Construction of Optimized Bispecific Antibodies for Selective Activation of the Death Receptor CD95

Tanja Herrmann,[1] Ludger Große-Hovest,[1] Tina Otz,[1] Peter H. Krammer,[2] Hans-Georg Rammensee,[1] and Gundram Jung[1]

[1]Department of Immunology, University of Tübingen, Tübingen, Germany and [2]Tumor Immunology Program, Division of Immunogenetics, German Cancer Research Center, Heidelberg, Germany

Abstract

We have previously reported that bispecific antibodies directed to different target antigens on lymphoma cells and to the death receptor CD95/Fas/Apo-1 selectively kill these cells, thus providing an attractive strategy for the selective stimulation of CD95 on the surface of tumor cells. Here, we further explore the general applicability of this approach under more stringent conditions using various bispecific antibodies directed to different target antigens on glioblastoma cells which express relatively low levels of CD95. We found that bispecific CD95 antibodies targeting the neuronal glial antigen-2 induce CD95-mediated apoptosis selectively in glioblastoma cells expressing this target antigen. A recombinant bispecific single-chain antibody was as effective as a chemically hybridized $F(ab')_2$ fragment with identical specificities. In contrast, a bispecific $F(ab')_2$ fragment binding to the epidermal growth factor receptor on the glioblastoma cells failed to induce apoptosis. This is most likely due to the exclusively unicellular binding of this particular fragment to target cells expressing both the epidermal growth factor receptor and CD95. If this type of binding in a *cis* configuration is favored by a particular bispecific antibody, rather than a bicellular binding in *trans*, effective cross-linking of CD95 does not occur and apoptosis is not induced. To facilitate bicellular binding in a *trans* configuration, we constructed a bispecific antibody directed to the extracellular matrix protein tenascin. As expected, this reagent was the most effective of all the antibodies tested. The presence of sensitizing reagents such as cycloheximide and various cytostatic drugs further enhanced antibody-mediated killing of the tumor cells. We believe that these results may point the way to a successful application of bispecific CD95 antibodies in experimental tumor therapy. [Cancer Res 2008;68(4):1221–7]

Introduction

CD95/Fas/Apo-1 is a cell surface receptor capable of inducing apoptotic death of human cells (1, 2). Similar to the physiologic ligand of this receptor, CD95L, agonistic anti-CD95 antibodies may induce apoptosis if binding to CD95 occurs in a multimeric format, e.g., as pentameric IgM or self-aggregating IgG_3. Alternatively, anti-CD95 antibodies may be cross-linked by Fc receptors on neighboring cells or by secondary antibodies to achieve agonistic activity (3).

Because many tumor cells express CD95, the use of agonistic anti-CD95 antibodies for tumor therapy has been vigorously pursued after initial characterization of prototypic CD95 antibodies (4, 5). However, it soon became obvious that, at least in its most simple form of applying agonistic anti-CD95 antibodies or recombinant CD95L to patients, this approach fails because many normal cell types express functional CD95 and may be killed by agonistic antibodies. In mice, for example, agonistic anti-CD95 antibodies induce severe liver cell apoptosis and acute hepatic failure (6). Several strategies have been suggested to overcome the toxic side effects induced by such antibodies, e.g., by using compounds which attenuate the liver toxicity of these reagents (7, 8).

We have previously shown that bispecific $F(ab')_2$ fragments [bs-$F(ab')_2$] with specificity for CD95 and different target antigens on lymphoma cells, such as CD20 and CD40, induce the apoptosis of cells positive for CD95 and the respective target antigen. Lymphoma cells expressing CD95 but no target antigen were not killed (9). Subsequently, Samel et al. reported similar results using recombinant fusion proteins consisting of a single-chain antibody directed to the tumor-associated fibroblast-activating protein and the CD95L protein (10). In our view, bispecific reagents of the described kind may constitute an attractive strategy to increase the specificity of agonistic anti-CD95 antibodies. It is obvious, however, that using those antibodies for tumor cell killing faces not only a specificity, but also a sensitivity problem: many tumor cells have lost their susceptibility to CD95-mediated cell death either by down-regulation of CD95 or by tilting the balance of proapoptotic and antiapoptotic intracellular proteins towards resistance (11, 12). In fact, the apoptosis-resistant phenotype has been linked to an increased metastatic potential in tumor cells (13). This sensitivity problem is now well recognized and numerous compounds have been described which are capable of enhancing the susceptibility of tumor cells towards apoptotic cell death in general and towards CD95-mediated apoptosis in particular (14–18).

In this article, we extend the approach of selective CD95 stimulation with bispecific antibodies using various target antigens expressed on glioblastoma cells. We have noted in preliminary experiments that CD95 expression on such cells, as well as on other solid tumor cells, is considerably lower than that found on the lymphoma cell lines used in our previous experiments. This necessitated the exploration of the activity of CD95-triggering bispecific antibodies under more stringent conditions. In particular, we assessed the influence of antibody format, nature of the target antigen, and combination with sensitizing reagents, with the aim of identifying compounds which are optimally suited for the induction of selective CD95-mediated apoptosis in tumor cells.

