng/ml of DCH and DCH-mu, respectively (data not shown). These data indicate that under the cell culture conditions used, lysis of Kato III cells in the presence of PBMCs was enhanced by the combination of GM-CSF and IL-2 in DCH

fusion proteins. Cell lysis, however, was largely independent of targeting of IL-2 and GM-CSF to Ep-CAM, most likely due to general activation of immune effector cells by way of cross-linking of cytokine receptors.

The cytotoxic potential of DCH (10 µg/ml) against Kato III tumor cells was also assessed with isolated T cells at an E/T ratio of 10:1. T cells were isolated from human PBMCs by retaining non-T cell populations on specific Ab columns. Furthermore, 10 µg/ml of variant proteins and parental HMB, as well as 1.4 µg/ml of the two cytokines either singly or in combination, were evaluated under these conditions. DCH was highly efficient at inducing tumor cell lysis in the presence of isolated T cells (Figure 5B). Incubation of T cells and Kato III cells with the parental HMB in the absence of cytokines, the SCH fusion protein lacking IL-2, and an equivalent amount of free GM-CSF did not result in any specific cytotoxicity (Figure 5B). Equimolar concentrations of free recombinant IL-2 and the Ep-CAM binding mutated DCH-mu control protein were able to stimulate some tumor cell lysis by means of isolated T cells, but cytotoxic activity was approximately two-fold lower than with DCH (Figure 5B). Addition of free GM-CSF to IL-2 had no effect on T cell-dependent tumor cell lysis (Figure 5B). Dose-dependent lysis of Kato III and SW480 tumor cells through DCH-redirection T cells resulted in EC50 values of 1502 ng/ml and 475 ng/ml, respectively (data not shown). In summary, DCH caused a redirected tumor cell lysis in the presence of purified T cells, which required targeting of IL-2 to the Ep-CAM antigen on tumor cells.

Analysis of T cell subsets involved in DCH-mediated cellular cytotoxicity

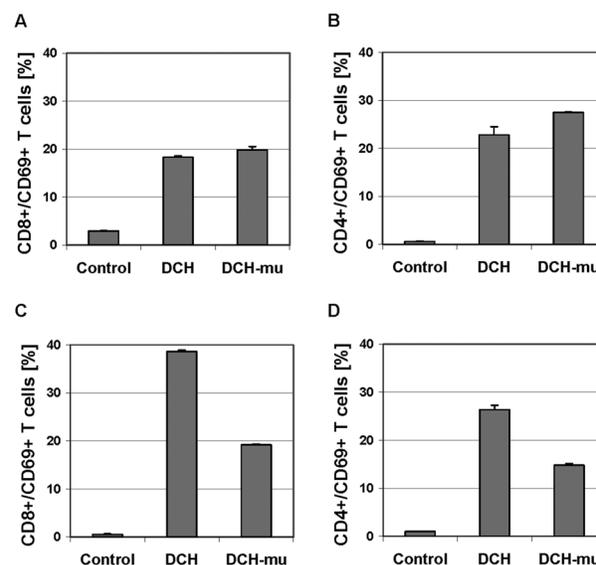
Activation of CD4+ and CD8+ T cells was analyzed by flow cytometric analysis of CD69 expression after incubation of PBMCs or enriched T cells with Kato III cells and 10 µg/ml DCH and DCH-mu. Using PBMCs, similar activation levels of CD4+ and CD8+ T cells through DCH and DCH-mu were seen (Figure 6, panels A and B). A control reaction in the absence of cytokine fusion proteins did not show activation of T cells. DCH was highly efficient in activating CD8+ and CD4+ T cell subsets after a 16-h incubation of purified T cells with Kato III cells (Figure 6, panels C and D). The Ep-CAM binding-deficient DCH-mu control protein also activated CD4+ and CD8+ T cells, but was clearly less effective than the Ep-CAM binding DCH (Figure 6, panels C and D). Both isolated CD4+ and CD8+ human T cells were able to mediate cellular cytotoxicity when incubated with 10 µg/ml DCH and PKH26-labeled Kato III tumor cells at E/T ratios of 10:1 (Figure 7). However, CD8+ T cells showed a two-fold higher cytotoxic activity than isolated CD4+ T cells.

Discussion

DCH is a novel single-chain Ab-based dual cytokine fusion protein designed to deliver IL-2 and GM-CSF to the surface of Ep-CAM-positive tumor cells simultaneously in order to induce a tumor-specific immune response. We will discuss differences between DCH and conventional Ab cytokine fusions with respect to production, Ep-CAM binding affinity, potential issues of safety and immunogenicity, contribution of Fcγ domains to efficacy, mode of action, serum half-life, size, and potential tumor penetration, and the consequences of combining two cytokines in one protein.

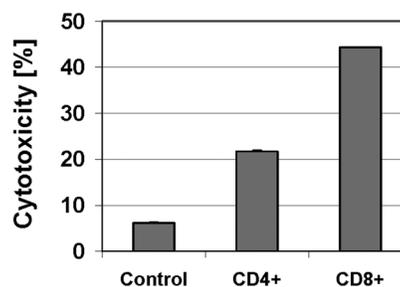
DCH was found to be glycosylated, secreted, and well produced by CHO cells. When fused to the C-termini of the

Figure 6



Flow cytometric analysis of T cell activation. (A) Human PBMCs were incubated for 16 h with Kato III cells at an E/T ratio of 20:1 in the presence of 10 µg/ml DCH, DCH-mu, or cell culture medium (control). CD69 expression on CD8+ T cells was detected by anti-CD69 FITC-conjugated and anti-CD8 PE-conjugated mouse mAb staining. (B) CD69 expression on CD4+ T cells within the PBMC population was detected by anti-CD69 FITC-conjugated and anti-CD4 PE-conjugated mouse mAb staining. (C) Purified human T cells were incubated for 16 h with Kato III cells at an E/T ratio of 10:1 in the presence of 10 µg/ml DCH, DCH-mu, or medium (control). CD69 expression on CD8+ T cells was detected as above. (D) CD69 expression on CD4+ T cells within isolated human T cells was detected as above. Error bars indicate standard deviations from duplicate determinations.

