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ORIGINAL ARTICLE

Nicole M. Haynes · Mark J. Smyth
Michael H. Kershaw · Joseph A. Trapani
Phillip K. Darcy

Fas-ligand-mediated lysis of erbB-2-expressing tumour cells by redirected cytotoxic T lymphocytes

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Abstract A chimeric receptor, consisting of the single-chain variable (scFv) domains of an anti-erbB-2 mAb linked via a CD8 membrane-proximal hinge to the Fc receptor γ chain, was expressed in the mouse cytotoxic T lymphocyte (CTL) hybridoma cell line, MD45. This cell line was grafted with the additional specificity to recognise and bind erbB-2-expressing breast carcinoma target cells T47D, MCF-7 and BT-20 in a non-MHC-restricted manner. Tumour cell lysis was antigen-specific since erbB-2-negative tumours were insensitive to lysis by MD45-scFv-anti-erbB-2-γ clones, and lysis of erbB-2$^+$ tumour targets was inhibited in the presence of an anti-erbB-2 mAb. Furthermore, target cell death correlated with the level of chimeric receptor expression on the effector MD45 subclones. Redirected MD45 CTL utilised Fas ligand to induce target cell death since soluble Fas-Fc fusion protein completely inhibited cytolysis. The sensitivity of tumour target cells to Fas ligand was further enhanced by treating them with interferon-γ, a regulator of Fas and downstream signalling components of the Fas pathway. Overall, this study has demonstrated the requirement for successful activation of Fas ligand function in conjunction with cytokine treatment for effective lysis of breast carcinoma target cells mediated by redirected CTL.

Key words Single-chain antibody · Redirected cytotoxicity · Fas ligand · Breast carcinoma · Immunotherapy

N.M. Haynes · M.J. Smyth · M.H. Kershaw
J.A. Trapani · P.K. Darcy (✉)
Cellular Cytotoxicity Laboratory,
The Austin Research Institute,
Studley Road, Heidelberg,
3084, Victoria, Australia
e-mail: p.darcy@ari.unimelb.edu.au
Tel.: +61-3-92870695; Fax: +61-3-92870600

Introduction

Cytotoxic lymphocytes (CL) have been used successfully to treat some cancer patients [38, 39], however their use in adoptive immunotherapy has often suffered from the difficulty in obtaining T cells with specific reactivity against common carcinomas such as breast and colon cancer. Methods to target lytic cells of the immune system to destroy tumour cells have been derived through the application of tumour-reactive mAb [26] and bispecific antibodies [36]; however, the inefficient penetration of antibodies into solid tumours [12, 24] limits their therapeutic application. The ability to manipulate CL genetically to generate expression of integral membrane single-chain variable-domain (scFv) chimeric receptors, reactive to tumour antigens, provides a potentially more effective therapy that combines the antibody specificity of the scFv receptor with the tumour-penetrating and cytolytic potential of CL.

To date, the use of scFv chimeric receptors has demonstrated their potential to redirect the lytic activity of a variety of effector CL. These include cytotoxic T lymphocyte (CTL) cell lines [7, 10, 41] and primary mouse and human lymphocytes [1, 5, 19, 43], which have been targeted at predefined tumour targets in vitro. Studies in vivo have demonstrated the capacity of genetically redirected lymphocytes to traffic to tumours [2, 32] and their ability to inhibit growth of human tumours in nude mice [2, 15, 20]. CTL can mediate target cell lysis through the granular exocytosis pathway and/or the Fas-ligand (FasL)-mediated pathway [17]; however, to date the cytolytic mechanism(s) by which redirected lymphocytes trigger tumour cell death have not been fully characterised.

The c-erbB-2 gene, encoding a member of the tyrosinase kinase growth factor receptor family, is among those most frequently altered in human cancers [22], and is amplified and/or overexpressed in a high percentage of human adenocarcinomas arising at numerous sites, including the breast, ovary, lung, stomach and salivary

gland [21]. The accessiblity of the receptor, its high level of expression in metastatic lesions and low expression on normal tissue makes the erbB-2 antigen an ideal target for redirected CTL therapy. In this study we have genetically manipulated a mouse CTL hybridoma cell line, MD45, with a chimeric receptor consisting of a scFv anti-erbB-2 mAb, CD8 hinge/spacer and the lymphocyte signalling molecule FcR-γ. We demonstrate that MD45-scFv-anti-erbB-2-γ transfectants mediate non-MHC-restricted, erbB-2-specific lysis of erbB-2-expressing breast carcinoma cells in a FasL-dependent and interferon-γ (IFN-γ)-inducible manner.