Requests for reprints: Gundram Jung, Department of Immunology, University of Tübingen, Auf der Morgenstelle 15, D-72076 Tübingen, Germany. Phone: 49-7071-29-87621; Fax: 49-7071-29-5653; E-mail: gundram.jung@uni-tuebingen.de.

doi:10.1158/0008-5472.CAN-07-6175

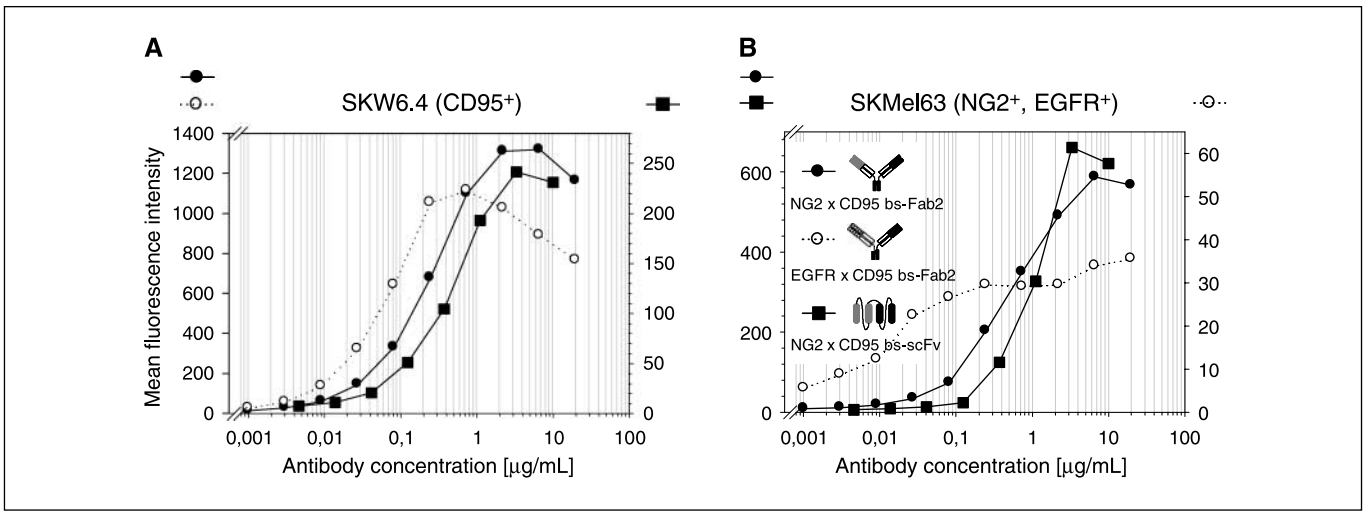

Figure 1. Binding of the NG2 × CD95 bs-F(ab′)$_2$ (●), the NG2 × CD95 bs-cFv (■), and the EGFR × CD95 bs-F(ab′)$_2$ (○) to CD95 expressing SKW6.4 cells (*A*) and to SKMel63 cells expressing NG2 and EGFR (*B*). Scales for the mean intensity fluorescence values are assigned according to the symbols placed above the respective ordinate.

Materials and Methods

Cells and reagents. The SKMel63 melanoma cell line was a gift from Dr. Brigitte Gückel (Department of Gynecology, University of Tübingen, Tübingen, Germany). The glioblastoma cell lines, U87MG and LN-18, were kindly provided by M. Weller, Department of Neurology, University of Tübingen, Tübingen, Germany. SKMel63 cells and the lymphoma cell line SKW6.4 (American Type Culture Collection) were kept in RPMI 1640 and both glioblastoma cell lines in DMEM, supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μmol/L of β-mercaptoethanol, 1 mmol/L of sodium pyruvate, and 10% heat-inactivated fetal bovine serum. Hybridomas producing monoclonal antibodies directed at neuronal glial antigen-2 (NG2; 9.2.27) and CD40 (G28-5) were kindly provided by R.A. Reisfeld, La Jolla, CA and purchased from the American Type Culture Collection, respectively. Antibodies were purified from hybridoma supernatant using protein A affinity chromatography. The antibody directed to tenascin (BC24) was purified from mouse ascites (Sigma) also using protein A affinity chromatography. The purified antibody directed to epidermal growth factor receptor (EGFR; mAb 425) was kindly provided by Merck Darmstadt (Darmstadt, Germany). The CD95 antibody (Apo-1) and switch variants derived thereof have been previously described (3). The CD95 cross-linking antibody directed against mouse Fcγ portions was purchased from Dianova; it was used at 10 μg/mL. Purified CD95L and the pan-caspase inhibitor Z-VAD-FMK were obtained from Alexis Biochemicals, Gruenberg, Germany. Cycloheximide and EDTA were purchased from Sigma. The topoisomerase-I inhibitor Topotecan was obtained from SmithKline Beecham Pharma and the EGFR tyrosine kinase inhibitor AG1478 was from Calbiochem-Novabiochem. Agonistic peptides derived from the second mitochondria-derived activator of caspases (SMAC) with the sequences AVPIAQKRQIKIWFQNRRMKWKK and AVPIAQKGGGRRRRRRRRGC were synthesized as amides by Dr. M. Fotin-Mleczek, Institute for Cell Biology, University of Tübingen, Tübingen, Germany; histone deacetylase inhibitors valproic acid and soberoylanilide hydroxamic acid, were kindly provided by M. Bitzer, Medical Clinic III, University of Tübingen, Tübingen, Germany.