Figure 7



DCH-mediated cytotoxicity of isolated CD4+ and CD8+ T cells. A two-color FACS[®]-based assay was performed. Kato III target cells were prelabeled with PKH26 and mixed with isolated human CD4+ or CD8+ T cells at an E/T ratio of 10:1. We incubated 10 µg/ml DCH with the cells for 16 h at 37°C and 5% CO₂. A control reaction without fusion protein was included. PI was added to discriminate between live and dead target cells. Error bars indicate standard deviations from duplicate determinations.

heterodimerization domain, both cytokines fully retained their biological activities, and DCH showed Ep-CAM-specific binding to a human cancer cell line. This was not too surprising, since IL-2 and GM-CSF also retain their activities

when fused to the C-termini of CH3 domains in complete antibodies (26, 27). DCH may thus be similar to conventional Ab-cytokine fusions with respect to production by a well-established eukaryotic producer cell line and preservation of cytokine and target-binding activities.

Ep-CAM is a well characterized and clinically validated immunotherapeutic target that is overexpressed on a large variety of human carcinomas, including colorectal, lung, breast, stomach, prostate, pancreatic, ovarian, and squamous cell carcinoma of the head and neck (14, 28). Ep-CAM was also the target of choice for the development of fusions between murine mAb KS1/4 and IL-2, and for a humanized version of the KS1/4-IL2 fusion protein (7). Contrary to these established formats, we have constructed DCH using a fully human single-chain Ab with rather low affinity for Ep-CAM, with consequences on the construct size and potential implications for safety and immunogenicity. The binding affinity of the parental Ab HD70 to Ep-CAM is reported to have a K_D of 3.9×10^{-7} M (24). This affinity constant is similar to that of the well-tolerated Ab edrecolomab (24) and is therefore expected to cause much less toxicity than is reported for high-affinity Ep-CAM antibodies, which can elicit acute pancreatitis (29). The fully human nature of DCH is expected to translate into a lower immunogenicity, as is observed with conventional cytokine fusions using murine or humanized mAbs. In particular, protein fusions with human GM-CSF may potentiate immunogenicity because of the potent adjuvant property of the APC-stimulating cytokine, which is currently being exploited in numerous vaccination trials (10, 11, 30). Neutralizing antibodies against recombinant GM-CSF have been found in cancer immunotherapy studies in 40-60% of patients (31, 32).

Another difference between DCH and conventional IgG-cytokine fusion proteins is that DCH is devoid of Fc γ domains. This not only reduces the size of DCH but may further decrease its potential immunogenicity because the fusion protein will not be internalized via Fc γ receptors by APCs. On the other hand, the absence of C γ 1 and C γ 3 heavy chains may reduce the cytotoxic potential of DCH with respect to whole Ab cytokine fusion proteins because ADCC and CDC cannot add to the target cell lysis observed with DCH. It is interesting to note, however, that a humanized IgG4-IL-2 Ab fusion protein with almost no ADCC or CDC activity showed a higher antitumor activity in two syngeneic mouse tumor models than the corresponding humanized IgG1-IL-2 fusion protein (33). This would argue that ADCC and CDC do not make an important contribution to the activity of Ab-IL-2 fusion proteins.

Fc γ domains are also expected to mediate a relatively long half-life of conventional IgG-cytokine fusion proteins through recycling by specific IgG protection receptors (FcRp). Although this can be an advantage in permitting infrequent dosing, the fusion with whole IgG may create long-lived inflammatory cytokines with potential side effects that cannot be easily controlled once the Ab fusion is administered. Surprisingly, clinical trials with two Ab-IL-2 fusion proteins reported a serum half-life in cancer patients of only 4 h, which is a much faster clearance rate than that seen for regular mAb therapeutics (4, 5). We do not know the half-life of DCH, but it seems that the Fc γ portion is dispensable for providing a long half-life to Ab-cytokine fusion proteins.

Another difference between DCH and conventional Ab-cytokine fusions is size. Because it uses two single-chain antibodies for tumor targeting and small heterodimerization domains, DCH is approximately 50 kDa smaller in size than conventional Ab-cytokine fusion proteins. With a size of

approximately 125 kDa, DCH may be at the threshold where one study showed a steep improvement in tumor penetration of truncated Ab constructs (34). IL-2 was shown to enhance the access of fusion proteins to tumors by increasing vascular permeability, as has been reported for an antihuman B cell Ab-IL-2 fusion protein in a murine model (35). Only studies in cancer patients will allow researchers to investigate tumor penetration of the human-specific DCH protein.