Materials and methods

Cell lines, antibodies and reagents

MD45 is a murine allospecific CTL hybridoma cell line that can be induced by phorbol 12-myristate 13-acetate (PMA) to express FasL, but does not express perforin or granzymes [16]. COS-7, a simian kidney fibroblast cell line, and MD45 were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 2 mM glutamine, 0.1 μg/ml streptomycin, and 100 U/ml penicillin (Gibco, Grand Island, New York). d12S is the 12th serial subcloning of the rat × mouse hybridoma PC60 cell line [6] and was grown in DMEM (Gibco) supplemented with 5% FCS and additives as above. The human T cell leukemia, Jurkat, and the human breast carcinoma cell lines T47D, MCF-7 and BT-20 were maintained in RPMI medium supplemented as above. Anti-tag mAb (mouse IgG2a) detects an epitope contained within an 11-amino-acid sequence of the protein derived from the proto-oncogene c-*myc*. Mouse Fas-Fc (which also inhibits human FasL) and human p80 tumor-necrosis-factor-receptor (TNFR)–Fc fusion proteins were a kind gift from Dr. David Lynch, Immunex Corp., Seattle, Wash. CH-11 anti-(human Fas) IgM mAb was purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Human IFN-γ was provided by the Frederick Cancer Research and Development Center Biological Resources Branch, Frederick, Md. The mouse anti-(human granzyme M) mAb (IgG1) and mouse anti-(human granzyme B) mAb (IgG2a) were used as isotype controls. Before some cytotoxicity assays, d12S cells were incubated in medium supplemented with 5 ng/ml PMA (Sigma Chemical Co., St Louis, Mo., 5 ng/ml) and the Ca^{2+} ionophore ionomycin (Calbiochem Corp., San Diego, Calif., 1 μg/ml) [40].

Chimeric receptor gene constuction

A 767-bp fragment of DNA, coding for scFv of anti-erbB-2 and a marker epitope from c-myc (a kind gift from Dr. Winfried Wels, Institute of Experimental Cancer Research, Germany), was amplified by the polymerase chain reaction from the pSW50-5 vector using the following primers: forward, 5′-ATAATGTCTAGAC-AGGTACAACTGCAG-3′ (779) incorporating an *Xba*I restriction site (in bold type), and reverse, 5′-AGAGACGGTGACCC-CATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCGATCT-CCAATTTTGT-3′ (601) incorporating the sequence for the c-myc tag epitope at the 3′ end of the scFv fragment and a *Bst*EII restriction site (bold type). This fragment was subcloned into *Xba*I/*Bst*EII-digested pRSVscFvRγ (a kind gift from Dr. Zelig Eshhar, Weismann Institute, Rehovot, Israel). The final construct was made up of an extracellular region composed of variable heavy and light regions of anti-erbB-2 mAb joined via a flexible linker, a membrane-proximal hinge region of human CD8 and the transmembrane and cytoplasmic regions of the human FcR-γ chain. The leader was from the immunoglobulin κ light chain. Expression was driven by the Rous sarcoma virus long terminal repeat.

Gene transfection

COS-7 cells were transiently transfected by the DEAE-dextran method as previously described [3]. MD45 cells (10^7 in 0.25 ml) were stably transfected by incubation for 10 min at 4 °C in a 0.4-cm cuvette (Bio-Rad, Richmond, Calif.) with 20 μg *Hpa*I-linearized plasmid DNA. Electroporation was then carried out (Genepulsar, Bio-Rad) at 250 V and 960 μF. Stably transfected clones were selected in complete DMEM containing 2.0 mg/ml geneticin (Gibco BRL, N.Y.).

Immunofluorescence studies

Detection of cell surface chimeric receptor was achieved by indirect immunofluorescence following incubation of cells with anti-tag mAb, specific for a c-myc epitope located within the chimeric receptor. Transiently transfected COS-7 cells were incubated for 1 h at 4 °C with a 1/10 dilution in phosphate buffered saline/0.5% bovine serum albumin (PBS/BSA) of tissue culture supernatant from a hybridoma cell line secreting anti-tag-mAb. Cells were then washed in PBS/BSA, incubated for 1 h in a 1/200 dilution of fluorescein-isothiocyanate (FITC)-conjugated $F(ab')_2$ sheep anti-(mouse Ig) (Silenus, Hawthorn, Australia), washed a further three times, and viewed under a fluorescent microscope. Stably transfected MD45 CTL and breast carcinoma target cells were treated as for COS-7 cells and fluorescence was assessed by flow cytometry (FacsCalibur, Becton Dickinson, San Jose, Calif.). Tumour cell lines were assayed for erbB-2 expression by incubation with anti-erbB-2 mAb followed by FITC-conjugated sheep anti-(mouse Ig). The isotype control mAb used was anti-(human granzyme M). Expression of Fas on tumour cell lines was determined following incubation with polyclonal rabbit anti-(human Fas) and FITC-conjugated goat anti-(rabbit Ig). Non-immune rabbit serum was used as a control.