Generation of bispecific antibody fragments. Bispecific F(ab′)$_2$ fragments were prepared by selective reduction and reoxidation of hinge region disulfide bonds as described previously. The reaction conditions used prevent the formation of homodimers and allow effective hybridization of modified Fab′ fragments of the parental antibodies (19). For this study, we hybridized the IgG$_{2a}$ variant of the Apo-1 (CD95) antibody to antibodies directed against the glioblastoma-associated antigens NG2, EGFR, CD40, and tenascin and obtained four bispecific constructs termed NG2 × CD95, EGFR × CD95, CD40 × CD95, and TEN × CD95. An equimolar mixture of modified, nonhybridized Fab′ fragments of the parental anti-NG2 and anti-CD95 antibodies was used as a control in some experiments (Fab′ mix).

Generation and characterization of the bispecific single-chain antibody bsscFv NG2 × CD95. mRNA from the mouse IgG$_{2a}$/κ producing hybridoma anti–Apo-1 was isolated using the Qiagen RNeasy Kit (Qiagen). Oligo-dT (15) primer and the cDNA synthesis kit (Roche) were used for the generation of double-stranded cDNA. The blunted double-stranded cDNA was circularized with the T4-DNA-Ligase (Roche) at 16°C. For the amplification of both unknown variable antibody gene segments, oligonucleotides specific for the constant region of γ2a (2a-back, 5′-catgcaaatgcccagcacctaacctcttgggtg-3′; 2a-forward, 5′-gaggacagggcttgattgtgggccctctgggct-3′) and κ (CK-back, 5′-acttctaccccaaagacatcaatgtcaag-3′; CK-forward, 5′-tgttcaagaagcacacgactgaggcacctcc-3′) were used in an inverse PCR. Specific amplicons (heavy 1,600 bp; κ 900 bp) were cloned into the TOPO pCR2.1 vector (Invitrogen) and the unknown V_H and V_L domains were sequenced. For the generation of a single-chain Fv fragment, V_H and V_L domains were amplified with overlapping primers adding an oligonucleotide stretch coding for a glycin-serin linker, $(G_4S)_3$, and assembled in a SOE-PCR. *Age*I and *Sfi*I restriction sites were added 5′ and 3′, respectively, for an in-frame insertion in an eukaryotic expression vector for bispecific fusion proteins as previously described (20). This resulted in an expression vector with an insert coding for a 57-kDa bispecific single-chain Fv fragment containing the variable regions of the anti–Apo-1 (CD95)- and the NG2 antibody 9.2.27 in the following 5′ to 3′ orientation: $V_{H\text{-}CD95}$–$V_{L\text{-}CD95}$–$V_{H\text{-}NG2}$–$V_{L\text{-}NG2}$. The mouse myeloma cell line Sp2/0 was transfected with the linearized vector and selected with G418 (1 mg/mL).

The bispecific single-chain antibody was purified from cell culture supernatant by protein L affinity chromatography and characterized as previously described for the bsscFv antibody r28M (21). Analysis on SDS page yielded a single band corresponding to the expected molecular weight of 57 kDa.

FACS analysis. To determine the antigen expression level on glioblastoma cells, these cells were incubated for 1 h at 4°C with a saturating concentration (5 μg/mL) of the respective parental antibodies, washed and stained with phycoerythrin-labeled F(ab′)$_2$ fragments of a goat anti-mouse IgG antibody (Dianova) at dilutions recommended by the manufacturer. Isotype controls were purchased from BD PharMingen. Cells were analyzed in a FACSCalibur equipped with the CellQuest Pro software (Becton Dickinson).

Cell killing assays. To measure direct apoptosis of U87MG cells, cells were labeled with ^{51}Cr (100 μCi/mL) for 1 h, washed, plated in triplicate in 96-well microtiter plates (3.5 × 10^4/well) and incubated for 20 h with bispecific tumor antigen × CD95 constructs. For bystander lysis experiments,

2×10^4 ^{51}Cr-labeled SKW6.4 cells per well were incubated with bispecific CD95 antibodies in the absence or presence of tumor cells expressing the relevant target antigens (4×10^4 / well) for 20 h. The percentage of killed tumor cells was calculated according to the standard formula ($cpm_x - cpm_{spont}$) / ($cpm_{max} - cpm_{spont}$) where cpm_{max} is radioactivity released by detergent-treated target cells and cpm_{spont} is spontaneous release in the absence of antibodies.

Alternatively, the viability of LN-18 cells was determined by seeding 3.5×10^4 tumor cells/well in 96-well plates. After antibody treatment in the presence or absence of cycloheximide, the cell culture medium was removed and surviving cells were stained with 0.5% crystal violet in 20% methanol for 20 min at room temperature. The plates were washed extensively under running tap water, air-dried, and surviving cells were quantified by measuring absorbance at 550 nm in an ELISA reader (Spectramax 340; Molecular Devices).