An important difference between DCH and conventional Ab-cytokine fusion proteins is the combination of IL-2 and GM-CSF in one protein. This was made possible by using a heterodimer composed of human IgG1 CH1 and C kappa domains presenting four positions for fusion of other proteins by way of linkers and hinge regions. CH1 and C kappa domains have a strong tendency to heterodimerize, but C kappa domains can also homodimerize to some extent. However, since the hexahistidine tag was placed on the CH1 chain, only heterodimerized species were affinity-purified. Combining IL-2 and GM-CSF in one molecule has the obvious advantage of delivering equal amounts of the two cytokines to tumor cells without the problem of binding competition by single-cytokine fusion proteins and the expense and effort of developing two fusion proteins in parallel. Systemic administration of high-dose IL-2 and GM-CSF has been associated with severe toxicities in several clinical studies (1, 36), whereas local treatment with subcutaneous injections of low doses of both cytokines was well tolerated (37). Therefore, specific targeting of IL-2 and GM-CSF to the tumor microenvironment by way of the DCH should increase local cytokine concentrations and reduce the side effects of systemic administration. Another advantage of cytokine fusion proteins is the increased half-life of cytokines due to larger size and slower renal clearance. IL-2 has a short distribution half-life $T_{1/2\alpha}$ of approximately 10 min and an elimination half-life $T_{1/2\beta}$ of 85-180 min after bolus injection (3). A distribution half-life $T_{1/2\alpha}$ of 10 min and an elimination half-life $T_{1/2\beta}$ of 50-85 min have been reported for GM-CSF (38). In DCH, the half-lives of the two cytokines should not only be equal, but should also be prolonged due to the size of DCH, which is above the cut-off for renal clearance.

Our study shows that DCH strongly stimulates PBMCs to lyse tumor cells *in vitro*. The combination of GM-CSF and IL-2 in one molecule enhances cytotoxic activity over that of equimolar ratios of free cytokines. High IL-2 concentrations (>500 units/ml) reportedly generate LAK cells from PBMCs within 18 h (39). LAK cells represent a mixture of NK and cytotoxic T cells with the capacity to lyse malignant cells without MHC restriction (40). We reproduced unspecific tumor cell lysis through LAK cells by incubating Kato III cells and human PBMCs in the presence of 1.4 μ g/ml recombinant human IL-2, corresponding to 1400 units/ml. The addition of free GM-CSF to IL-2 did not result in a significant increase in tumor cell lysis, whereas addition of GM-CSF to IL-2 within DCH fusion proteins clearly increased PBMC-dependent cytotoxicity. Activation of PBMCs to lyse tumor cells was independent of Ep-CAM targeting, as was demonstrated by a control protein with mutated Ep-CAM binding sites. These results indicate cross-linking of IL-2 and GM-CSF receptors on adjacent cells or the same cell by DCH. GM-CSF receptors are mainly expressed on monocytes, macrophages, and DCs within the PBMC population (9), whereas IL-2 receptors are present on T cells, NK cells, B cells, and macrophages (41).

DCH was also capable of redirecting polyclonal T cells for the lysis of human gastric and colon carcinoma cells, which required targeting of IL-2 to the Ep-CAM antigen on tumor cells.

Redirection of nontumor-specific T cells was described for an anti-Her2/neu scFv IL-2 fusion protein that was shown to transiently link tumor cells and IL-2 receptor-positive T cells, thereby facilitating tumor cell killing in a non-MHC-restricted manner (42). The investigators demonstrated redirected lysis of tumor cells with cloned CD4+ and CD8+ T cell lines derived from transgenic mice and described a Fas ligand-dependent killing mechanism using a T cell clone obtained from Fas ligand-negative mice. In contrast, we used polyclonal T cells isolated from human donors and showed specific activation of CD4+ and CD8+ T cell subsets through DCH in the presence of tumor cells. Both CD4+ and CD8+ T cells were able to induce redirected T cell cytotoxicity, with isolated CD8+ T cells being more active than CD4+ T cells, however. Redirected lysis of tumor cells was highly specific for the Ep-CAM target antigen, as was demonstrated by reduced cytotoxicity of a control protein with abrogated Ep-CAM binding specificity or by equimolar amounts of recombinant human IL-2. Interestingly, in the study by Lustgarten *et al.* (42), free IL-2 induced tumor cell lysis at a level similar to that obtained with their Ab-IL-2 fusion protein. These results indicate that DCH fusion proteins may act by a different mechanism. Several studies have shown that IL-2 stimulates the transcriptional activation of genes involved in cytotoxicity, such as perforin, granzymes, and Fas ligand (43, 44), but the precise mechanism of redirected tumor cell lysis through DCH fusion proteins, which is independent of specific TCR engagement, remains to be elucidated.

Preclinical studies with Ab-IL-2 and scFv-IL-2 fusion proteins directed against tumor antigens in murine tumor models demonstrated that high local concentrations of IL-2 at the site of the tumor can effectively stimulate the host immune system to eradicate tumor cells (7, 45). In addition, targeted IL-2 has been shown to stimulate the proliferation and differentiation of tumor-specific T cells leading to long-term tumor protective immunity in immune competent mice (46, 47).

In the present study, a SCH fusion protein carrying GM-CSF in the absence of IL-2 did not show significant cytotoxic activity *in vitro*. One reason may be that granulocytes, whose cytolytic activity can be stimulated by GM-CSF, are absent from PBMCs. Moreover, the adjuvant activity of GM-CSF by way of DC activation, T cell priming, and induction of Ab responses cannot be recapitulated *in vitro*. Potent antitumor activity of Ab-GM-CSF fusion proteins specific for tumor-associated antigens has been demonstrated *in vivo* in several mouse tumor models (12, 13). Combined GM-CSF and IL-2 in DCH fusion proteins, however, may strongly synergize in stimulating the immune system. By activating DCs and other APCs, GM-CSF may render weakly immunogenic tumor cells highly immunogenic, as has been demonstrated in vaccination studies with GM-CSF-secreting irradiated tumor cells in cancer patients (10, 11, 30).