Rosetting

The binding capacity of the scFv-anti-erbB-2-γ-expressing MD45 CTL was determined in a rosetting assay. Stably transfected MD45 CTL (1×10^6 cells/ml) were added to transiently transfected COS-7 cells or tumour cell lines grown to 50–70% confluence in six-well plates. The plates were then incubated at 37 °C for 10 min, centrifuged (800 *g* for 3 min) and incubated for 30 min on ice. The cells were then washed 10–15 times with PBS/BSA and examined microscopically for rosette formation following staining with 0.1% ethyl violet. A rosette was defined as an erbB-2-expressing COS-7 cell or tumour cell that bound more than five scFv-anti-erbB-2-γ-expressing MD45 CTL. The percentage rosette formation was estimated by counting 100 cells in randomly chosen microscopic fields.

Western blot analysis

Cell lysates of the MD45 CTL cell line were analysed by Western blotting to verify expression of the scFv anti-erbB-2-γ protein. Cells were harvested and resuspended at 5×10^7 cells/ml in NP-40 lysis buffer. Cell debris was pelleted by centrifugation and supernatant was isolated. Lysates were diluted in an equal volume of 2 × electrophoresis sample buffer containing sodium dodecyl sulphate, boiled at 95 °C for 5 min, and placed on ice for 5 min prior to being loaded on a 10% (w/w) sodium dodecyl sulphate/polyacrylamide (SDS-PAGE) gel. Protein samples were electrophoretically transferred from the SDS/PAGE gel onto a nitrocellulose Immobilon membrane using the Trans-Blot Semi-Dry Transfer Cell as specified by the manufacturer. After transfer, the membrane was blocked for 16 h in a 5% skim-milk solution. The membrane was probed with undiluted anti-tag mAb for 1 h prior to being washed in Western washing buffer. Bound antibody was made visible with horseradish peroxidase-conjugated rabbit anti-(mouse Ig) diluted 1/8000 in

Western washing buffer and the enhanced chemiluminescence detection system. Protein detection was by autoradiography on OMAT XAR film.

^{51}Cr-release assay

The cytotoxic capacity of scFv-anti-erbB-2-γ-expressing CTL was determined in a ^{51}Cr-release assay. Adherent target cells (1×10^6 cells/ml) were harvested and washed three times in PBS, resuspended in 100 µl and incubated for 1 h at 37 °C with 100 µCi sodium [^{51}Cr]chromate. Free ^{51}Cr was removed by washing the cells, which were then placed in 96-well plates (2×10^4/well) and incubated for 16 h/37 °C with effector cells. In the case of antibody (anti-erbB-2) or Fas-Fc inhibition experiments, target or effector cells were preincubated with reagent for 30 min at 4 °C and then added to effector or target cells respectively. The effector cells were harvested, washed and used in 16-h ^{51}Cr-release assays against breast carcinoma targets. Cytotoxicity was expressed as the percentage specific ^{51}Cr release after subtraction of spontaneous ^{51}Cr release, which was 5–10% of the total release. The spontaneous release of ^{51}Cr was determined by incubating the target cells (2×10^4) with medium alone (200 µl), whereas the maximum release was determined by adding SDS to a final concentration of 5%. The percentage specific lysis was calculated as follows: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. All determinations were performed at least twice in triplicate.

Results

Construction and expression of the chimeric scFv-anti-erbB-2-γ receptor gene

A chimeric receptor gene consisting of the scFv of the anti-erbB-2 mAb linked to the FcR-γ chain via a CD8 hinge was cloned into the pRSV vector. The scFv specifically recognises the extracellular domain of erbB-2, and the CD8 hinge provides a spacer region allowing optimal binding of the target antigen as shown previously [31]. The signalling component of the receptor gene consisted of the transmembrane and cytoplasmic domain of the FcR-γ chain. Expression of the chimeric gene for scFv-anti-erbB-2-γ receptor was initially demonstrated in COS-7 cells after transient transfection (data not shown) and in the mouse CTL hybridoma cell line, MD45, after stable transfection by indirect immunofluorescence using an anti-c-myc tag mAb (Fig. 1a). Flow cytometry demonstrated subclones expressing both high (H) and low (L) levels of the chimeric receptor compared to background fluorescence observed for the parental MD45. Western-blot analysis, performed under reducing conditions on cell lysates of the E6H scFv-anti-erbB-2-γ clone, revealed two specific proteins not evident in the parental MD45 cell lysate (Fig. 1b). The smaller fragment confirmed the presence of the full-length integral membrane receptor protein with a calculated molecular mass of 48.7 kDa, consistent with a previous report [32]. The more slowly migrating species may be explained by either (i) complex glycosylation of the scFv and hinge region or (ii) constitutive glycosylation of the γ chain, although both of these possibilities require further investigation.