Results

Selective killing of tumor target cells by bispecific antibodies does not depend on the antibody format but on the nature of the target antigen used. We first constructed bispecific CD95 antibodies directed to three different target antigens on glioblastoma cells: NG2, EGFR, and CD40. The antibodies were generated as chemically hybridized bispecific F(ab′)$_2$ fragments [bs-F(ab′)$_2$]. The antibody targeting NG2 was constructed in addition as a recombinant bispecific single-chain Fv antibody (bs-scFv) as described in Materials and Methods. In Fig. 1, binding of the NG2- and EGFR-targeting constructs to CD95 expressing SKW6.4 lymphoma cells and to SKMel63 cells expressing the target antigens NG2 and EGFR is depicted. A somewhat reduced binding to CD95 was noted for the recombinant bispecific single-chain antibody (*A*). Likewise, binding of this reagent to its target antigen NG2 was slightly reduced if compared with the bs-F(ab′)$_2$ with NG2 × CD95 specificity (*B*). Obviously, the expression of NG2 on the SKMel63 cells largely exceeds that of EGFR (note the different scales for the mean fluorescence intensity values in Fig. 1*B*). However, in the context of the following experiments, it is more interesting that the avidity of the EGFR targeting bs-F(ab′)$_2$ seems to be superior to that of the NG2 targeting antibodies.

We then tested the capability of these antibodies and the bs-F(ab′)$_2$ targeting CD40 (CD40 × CD95) to induce apoptosis in two glioblastoma cell lines with different antigenic profiles. The U87MG cell line expresses CD95 and the three target antigens used for the construction of bispecific antibodies in these experiments: NG2, EGFR, and CD40. In contrast, the LN-18 cell line expresses only one of these antigens, EGFR. On both cell lines, CD95 expression is more than one magnitude lower (Fig. 2*A* and *B*) than previously observed on various lymphoma cells (9). In all experiments, both constructs containing a NG2 specificity, the bispecific single-chain and the F(ab′)$_2$ fragment, induced significant and comparable killing of U87MG cells (Fig. 2*C*) indicating that the somewhat reduced binding activity of the single-chain

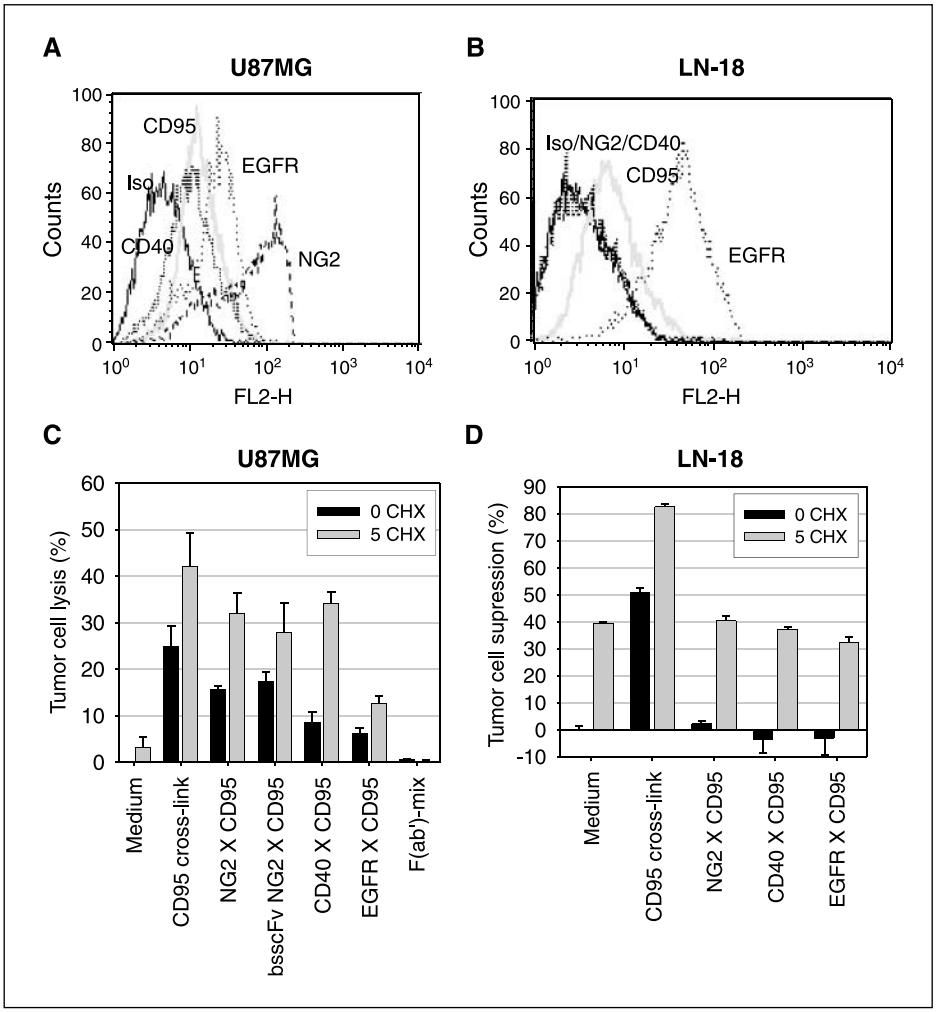

Figure 2. *A* and *B,* flow cytometric analysis of U87MG (*A*) and LN-18 glioblastoma cells (*B*) incubated with saturating doses (5 μg/mL) of antibodies directed to CD95 and the target antigens NG2, EGFR, and CD40. *Iso,* a control antibody of the IgG_{2a} subtype. *C* and *D,* induction of apoptosis in these glioblastoma cells by bispecific CD95 antibodies directed to the abovementioned target antigens. Target cells and antibodies (1 μg/mL) were incubated for 20 h and cell death was determined by labeling of the target cells with ^{51}Cr (*C*) or crystal violet (*D*) as described in Materials and Methods. *Gray columns,* the presence of cycloheximide (5 μg/mL) during the assay. Nonhybridized F(ab′) fragments of the parental anti-NG2 and anti-CD95 antibodies were used as control [*F(ab′) mix*]. The CD95 antibody was cross-linked with 10 μg/mL of secondary goat anti-mouse antibody. One representative out of four independent experiments.