A major issue with dual cytokine fusion proteins may be the potential to link cytokine receptors on adjacent cells, and where coexpressed, perhaps on the same cell. Cytokines bind their receptors with extremely high affinity, and fusion proteins may become targeted to immune cells expressing high-affinity cytokine receptors rather than to tumor cells. Thus, higher target affinity may be needed in order to achieve better tumor targeting of DCH fusion proteins *in vivo*. A murine-specific analogue of DCH with scFvs derived from a high-affinity anti-Ep-CAM murine mAb was therefore constructed and is currently being compared to the low-affinity murine DCH fusion protein regarding its ability to eradicate established lung metastases in mice. These *in vivo* experiments will determine the therapeutic potential of the approach and will explore

cytokine targeting, as well as potential systemic side effects of dual cytokine fusion proteins.

In summary, further preclinical development of DCH requires *in vivo* studies determining antitumor activity, cytokine targeting, and toxicology. Because DCH is human-specific, only rodent-specific analogues can address these questions. Several murine-specific analogues of DCH with various binding affinities for the Ep-CAM antigen were constructed and characterized, and are currently being tested in a mouse tumor model.

Abbreviations

DCH, dual cytokine heterominibody; Ep-CAM, epithelial cell adhesion molecule; IMAC, immobilized metal ion affinity chromatography; mu, mutant; SCH, single cytokine heterominibody

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References

- Rosenberg SA, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA* 1994; **271**: 907-913. (PMID: 8120958)
- Rosenberg SA. A new era of cancer immunotherapy: converting theory to performance. *CA Cancer J Clin* 1999; **49**: 70-73, 65. (PMID: 11198888)
- Smith K. IL-2. In: *Cytokine reference, volume 1*. Oppenheim J. (Ed.) New York: Academic Press; 2000; 113-125.
- Ko YJ, Bublely GJ, Weber R, Redfern C, Gold DP, Finke L, Kovar A, Dahl T, Gillies SD. Safety, pharmacokinetics, and biological pharmacodynamics of the immunocytokine EMD 273066 (huKS-IL2): results of a phase I trial in patients with prostate cancer. *J Immunother* 2004; **27**: 232-239. (PMID: 15076141)
- King DM, Albertini MR, Schalch H, Hank JA, Gan J, Surfus J, Mahvi D, Schiller JH, Warner T, Kim K, Eickhoff J, Kendra K, Reisfeld R, Gillies SD, Sondel P. Phase I clinical trial of the immunocytokine EMD 273063 in melanoma patients. *J Clin Oncol* 2004; **22**: 4463-4473. (PMID: 15483010)
- Lode HN, Xiang R, Dreier T, Varki NM, Gillies SD, Reisfeld RA. Natural killer cell-mediated eradication of neuroblastoma metastases to bone marrow by targeted interleukin-2 therapy. *Blood* 1998; **91**: 1706-1715. (PMID: 9473237)
- Xiang R, Lode HN, Dolman CS, Dreier T, Varki NM, Qian X, Lo KM, Lan Y, Super M, Gillies SD, Reisfeld RA. Elimination of established murine colon carcinoma metastases by antibody-interleukin 2 fusion protein therapy. *Cancer Res* 1997; **57**: 4948-4955. (PMID: 9354462)
- Nicola N. *Cytokine reference, volume 1*. Oppenheim J. (Ed.) New York: Academic Press; 2000; 899-910.