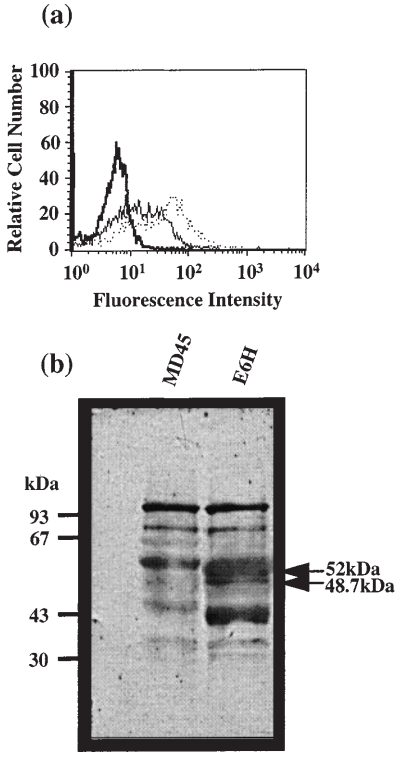

Fig. 1a,b Expression and detection of the scFv-anti-erbB-2-γ chimeric receptor in the MD45 CTL cell line. **a** MD45 cells were stably transfected with the scFv-anti-erbB-2-γ receptor cDNA under G418 selection. Four transfectants were cloned by limiting dilution and analysed for chimeric receptor expression by flow cytometry following staining with mouse anti-tag mAb and fluorescein-isothiocyanate(FITC)-conjugated sheep anti-(mouse Ig). High (*H*)- and low(*L*)-expression subclones of each transfectant were isolated and expression of the E6H (···) and E6L (—) subclones are shown here. No specific binding of the anti-tag mAb was detected in parental MD45 cells (—). **b** The scFv-anti-erbB-2 protein in MD45 transfectants was determined by Western blot analysis. Cell lysates of parental MD45 and E6H were subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis under reducing conditions, electroblotted and probed with anti-tag mAb. Two specific bands were detected in the stably transfected E6H clone (*arrows*); the smaller fragment represents the scFv-anti-erbB-2-γ protein (48.7 kDa), the more slowly migrating protein (52 kDa) possibly being a more complex form of the receptor. Expression of these proteins was not evident in the parental MD45 cell line. *Left* molecular mass standards (kDa)

MD45-scFv-anti-erbB-2-γ clones bind erbB-2-expressing target cells

The ability of MD45 expressing the scFv anti-erbB-2-γ chimeric receptor to bind erbB-2-expressing target cells was initially demonstrated using COS-7 cells transiently transfected with erbB-2 cDNA. The E6H (Fig. 2C) and E6L (Fig. 2D) scFv-anti-erbB-2-γ subclones, but not the parental MD45 cell line (Fig. 2A) were shown to conjugate effectively to erbB-2-expressing COS-7 cells to form defined rosettes. The E6H subclone was unable to conjugate to untransfected COS-7 cells (Fig. 2B). Interestingly, the level of expression of the chimeric receptor correlated with the level of rosette formation. The