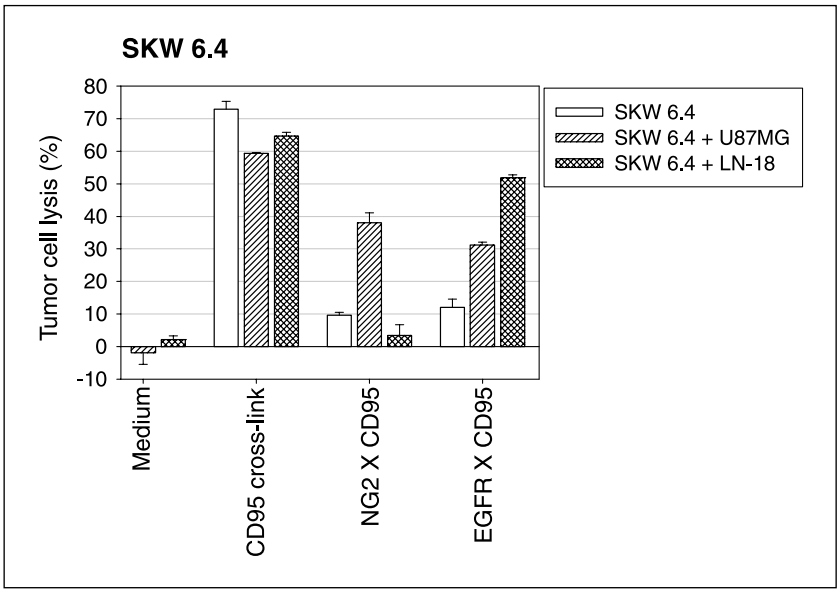

Figure 3. Lysis of ^{51}Cr-labeled SKW6.4 bystander cells (20,000/well) by bispecific antibodies (1 μg/mL; *white columns*). Shading indicates the presence of U87MG (*striped columns*) and LN-18 (*hatched columns*) glioblastoma cells, respectively (40,000/well) during the 20-h assay. One representative out of three independent experiments.

antibody does not affect its function substantially. In general, the extent of apoptosis induction by the NG2 targeting antibodies was somewhat lower than that induced by the cross-linked parental anti-CD95 antibody. Without sensitization, apoptosis reached 10% to 30% after 20 h depending on the somewhat variable sensitivity of the tumor cells in different experiments. For all antibodies, maximal killing required sensitization by the protein synthesis inhibitor cycloheximide. In the presence of cycloheximide, a moderate induction of apoptosis was noted even for the bispecific antibody targeting CD40, an antigen expressed at relatively low levels on the U87MG cells. Bispecific antibodies recognizing EGFR were even less effective than those targeting CD40 (Fig. 2*C*), although the expression of EGFR exceeds that of CD40 on this cell line (Fig. 2*A*).

The EGFR targeting bs-F(ab′)$_2$ also failed to induce apoptosis in LN-18 cells. On these cells, EGFR expression is higher than on U87MG, whereas NG2 and CD40 are not expressed (Fig. 2*B*). Thus, although more sensitive to CD95-mediated apoptosis than U87MG cells, the LN-18 cells are not killed by any of the bispecific antibodies discussed thus far. This remained true in the presence of cycloheximide: the standard concentration of this reagent alone induced ~40% apoptosis, and none of the bispecific antibodies further increased this percentage (Fig. 2*D*).

In all experiments described above, inhibition of killing by the caspase inhibitor Z-VAD-FMK confirmed that cell death was due to the induction of apoptosis (data not shown). Taken together, these findings show that (*a*) bispecific antibodies with NG2 × CD95 specificity in two different formats induce apoptosis selectively in cells carrying the NG2 antigen and (*b*) failure of the EGFR targeting bispecific antibody cannot be attributed to an insufficient binding avidity or to a low EGFR expression level on the target cells.

In subsequent experiments, we ruled out that failure of the EGFR targeting bs-F(ab′)$_2$ was due to modulation of the EGFR or to the stimulation of antiapoptotic pathways by this receptor. Using flow cytometry, we could not detect the down-regulation of EGFR expression under the experimental conditions used. Moreover, AG1478, an inhibitor of intracellular EGFR signaling, did not enhance the activity of the EGFR × CD95 bs-F(ab′)$_2$ (data not shown).