9. Armitage JO. Emerging applications of recombinant human granulocyte-macrophage colony-stimulating factor. *Blood* 1998; **92**: 4491-4508. (PMID: 9845514)
10. Dummer R. GVAX (Cell Genesys) [review]. *Curr Opin Investig Drugs* 2001; **2**: 844-848. (PMID: 11572668)
11. Nemunaitis J, Nemunaitis J. Granulocyte-macrophage colony-stimulating factor gene-transfected autologous tumor cell vaccine: focus[correction to fcous] on non-small-cell lung cancer. *Clin Lung Cancer* 2003; **5**: 148-157. (PMID: 14667270)
12. Dreier T, Lode HN, Xiang R, Dolman CS, Reisfeld RA, Kang AS. Recombinant immunocytokines targeting the mouse transferrin receptor: construction and biological activities. *Bioconjug Chem* 1998; **9**: 482-489. (PMID: 9667950)
13. Dela Cruz JS, Trinh KR, Morrison SL, Penichet ML. Recombinant anti-human HER2/neu IgG3-(GM-CSF) fusion protein retains antigen specificity and cytokine function and demonstrates antitumor activity. *J Immunol* 2000; **165**: 5112-5121. (PMID: 11046042)
14. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17-1A antigen (Ep-CAM) [review]. *J Mol Med* 1999; **77**: 699-712. (PMID: 10606205)
15. Went PT, Lugli A, Meier S, Bundi M, Mirlacher M, Sauter G, Dirnhofner S. Frequent EpCam protein expression in human carcinomas. *Hum Pathol* 2004; **35**: 122-128. (PMID: 14745734)
16. Riethmuller G, Holz E, Schlimok G, Schmiegler W, Raab R, Hoffken K, Gruber R, Funke I, Pichlmaier H, Hirche H, Buggisch P, Witte J, Pichlmayr R. Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol* 1998; **16**: 1788-1794. (PMID: 9586892)
17. Naundorf S, Preithner S, Mayer P, Lippold S, Wolf A, Hanakam F, Fichtner I, Kufer P, Raum T, Riethmuller G, Baeuerle PA, Dreier T. *In vitro* and *in vivo* activity of MT201, a fully human monoclonal antibody for pancreatic carcinoma treatment. *Int J Cancer* 2002; **100**: 101-110. (PMID: 12115595)
18. Kroesen BJ, Nieken J, Sleijfer DT, Molema G, de Vries EG, Groen HJ, Helfrich W, The TH, Mulder NH, de Leij L. Approaches to lung cancer treatment using the CD3 x EGP-2-directed bispecific monoclonal antibody BIS-1. *Cancer Immunol Immunother* 1997; **45**: 203-206. (PMID: 9435874)
19. Gastl G, Spizzo G, Obrist P, Dunser M, Mikuz G. Ep-CAM overexpression in breast cancer as a predictor of survival. *Lancet* 2000; **356**: 1981-1982. (PMID: 11130529)
20. Osta WA, Chen Y, Mikhitarian K, Mitas M, Salem M, Hannun YA, Cole DJ, Gillanders WE. EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. *Cancer Res* 2004; **64**: 5818-5824. (PMID: 15313925)
21. Munz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. *Oncogene* 2004; **23**: 5748-5758. (PMID: 15195135)
22. Kim JH, Herlyn D, Wong KK, Park DC, Schorge JO, Lu KH, Skates SJ, Cramer DW, Berkowitz RS, Mok SC. Identification of epithelial cell adhesion molecule autoantibody in patients with ovarian cancer. *Clin Cancer Res* 2003; **9**: 4782-4791. (PMID: 14581349)
23. Kufer P, Zettl F, Borschert K, Lutterbuse R, Kischel R, Riethmuller G. Minimal costimulatory requirements for T cell priming and TH1 differentiation: activation of naive human T lymphocytes by tumor cells armed with bifunctional antibody constructs. *Cancer Immunol* 2001; **1**: 10. (PMID: 12747771)
24. Raum T, Gruber R, Riethmuller G, Kufer P. Anti-self antibodies selected from a human IgD heavy chain repertoire: a novel approach to generate therapeutic human antibodies against tumor-associated differentiation antigens. *Cancer Immunol Immunother* 2001; **50**: 141-150. (PMID: 11419181)
25. Moonen P, Mermod JJ, Ernst JF, Hirschi M, DeLamararter JF. Increased biological activity of deglycosylated recombinant human granulocyte/macrophage colony-stimulating factor produced by yeast or animal cells. *Proc Natl Acad Sci U S A* 1987; **84**: 4428-4431. (PMID: 3299366)
26. Gillies SD, Reilly EB, Lo KM, Reisfeld RA. Antibody-targeted interleukin 2 stimulates T cell killing of autologous tumor cells. *Proc Natl Acad Sci U S A* 1992; **89**: 1428-1432. (PMID: 1741398)
27. Metelitsa LS, Gillies SD, Super M, Shimada H, Reynolds CP, Seeger RC. Antidisialoganglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophilic granule exocytosis. *Blood* 2002; **99**: 4166-4173. (PMID: 12010822)
28. Armstrong A, Eck SL. EpCAM: A new therapeutic target for an old cancer antigen [review]. *Cancer Biol Ther* 2003; **2**: 320-326. (PMID: 14508099)
29. de Bono JS, Tolcher AW, Forero A, Vanhove GF, Takimoto C, Bauer RJ, Hammond LA, Patnaik A, White ML, Shen S, Khazaeli MB, Rowinsky EK, LoBuglio AF. ING-1, a monoclonal antibody targeting Ep-CAM in patients with advanced adenocarcinomas. *Clin Cancer Res* 2004; **10**: 7555-7565. (PMID: 15569986)
30. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy [review]. *Nat Rev Cancer* 2004; **4**: 11-22. (PMID: 14708024)
31. Rini B, Wadhwa M, Bird C, Small E, Gaines-Das R, Thorpe R. Kinetics of development and characteristics of antibodies induced in cancer patients against yeast expressed rDNA derived granulocyte macrophage colony stimulating factor (GM-CSF). *Cytokine* 2005; **29**: 56-66. (PMID: 15598439)
32. Wadhwa M, Bird C, Fagerberg J, Gaines-Das R, Ragnhammar P, Mellstedt H, Thorpe R. Production of neutralizing granulocyte-macrophage colony-stimulating factor (GM-CSF) antibodies in carcinoma patients following GM-CSF combination therapy. *Clin Exp Immunol* 1996; **104**: 351-358. (PMID: 8625532)
33. Gillies SD, Lan Y, Lo KM, Super M, Wesolowski J. Improving the efficacy of antibody-interleukin 2 fusion proteins by reducing their interaction with Fc receptors. *Cancer Res* 1999; **59**: 2159-2166. (PMID: 10232603)