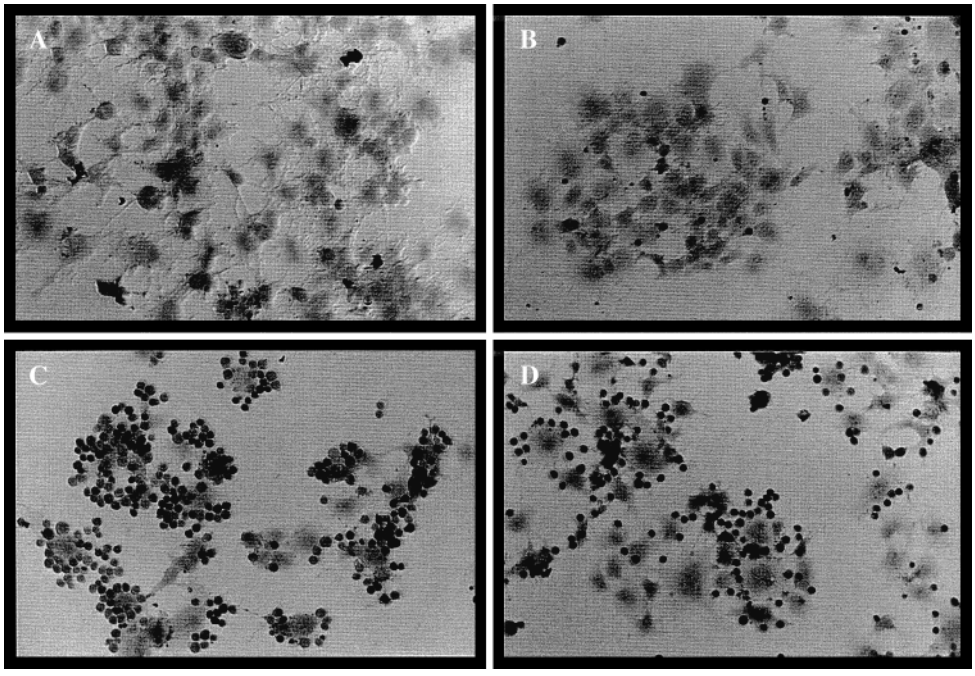

Fig. 2A–D MD45-scFv-anti-erbB-2-γ clones bind COS-7 cells transiently transfected with erbB-2 cDNA. The binding potential of MD45 expressing the scFv-anti-erbB-2-γ chimeric receptor was determined by their ability to mediate rosette formation with COS-7 cells transiently transfected with erbB-2 cDNA. The E6H (**c**) and E6L (**d**) subclones were able to conjugate and form rosettes with erbB-2-expressing COS-7 cells, and formation of rosettes correlated with the level of chimeric receptor expression (percentage of rosettes formed between E6H and E6L and COS-7 cells expressing erbB-2 was 65% and 40% respectively). Parental MD45 cells (**a**) could not bind to erbB-2-transfected COS-7 cells and, in the absence of erbB-2 expression, the E6H subclone (**b**) was unable to conjugate to COS-7 cells as shown

percentage of rosettes formed between E6H and erbB-2-transfected COS-7 cells was 65% compared to 40% observed for E6L. For the purpose of tumour therapy it was important to demonstrate that the MD45-scFv anti-erbB-2-γ clones could also bind tumour targets expressing the erbB-2 antigen. The E6H subclone was better able to congugate effectively to erbB-2^+ human breast carcinoma cell lines T47D and MCF-7 (Fig. 3B,D) in rosetting assays (percentage rosettes formed were 55% and 45% respectively) than was the low-erbB-2-expressing cell line BT-20 (Fig. 3F) (percentage rosettes formed was 10%) and again rosetting of E6L with these targets was comparatively reduced (data not shown). The parental MD45 cell line was unable to bind to T47D, MCF-7 or BT-20 cells (Fig. 3A,C,E). Collectively these results demonstrate that the specificity of MD45 CTL can be altered by expression of a chimeric receptor gene engrafting them with the ability to recognise and bind target cells expressing the erbB-2 antigen.

Tumour cell lysis redirected by scFv-anti-erbB-2-γ is antigen-dependent

The ability of MD45 expressing scFv-anti-erbB-2-γ receptor to mediate specific target cell lysis was determined by a ^{51}Cr-release assay. Significant erbB-2 expression was detected on the human breast carcinoma cell lines T47D and MCF-7 (Fig. 4a,b) but not on BT-20 (Fig. 4c) or the negative-control human Jurkat cells (Fig. 4d). The E6H scFv-anti-erbB-2-γ transfectant was demonstrated to lyse T47D target cells specifically in a 16-h ^{51}Cr-release assay (Fig. 4e). Lysis of T47D cells was