Successful killing of target cells with bispecific antibodies requires *trans* engagement of CD95 and the target antigen. To determine whether the EGFR × CD95 bs-F(ab′)$_2$, is capable of inducing apoptosis in innocent bystander cells, we labeled CD95-sensitive SKW6.4 lymphoma cells which neither express NG2 nor EGFR with ^{51}Cr. Bystander lysis of these cells was then assessed in the presence and absence of the two glioblastoma cell lines used as targets in the experiments described above. In these experiments, CD95 and the target antigen are provided on different cells and the glioblastoma cells expressing NG2 and EGFR, respectively, allow for a bicellular binding of the bispecific antibodies, and thus, for effective cross-linking of CD95 on the SKW6.4 bystander cells. Figure 3 shows that in these experiments, the EGFR × CD95 bs-F(ab′)$_2$ induces apoptosis in SKW6.4 bystander cells if EGFR-expressing glioblastoma target cells were also present. In fact, the bispecific F(ab′)$_2$ fragment targeting EGFR was even more effective than the one binding to NG2 if LN-18 cells were used as "*trans*-presenting cells." As expected, bs-F(ab′)$_2$ targeting the NG2 antigen failed to induce bystander killing in the presence of LN-18 cells because these cells do not express NG2. In conclusion, these experiments confirm that the EGFR × CD95 bs-F(ab′)$_2$ is functionally active in a bicellular *trans* configuration and that it fails, however, if bound in a unicellular fashion to target cells expressing EGFR and CD95 on the same cell surface (see Fig. 4). Obviously, the ability to cross-link CD95 in a *trans* configuration varies with the target antigen used.

We then reasoned that target antigens that are expressed on the extracellular matrix rather than on the target cell itself might be particularly suited for *trans* presentation of anti-CD95 within a bispecific construct. Therefore, we generated a bispecific CD95 antibody targeting tenascin, an extracellular matrix protein known to be produced by most glioblastoma cells. In Fig. 5, it is shown that this antibody induces apoptosis in both cell lines, U87MG and LN-18. The efficiency of killing was comparable to that of cross-linked parental anti-CD95 antibody. In most experiments, it exceeded that of the NG2 targeting bs-F(ab′$_2$) (Fig. 6). In contrast,

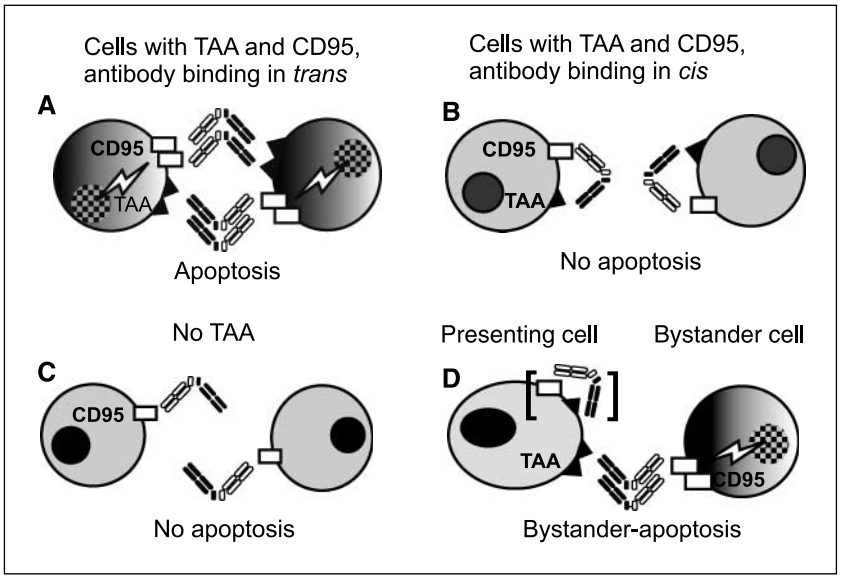

Figure 4. Induction of apoptosis by bispecific antibodies directed to a tumor-associated antigen (*TAA*) and the death receptor CD95 requires cross-linking of CD95, that is binding in a *trans* (*A*) rather than in a *cis* (*B*) configuration. In the absence of the tumor-associated antigen, neither of these is possible and apoptosis does not occur (*C*). TAA-negative but CD95-positive cells may be killed in the presence of tumor-associated antigen–positive cells "presenting" the bispecific antibody in *trans*. Note that effective *trans* presentation can occur despite the presence of CD95 and possibly *cis* binding on the presenting cells provided that the expression of the tumor-associated antigen exceeds that of CD95 (*D*). In analogy to the situation in *D*, *trans* presentation may be preferred if a tumor-associated antigen is expressed on the extracellular matrix rather than on the tumor cell itself.

killing of CD95-sensitive SKW6.4 cells by the TEN × CD95 construct was negligible (data not shown).

Various cytostatic drugs enhance CD95-induced apoptosis of tumor cells. In most of the experiments described above, induction of maximal apoptosis in tumor cells by the bispecific antibodies required sensitization by cycloheximide, a reagent inhibiting protein synthesis. In initial experiments, we recognized that the sensitizing function of this reagent could be substituted by several cytostatic drugs of which the anthracycline doxorubicin and the topoisomerase inhibitor topotecan were the most effective (data not shown). Figure 6 shows that topotecan also enhances apoptosis induction by the bispecific antibodies targeting NG2 and tenascin, respectively.

In contrast, several other reagents reported to enhance the sensitivity of cells towards TRAILR- or CD95-mediated apoptosis, such as HDAC inhibitors or agonistic SMAC peptides, failed to enhance the apoptotic activity of the NG2 × CD95–bispecific antibodies (data not shown).