34. Hudson PJ. Recombinant antibody constructs in cancer therapy [review]. *Curr Opin Immunol* 1999; **11**: 548-557. (PMID: 10508712)
35. Hu P, Hornick JL, Glasky MS, Yun A, Milkie MN, Khawli LA, Anderson PM, Epstein AL. A chimeric Lym-1/interleukin 2 fusion protein for increasing tumor vascular permeability and enhancing antibody uptake. *Cancer Res* 1996; **56**: 4998-5004. (PMID: 8895756)
36. Ruef C, Coleman DL. Granulocyte-macrophage colony-stimulating factor: pleiotropic cytokine with potential clinical usefulness [review]. *Rev Infect Dis* 1990; **12**: 41-62. (PMID: 2405468)
37. O'Day SJ, Boasberg PD, Piro L, Kristedja TS, Wang HJ, Martin M, Deck R, Ames P, Shinn K, Kim H, Fournier P, Gammon G. Maintenance biotherapy for metastatic melanoma with interleukin-2 and granulocyte macrophage-colony stimulating factor improves survival for patients responding to induction concurrent chemotherapy. *Clin Cancer Res* 2002; **8**: 2775-2781. (PMID: 12231516)
38. Cebon J, Dempsey P, Fox R, Kannourakis G, Bonnem E, Burgess AW, Morstyn G. Pharmacokinetics of human granulocyte-macrophage colony-stimulating factor using a sensitive immunoassay. *Blood* 1988; **72**: 1340-1347. (PMID: 3048442)
39. Phillips JH, Lanier LL. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. *J Exp Med* 1986; **164**: 814-825. (PMID: 3489062)
40. Falk CS, Noessner E, Weiss EH, Schendel DJ. Retaliation against tumor cells showing aberrant HLA expression using lymphokine activated killer-derived T cells. *Cancer Res* 2002; **62**: 480-487. (PMID: 11809699)
41. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. *Cell* 1993; **73**: 5-8. (PMID: 8462103)
42. Lustgarten J, Marks J, Sherman LA. Redirecting effector T cells through their IL-2 receptors. *J Immunol* 1999; **162**: 359-365. (PMID: 9886407)
43. Liu CC, Rafii S, Koizumi H, Granelli-Piperno A, Young JD. Perforin gene expression in stimulated human peripheral blood T cells studied by *in situ* hybridization and northern blotting analysis. *Immunol Lett* 1992; **33**: 79-85. (PMID: 1427993)
44. Refaeli Y, Van Parijs L, London CA, Tschopp J, Abbas AK. Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. *Immunity* 1998; **8**: 615-623. (PMID: 9620682)
45. Carnemolla B, Borsi L, Balza E, Castellani P, Meazza R, Berndt A, Ferrini S, Kosmehl H, Neri D, Zardi L. Enhancement of the antitumor properties of interleukin-2 by its targeted delivery to the tumor blood vessel extracellular matrix. *Blood* 2002; **99**: 1659-1665. (PMID: 11861281)
46. Xiang R, Lode HN, Dreier T, Gillies SD, Reisfeld RA. Induction of persistent tumor-protective immunity in mice cured of established colon carcinoma metastases. *Cancer Res* 1998; **58**: 3918-3925. (PMID: 9731503)
47. Becker JC, Varki N, Gillies SD, Furukawa K, Reisfeld RA. Long-lived and transferable tumor immunity in mice after targeted inter-

leukin-2 therapy. *J Clin Invest* 1996; **98**: 2801-2804. (PMID: 8981927)

Materials and methods

Construction of DCH and variants

DNA sequences encoding scFv fragments of human mAb HD70 were generated by fusion PCR with overlapping primers from HD70 IgG1 expression vectors pEF-dhfr and pEF-ada (24). The pEF-dhfr-M79scFv-IgG3hinge-CH1 and pEF-ada-M79scFv-IgG3hinge-Ck expression vectors (23) were digested with the restriction enzymes *Bsr* GI and *Bsp* EI to remove M79 scFv encoding DNA fragments and sequences coding for HD70 scFv inserted as *Bsr* GI-*Bsp* EI fragments, generating the scFv HD70-CH1 and HD70-C kappa DNA sequences. Human IL-2 was amplified from the plasmid pB11-IL2-Tet (Micromet, Munich, Germany) by PCR. PCR primers were designed with DNA sequences encoding the 15-aa C-terminal end of the C kappa domain, a glycine serine (G₄S) linker peptide, a *Sac* I (5'-primer), and a *Sal* I (3'-primer) restriction enzyme recognition site. The *Sac* I-*Sal* I DNA fragment was inserted into the pEF-ada-HD70-IgG3hinge-Ck plasmid to yield the scFv HD70-IL-2 DNA sequence (Figure 1A). Human GM-CSF cDNA was obtained by RT-PCR from RNA isolated from phorbol myristate acetate (Sigma, Taufkirchen, Germany) stimulated PBMCs. PCR primers were designed with DNA sequences coding for an N-terminal glycine serine (G₄S) linker, a *Bcu* I restriction enzyme recognition site (5'-primer), a C-terminal polyhistidine tag, and a *Sal* I recognition site (3'-primer). GM-CSF was inserted as a *Bcu* I-*Sal* I fragment into the pEF-dhfr-HD70-IgG3hinge-CH1 plasmid to obtain the final scFv HD70-GM-CSF DNA sequence (Figure 1A). DNA constructs encoding mutated HD70 scFv fragments were generated by site-directed mutagenesis. ScFv HD70-IL-2, mutant scFv HD70-IL-2, and scFv HD70-C kappa DNA constructs were each subcloned into the mammalian cell expression vector pEF-ada. ScFv HD70-GM-CSF and mutant scFv HD70-GM-CSF DNA constructs were subcloned into the mammalian cell expression vector pEF-dhfr. The expression of the inserted DNA constructs in both vectors is linked to the expression of a selection marker gene (*ada* or *dhfr*) by way of an internal ribosomal entry site. All DNA inserts were designed with a 5'-end DNA sequence coding for a human IgG1 leader peptide, which targets proteins for secretion in eukaryotic cells.