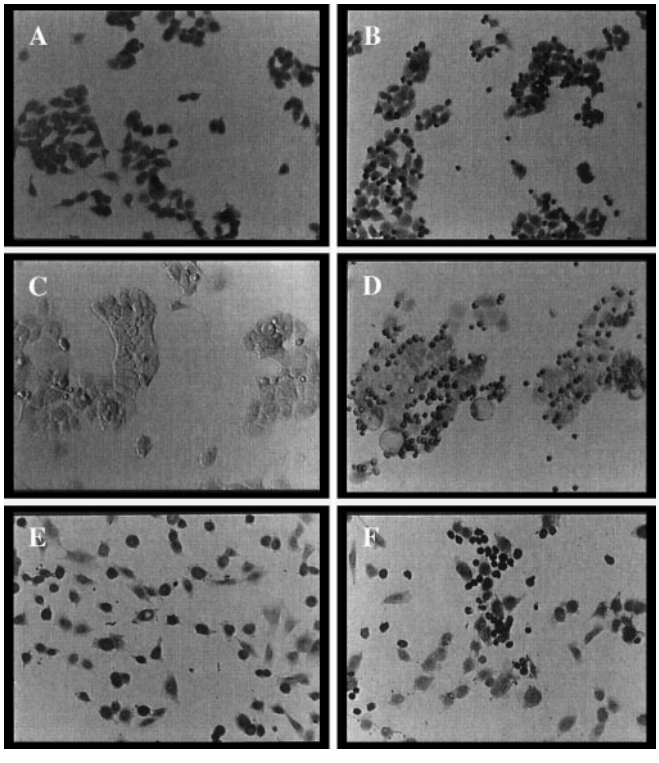

Fig. 3A–F MD45-scFv-anti-erbB-2-γ clones bind erbB-2^+ tumour cell lines. The ability of MD45 cells expressing the scFc-anti-erbB-2-γ chimeric receptor to recognise and bind erbB-2^+ tumour targets was determined in a rosetting assay as described in Materials and methods. The E6H subclone was able to conjugate and form rosettes with erbB-2-expressing human breast carcinoma cell lines T47D (**b**) and MCF-7 (**d**) but rosette formation was significantly reduced with the low-expression erbB-2 cell line BT-20 (**f**). The MD45 parental cell line could not bind either T47D (**a**) MCF-7 (**c**) or BT-20 (**e**) cells as shown

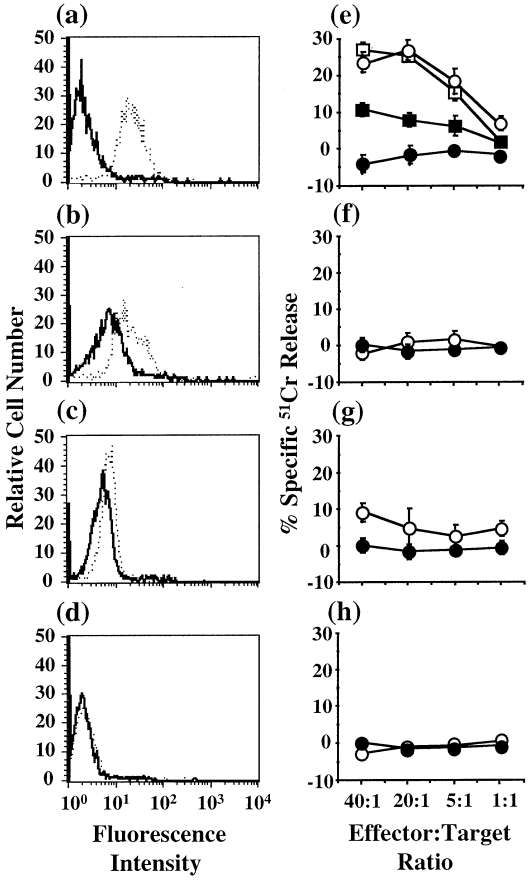

Fig. 4a–h Redirected lysis by cytotoxic T lymphocyte (CTL) transfectants was erbB-2-specific. Cells were incubated with mouse anti-erbB-2 mAb (···) or isotype control (—), followed by FITC-conjugated sheep anti-(mouse Ig), and erbB-2 expression was detected by flow cytometry: **a** T47D, **b** MCF-7, **c** BT-20, **d** Jurkat. The sensitivity of these target cells to MD45- and MD45-scFv-anti-erbB-2-γ-mediated lysis was determined by a 16-h ^{51}Cr-release assay (**e**–**h**). The MD45-scFv-anti-erbB-2-γ clone (E6H) or the parental MD45 were pretreated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml) for 3 h, washed twice and incubated with T47D (**e**) target cells in the absence (○ E6H, ● MD45) or presence of anti-erbB-2 mAb (■ E6H), or MCF-7 (**f**), BT-20 (**g**) or Jurkat (**h**) target cells. An isotype control antibody did not inhibit E6H lysis (□) in **e**. Results are expressed as specific ^{51}Cr release ± SE(%) for triplicate samples and are representative of two experiments performed