Discussion

The starting point of this study was our previous finding that bispecific antibodies directed to CD95 and different target antigens on lymphoma cells induce apoptosis selectively in cells carrying the respective target antigen. Because CD95 expression on solid tumor cells is generally lower than on lymphatic cells, the activity of bispecific antibodies targeting antigens on such cells was unclear. We found that bispecific CD95 antibodies directed to NG2 as a target antigen could induce selective apoptosis under these more stringent conditions. This holds true not only for the bispecific $F(ab')_2$ format used in most of our experiments: we could show for the first time that a bispecific CD95 antibody in a recombinant

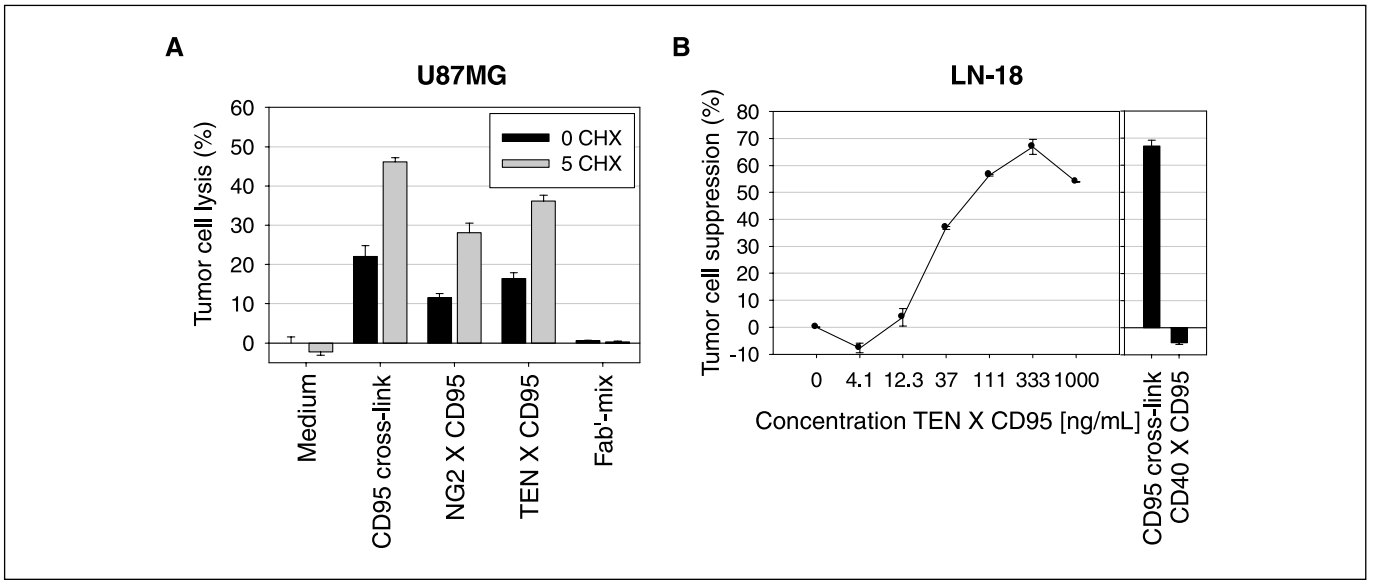

Figure 5. Effective lysis of both U87MG (^{51}Cr release assay; *A*) and LN-18 glioblastoma cells (crystal violet stain; *B*), by a bs-$F(ab')_2$ with tenascin × CD95 specificity (assay time, 20 h). The cross-linking goat anti-mouse antibody was used at 10 μg/mL. Antibody concentration in *A* was 1 μg/mL.

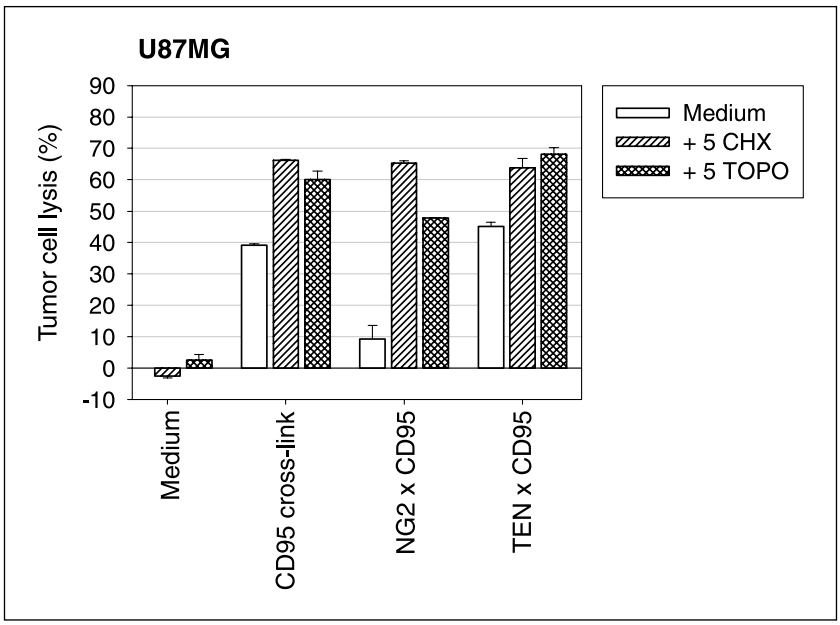

Figure 6. The topoisomerase inhibitor topotecan (5 μg/mL) enhances the apoptosis of U87MG glioblastoma cells induced by the NG2 × CD95 and tenascin × CD95 bs-F(ab′)$_2$ (assay time, 20 h). Antibody concentration was 1 μg/mL.

single-chain format exerts comparable activity. Recombinant bispecific antibodies are viewed as an attractive alternative option for large-scale production, which is still a considerable hurdle for the clinical evaluation of bispecific antibodies.