Transfection and gene amplification of DCH and variants

CHO *dhfr*- cells (provided by American Type Cell Culture Collection, Rockville, MD, USA) were maintained in MEM α (Invitrogen, Karlsruhe, Germany), 10% dialyzed FCS (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Sigma, Taufkirchen, Germany), and 1x HT media supplement (Sigma, Taufkirchen, Germany). Cells were seeded at 2.5×10^5 /well in 6-well tissue culture plates and incubated at 37°C overnight. Two-and-a-half micrograms of each fusion protein-encoding pEF-dhfr and pEF-ada vector were combined with 1 ml MEM α and 15 μ l TransFast™ transfection reagent (Promega, Mannheim, Germany), incubated for 15 min at room temperature, and added to the cells. Following a 1-h incubation at 37°C, cells were washed twice in medium, and 2 ml culture medium was added to each well. After 2 d at 37°C, the transfected cells were trypsinized, transferred to a cell culture flask, and selected for expression of the *ada* and *dhfr* genes in MEM α , 10% dialyzed FCS, 1% penicillin/streptomycin, 1.1 mM

adenosine (Sigma, Taufkirchen, Germany), 0.05 mM L-alanosine (NIH, Bethesda, MD, USA), 1 mM uridine (Sigma, Taufkirchen, Germany), and 0.1 μ M dCF (pentostatin, deoxycoformycin, Nipent; Wyeth Pharma, Muenster, Germany). The use of selective medium was continued for 2-3 wk with frequent changes of medium until confluent cell growth was obtained. Stable transfectants with confluent growth over several passages were cultured in selective medium supplemented with 0.3 μ M dCF and 20 nM MTX (methotrexate; Sigma, Taufkirchen, Germany) to amplify cytokine fusion protein-encoding genes. Confluent cells were propagated under these conditions. In a final round of amplification, medium concentrations were increased to 1 μ M dCF and 100 nM MTX.

Production and purification of DCH and variants

Production of cytokine fusion proteins was performed in serum-free RenCyte™ CHO K1 medium (Medicult, Jyllinge, Denmark). Confluent adherent cells were adapted to serum-free medium, transferred to tissue culture roller bottles, and cultured in 400 ml serum-free medium for 8-10 d at 37°C. Cells were sedimented by centrifugation, and supernatants were collected.

DCH and variants were purified from cell supernatants using a three-step purification procedure that involved Q-Sepharose FF (Amersham Pharmacia, Freiburg, Germany) anion exchange chromatography; immobilized metal ion adsorption chromatography (IMAC, HiTrap chelating, Ni²⁺; Amersham Pharmacia, Freiburg, Germany); and gel filtration (Superdex 200 prep grade; Amersham Pharmacia, Freiburg, Germany). Anion exchange chromatography was performed on a Q Sepharose FF column (37 ml) equilibrated with 20 mM Tris/HCl (pH 8.0). Cell culture supernatant was diluted 2:1 with distilled water and 1/50th volume of 1 M Tris/HCl (pH 8.5) to obtain a pH of 8.0, and loaded on the column at a flow rate of 4 ml/min. Nonadsorbed sample was washed out with the equilibration buffer, and bound fusion proteins were eluted at 60% of 1 M NaCl in 20 mM Tris/HCl (pH 8.0). Eluted protein fractions were pooled and applied to a HiTrap chelating column (5 ml) charged with 100 mM NiSO₄ and equilibrated with 20 mM Na₂HPO₄, 400 mM NaCl (pH 7.2). Unbound material was washed out with equilibration buffer; weakly adsorbed proteins were removed by washing with 10% 500 mM imidazole in 20 mM Na₂HPO₄, 400 mM NaCl (pH 7.2), and polyhistidine-tagged cytokine fusion proteins were eluted with 500 mM imidazole in 20 mM Na₂HPO₄, 400 mM NaCl (pH 7.2). Gel filtration chromatography was performed on a Superdex 200 (120 ml) column equilibrated with PBS. Pooled IMAC fractions were loaded on the column at a flow rate of 1 ml/min. Eluted protein samples were subjected to SDS-PAGE and Western blotting for fusion protein detection.

Parental heterominibody (HMB) was isolated in a three-step purification process using cation exchange chromatography, IMAC, and gel filtration. Cation exchange was performed on SP Sepharose FF column (Amersham Pharmacia, Freiburg, Germany) equilibrated with 20 mM MES (pH 5.5). Cell culture supernatant was diluted 2:1 with equilibration buffer and loaded onto the column (30 ml) at a flow rate of 5 ml/min. Bound HMB was eluted with 1 M NaCl, 20 mM MES (pH 5.5), and further purified by IMAC and gel filtration, as described above.

Fusion protein analysis

The molecular size and purity of the proteins was analyzed by SDS-PAGE (4-12% Bis-Tris gels; Invitrogen, Karlsruhe,

Germany) under reducing and nonreducing conditions. For Western blotting, proteins were blotted onto a nitrocellulose membrane and incubated with anti-human kappa light chain (alkaline phosphatase conjugate; Sigma, Taufkirchen, Germany) and anti-penta-His mouse IgG1 (Qiagen, Hilden, Germany) detection antibodies. The anti-penta-His Ab was detected with a secondary anti-mouse IgG1 alkaline phosphatase-conjugated Ab (Sigma, Taufkirchen, Germany). The nitrocellulose membrane was stained with a BCIP/NBT substrate solution (Sigma, Taufkirchen, Germany).

Determination of IL-2 and GM-CSF bioactivities

Biological activity of cytokines within recombinant fusion proteins was measured by proliferation of cell lines that are dependent on IL-2 or GM-CSF for growth. The amount of viable cells in the proliferation assays was determined by the colorimetric WST-1 assay (Boehringer, Mannheim, Germany). The murine leukemia CTLL-2 cell line (provided by American Type Cell Culture Collection, Rockville, MD, USA) was used as an indicator cell line for human IL-2 bioactivity. CTLL-2 cells were washed twice in culture medium without IL-2. Next, 9000 cells in a volume of 90 μ l assay medium and 10 μ l of serially diluted IL-2 standard (Strathmann Biotec, Hamburg, Germany) or recombinant fusion proteins were added to each well of a 96-well plate. Cells were cultured for 48 h in a 37°C, 5% CO₂ humidified incubator.