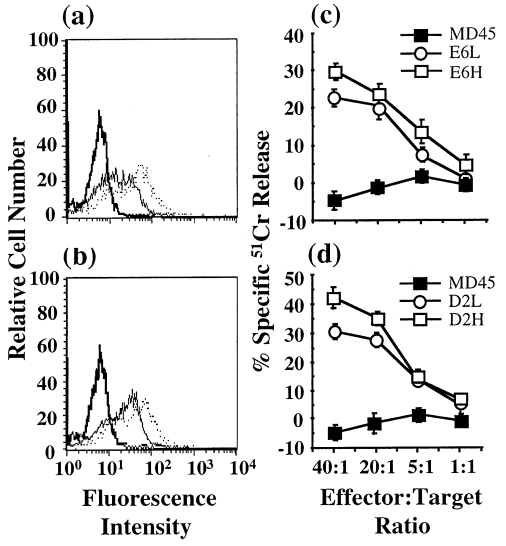

Fig. 5a–d The level of scFv-anti-erbB-2-γ chimeric receptor expression correlates with the degree of target cell lysis. Four MD45 transfectant subclones were analysed for chimeric receptor expression by flow cytometry following staining with mouse anti-tag mAb and FITC-conjugated sheep anti-mouse Ig (**a**,**b**). ··· High(*H*)-, — low(*L*)-expression subclones of transfectants (**a**) E6 and (**b**) D2. All subclones demonstrated only background levels of fluorescence when treated with an isotype control antibody. — Parental MD45 cells. The cytotoxic capacity of each clone (pretreated with 5 ng/ml PMA for 3 h) was also assessed in a 16-h ^{51}Cr-release assay against erbB-2^{+} T47D cells (**c**,**d**). High- (□) and low-expression (○) subclones are contrasted in each assay with control parental MD45 cells (■). Results are expressed as specific ^{51}Cr release ± SE(%) for triplicate samples and are representative of two experiments performed

erbB-2-specific since anti-erbB-2 mAb, but not isotype control mAb, substantially inhibited E6H-mediated lysis. The incomplete inhibition may be due to recognition of an alternative epitope by the anti-erbB-2 mAb on the extracellular domain of the erbB-2 antigen to that recognised by the scFv-anti-erbB-2 mAb, the close proximity of the two epitopes possibly resulting in steric hindrance. Parental MD45 was unable to lyse T47D cells (Fig. 4e) and other breast carcinoma targets (Fig. 4f,g) and Jurkat cells were not lysed by either E6H or parental MD45 (Fig. 4h). As demonstrated in the rosetting assays, the level of scFv-anti-erbB-2-γ receptor expression on both high- and low-expressing MD45 clones (Fig. 5a,b) was proportional to the level of binding and degree of lysis of T47D target cells (Fig. 5c,d). These data highlight the importance of achieving high levels of chimeric receptor expression when redirecting CTL to lyse tumour cells.

MD45-scFv-anti-erbB-2-γ clones mediate tumour cell lysis via FasL

It has previously been demonstrated that MD45 can be induced by PMA to express FasL as observed in other T/NK cell lines [9, 42], but does not express perforin or granzymes [16], suggesting that this CTL cell line potentially mediates cell death via a FasL-dependent death pathway. The breast carcinoma cell line MCF-7 expressing-erbB-2 (Fig. 4b) was shown to be insensitive to E6H-scFv-anti-erbB-2-γ-mediated lysis (Fig. 4f), which raised the possibility that MCF-7 might be insensitive to Fas-mediated lysis. To examine this possibility each of the breast carcinoma cell lines was initially characterised for Fas cell surface expression by flow cytometry. High levels of Fas were detected on T47D, BT-20 and Jurkat target cells (Fig, 6a,b,d); however, MCF-7 cells expressed no detectable levels of Fas (Fig. 6c). In addition, the non-MHC-restricted CTL hybridoma, d12S, lysed T47D and Jurkat cells but not MCF-7 target cells in a