Despite a clearly demonstrable effect, killing by all bispecific antibodies was not as effective as that reported previously using lymphoma cells as targets. To reach maximal apoptosis, the presence of the protein synthesis inhibitor cycloheximide was required. Given the relatively low amount of CD95 on the glioblastoma cells, these findings were not unexpected. What was surprising, however, was the almost complete failure of the EGFR × CD95 antibody. This failure could neither be explained by inferior EGFR expression or antibody affinity, nor was it due to the induction of antiapoptotic signaling after stimulation of the EGFR. The most likely explanation for this phenomenon was provided by experiments in which the EGFR × CD95 antibody was used to kill ^{51}Cr-labeled CD95-sensitive but EGFR-negative bystander cells in the presence of EGFR-positive "presenting cells" which allow bicellular binding of the bispecific antibody and effective cross-linking of CD95 on the bystander cells. In this situation, the bispecific EGFR × CD95 antibody turned out to be fully active in killing the EGFR-negative bystander cells. Therefore, we hypothesize that the failure of this construct to induce the apoptosis of EGFR-positive cells is due to its preferential binding in a "unicellular" *cis* configuration when EGFR and CD95 are expressed on the same cell. This prevents effective mutual cross-linking of CD95 in *trans*, which is obviously required for the effective stimulation of CD95 with bispecific antibodies (see Fig. 4).

In conclusion, the experiments discussed above indicate that the activity of bispecific antibodies stimulating CD95 does not only depend on the overall expression—but also on a particular architecture of the target antigen in the cell surface, allowing optimal "presentation" of the CD95 specificity in a *trans* configuration. Thus, antigens expressed by the extracellular matrix around tumors, such as tenascin and the fibroblast-activating protein used by Wajant et al. as a target antigen for the construction of bifunctional CD95L fusion proteins (10), might be particularly suited for effective targeting with bispecific CD95 antibodies. Tenascin is a well-known extracellular matrix protein produced by glioblastoma cells (22) and radiolabeled antibodies directed to this protein have been used for the immunotherapy of patients with gliomas (23). The bispecific tenascin × CD95 antibody we have constructed turned out to be the most effective of all bispecific CD95 antibodies tested. This does not formally prove, but strongly argues for, the hypothesis that successful stimulation of CD95 with bispecific antibodies requires mutual cross-linking by *trans*-bound antibodies. In this context, it should be noted that the activity of chemically hybridized bispecific F(ab′)$_2$ fragments was comparable to that of a bispecific single-chain antibody, indicating that the flexibility of the physiologic hinge region does not limit effective "*trans* presentation."

On the one hand, the hypothesis discussed above poses some restrictions in considering the general applicability of the concept of selective stimulation of the CD95 death receptor with bispecific antibodies, when the target antigen and CD95 are expressed on the same cell. Some, but not all, bispecific constructs might work in the desired way and allow effective CD95 cross-linking under these conditions. On the other hand, our results might provide some clues as to which target antigens might be particularly suitable for the selective induction of apoptosis with bispecific reagents.

Nevertheless, the direct apoptosis-inducing activity of agonistic CD95 antibodies faces another restriction by the well-established resistance of many tumor cells towards apoptosis in general. CD95-mediated killing of such cells requires sensitization with reagents blocking protein synthesis, such as cycloheximide. Because it has been reported that conventional cytostatic drugs may sensitize tumor cells towards apoptosis, we tested a panel of these reagents together with bispecific CD95 antibodies. We found that several of these drugs act synergistically with CD95 agonists, topotecan and doxorubicin being the most effective. We have also tested several compounds which have been reported to interfere with death receptor–mediated apoptosis in a more

defined way, such as (*a*) HDAC inhibitors (17), (*b*) specific peptides mimicking the function of the apoptosis-enhancing SMAC protein (24), and (*c*) the alkyl-lysophospholipid edelfosine (25). A clear-cut synergizing effect was *not* observed in any of these cases. Perhaps the most obvious explanation for this is that the complex interplay of proapoptotic and antiapoptotic signaling within the cell may differ considerably if different cell lines are used. Nevertheless, we have identified some conventional cytostatic drugs capable of enhancing the selective induction of apoptosis by bispecific CD95 antibodies. Thus, the concept of selective CD95 stimulation using bispecific antibodies or fusion proteins in combination with sensitizing reagents may be successfully used for tumor therapy.

Acknowledgments

Received 11/9/2007; accepted 11/15/2007.

Grant support: German Cancer Foundation (Stiftung Deutsche Krebshilfe) 10-2109-Ju5 (to G. Jung).

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We thank Dr. Mariola Fotin-Mleczec, Institute for Cell Biology, University of Tübingen, for synthesizing SMAC peptides.

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Announcements

Meeting of the Radiation Research Society

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

Erratum

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 821; substitute for the last paragraph:

The data in Table 3 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O_2 consumption, it is seen that the amount of glucose "cleavage products" *exceeds* the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and *is exceeded* by the glucose utilized by 16 per cent in CLL. If the assumption is made that, *in this respect*, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

$$0.65\ (+0.27) + 0.35\ (-0.16) = +0.12\ ,$$

a figure identical to the observed +0.12 for normal leukocytes.

Construction of Optimized Bispecific Antibodies for Selective Activation of the Death Receptor CD95

Tanja Herrmann, Ludger Große-Hovest, Tina Otz, et al.

Cancer Res 2008;68:1221-1227.