Bioactivity of human GM-CSF was measured by proliferation of the human erythroleukemia TF-1 cell line (provided by Deutsche Sammlung von Mikroorganismen und Zelllinien, DSMZ, Braunschweig, Germany). TF-1 cells were washed twice in assay medium to remove residual GM-CSF. The 9000 cells were then added to 96-well plates in a volume of 90 μ l, together with serial dilutions of recombinant human GM-CSF (Strathman Biotec, Hamburg, Germany) or recombinant fusion proteins, and incubated for 72 h at 37°C.

At the end of incubations with IL-2 or GM-CSF, 10 μ l of cell proliferation reagent WST-1 was added to each well and further incubated for 4 h at 37°C. Plates were read on a microplate spectrophotometer (Bio-Tek instruments, Bad Friedrichshall, Germany) at 450 nm (reference wavelength 620 nm). All samples were evaluated in triplicate.

Binding of DCH and variants to Kato III cells

Kato III (provided by European Collection of Cell Cultures, Salisbury, UK) is an Ep-CAM overexpressing cell line derived from a human gastric carcinoma. We added 1×10^5 Kato III cells to each well of a 96-well plate and incubated these with 50 μ l of serially diluted DCH fusion proteins in FACS® buffer for 30 min on ice. Next, the cells were centrifuged at 600 x g for 2 min, washed twice in 100 μ l FACS® buffer, and resuspended in 50 μ l of an anti-penta-His mouse IgG1 Ab (Qiagen, Hilden, Germany) solution in FACS® buffer (1:40). Following a 30-min incubation on ice, samples were washed twice and resuspended in 50 μ l of F(ab)₂ FITC-conjugated goat anti-mouse Ig (DAKO, Hamburg, Germany) diluted in FACS® buffer (1:25). Cells were incubated for another 30 min on ice, washed twice, and resuspended in 200 μ l FACS® buffer. The mean fluorescence intensity (MFI) of labeled cells at different DCH concentrations was analyzed by flow cytometry using a FACS Calibur (BD Biosciences, Heidelberg, Germany). All experiments were performed in duplicate.

Preparation of human immune effector cells

PBMCs were prepared by Ficoll-density centrifugation from enriched lymphocyte preparations (buffy coats or leukocyte removal filters). Isolated PBMCs were resuspended in 50 ml RPMI (Invitrogen, Karlsruhe, Germany)/10% FCS (Invitrogen, Karlsruhe, Germany) and cultured at 37°C and 5% CO₂. PBMCs were typically used between 3 and 4 d after preparation, without stimulation. Human T cells were purified from PBMCs by means of high-affinity negative selection using T cell enrichment columns (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Unstimulated CD4+ and CD8+ T cells were isolated from purified human T cells by magnetic cell sorting using Dynal CD4 and CD8 negative isolation kits according to the manufacturer's protocol (Dynal Biotech, Hamburg, Germany).

Cytotoxicity assay

Kato III cells were trypsinized, centrifuged, and resuspended in culture medium. We labeled 5×10^6 cells with 7 μ M PKH-26 (Sigma, Taufkirchen, Germany) for 2 min at room temperature. The reaction was stopped by addition of FCS. PKH-26-labeled target cells were washed twice with RPMI/10% FCS and adjusted to a concentration of 5×10^5 cells/ml in culture medium. We then added 40 μ l of the cell suspension (20,000 cells) into each well of a 96-well cell culture plate. Immune effector cells (PBMCs or isolated T cells) were sedimented by centrifugation, resuspended in culture medium, and mixed with the Kato III cells at an appropriate E/T ratio in a volume of 180 μ l. DCH, variants, and HMB were added in a volume of 20 μ l to 180 μ l cell suspension, resulting in final concentrations of 10 μ g/ml. Recombinant human GM-CSF and IL-2 were added at equivalent molar concentrations of 1.4 μ g/ml. Cells were incubated for 16 h at 37°C and 5% CO₂. Thereafter, cells were collected by centrifugation, resuspended in 200 μ l FACS[®] buffer containing 1 μ g/ml PI (Sigma, Taufkirchen, Germany), and analyzed by flow cytometry using a FACS Calibur instrument (BD Biosciences, Heidelberg, Germany) with an excitation wavelength of 488 nm and emission filters for orange (FL2, 585 nm) and red (FL3, 660 nm).

In all experiments, a control reaction without cytokine fusion proteins was included to measure unspecific background. Cytotoxicity (CT) was calculated as $CT = 100 \times (\text{dead target cells in sample}) / (\text{total number of target cells in control})$. Data analyses were carried out using Excel software (Microsoft, Munich, Germany). All experiments were performed in duplicate.

T cell activation assay

Reaction mixtures of Kato III cells, immune effector cells (PBMCs or CD3+ T cells at an appropriate E/T ratio), and 10 μ g/ml DCH or DCH-mu fusion proteins were prepared in a volume of 200 μ l and incubated for 16 h at 37°C and 5% CO₂. A control reaction without fusion protein was included to determine unspecific activation of immune cells. After the incubation, 100 μ l of the reaction was added to each well of a 96-well plate and centrifuged at 600 g for 2 min at 4°C. Cells were resuspended in 50 μ l FACS[®] buffer containing 10 μ l anti-CD69 FITC-conjugated mouse IgG1 Ab (BD Biosciences, Heidelberg, Germany) and 10 μ l of PE-conjugated anti-CD4 (BD Biosciences, Heidelberg, Germany) or anti-CD8 (Sigma, Taufkirchen, Germany) mouse mAbs. Following a 20-min incubation on ice, samples were washed twice in FACS[®] buffer,

resuspended in 200 μ l of FACS[®] buffer, and analyzed by flow cytometry using a FACS Calibur (BD Biosciences, Heidelberg, Germany) recording 10,000-20,000 events.

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