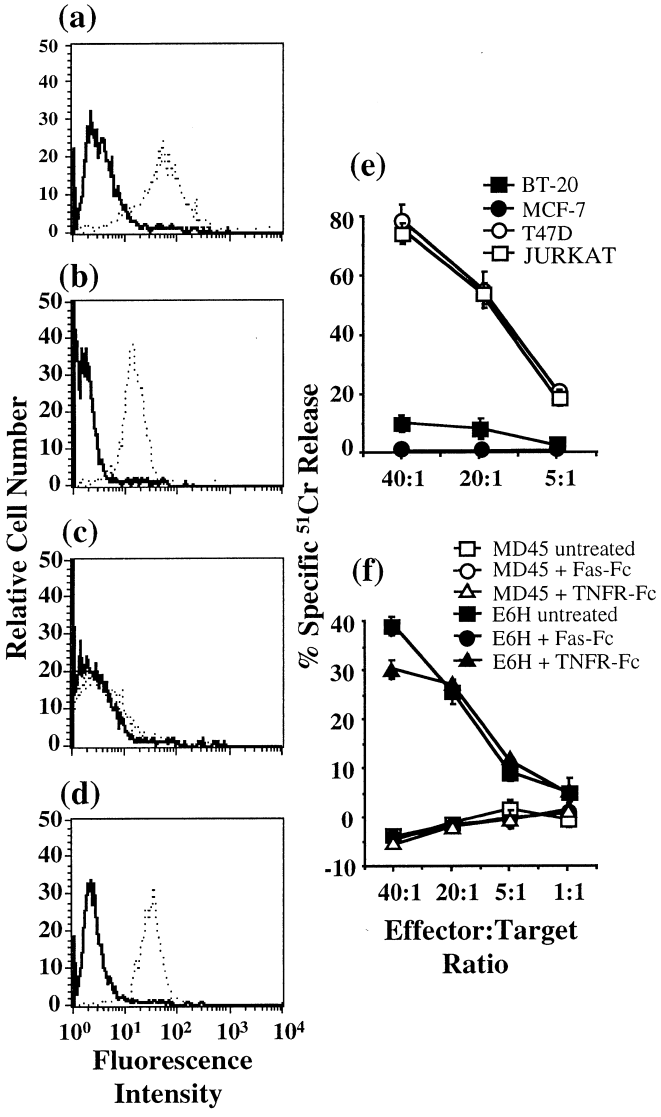

Fig. 6a–f Redirected lysis by CTL transfectants was FasL-mediated. Cells were incubated with rabbit anti-human Fas polyclonal (···) or control rabbit serum (—), followed by FITC-conjugated goat anti-(rabbit Ig), and Fas expression was detected by flow cytometry: **a** T47D, **b** BT-20, **c** MCF-7, **d** Jurkat. The sensitivity of these same targets to the CTL hybridoma cell line d12s was determined in a 16-h ^{51}Cr-release assay (**e**). MD45 and E6H (MD45-scFv-anti-erbB-2-γ) clones were pretreated with PMA (5 ng/ml) for 3 h and washed twice, and their lysis was determined in the absence or presence of soluble Fas-Fc or TNFR-Fc (5 μg/ml) in a 16-h ^{51}Cr-release assay (**f**). Results for (**e**, **f**) were expressed as specific ^{51}Cr-release ± SE(%) for triplicate samples and are representative of two experiments performed

Fas-mediated manner (Fig. 6e). BT-20 breast carcinoma cells were relatively insensitive to d12S killing, despite their high level of Fas expression, suggesting that the level of Fas expression is not necessarily indicative of Fas sensitivity. To support effector FasL interaction with target cell Fas, specific inhibition of T47D cell lysis by E6H was demonstrated by the addition of Fas-Fc, but not TNFR-Fc, fusion protein (Fig. 6f). These data confirmed that MD45-scFv-anti-erbB-2-γ clones use a FasL-mediated pathway to induce target cell lysis.

Target cell sensitivity to MD45-scFv-anti-erbB-2-γ cell-mediated lysis is IFN-γ-inducible

Some tumours (e.g. breast and colon carcinomas) have been observed to down-regulate Fas expression and/or partially lose Fas sensitivity as a means of evading immune surveillance and destruction [25, 30]. It has been previously demonstrated that IFN-γ can enhance leucocyte antigen surface expression [14, 29, 44] and, in particular, the sensitivity of some tumour cell lines to Fas-mediated apoptosis [25]. To determine whether IFN-γ could increase erbB-2 or Fas surface expression on T47D, BT-20 and MCF-7 target cells and enhance their sensitivity to CTL-redirected lysis, each was treated with or without 1000 U/ml IFN-γ for 16 h. Although IFN-γ did not increase either erbB-2 or Fas expression on each of the breast carcinoma cell lines, as demonstrated by flow cytometry (data not shown), T47D and, in particular, BT-20 were rendered more susceptible to E6H cell-mediated lysis (Fig. 7a,b). Interestingly, IFN-γ did not increase the sensitivity of MCF-7 breast carcinoma cells to FasL-mediated lysis, suggesting a complete lack of a functional Fas signalling pathway in these cells (Fig. 7c).

Discussion

The ability to manipulate CL genetically ex-vivo with gene constructs encoding chimeric cell-surface receptors has provided a promising means of specifically targeting common carcinomas, such as breast and colon cancer, that have remained refractory to other immunotherapies. However, one problem of redirecting CTL to tumours has been the lack of truly defined tumour-specific antigens. Despite this problem, the protein product of the c-erbB-2 proto-oncogene potentially serves as a better target than other tumour-associated antigens, since erbB-2 expression is more restricted in normal tissue [11, 23]. In addition, since the c-erbB-2 gene is amplified in certain malignancies, redirected CTL therapy against the erbB-2 antigen may achieve greater transduction selectivity than other target antigens and have indirect effects related to its effect on tumour growth [8, 18]. Thus, we have created a chimeric receptor gene construct encoding a scFv recognizing the tumour-associated antigen, erbB-2, linked via a flexible CD8 hinge to the lymphocyte signalling molecule FcR-γ. Expression of the scFv-anti-erbB-2-γ construct in the mouse CTL hybridoma cell line MD45 enabled specific redirection to tumour targets expressing the erbB-2 tumour-associated antigen. We showed that lysis of erbB-2$^+$ target cells was antigen-specific and that the extracellular scFv mAb component of the chimeric receptor endowed MD45 CTL with MHC-non-restricted specificity, as shown by

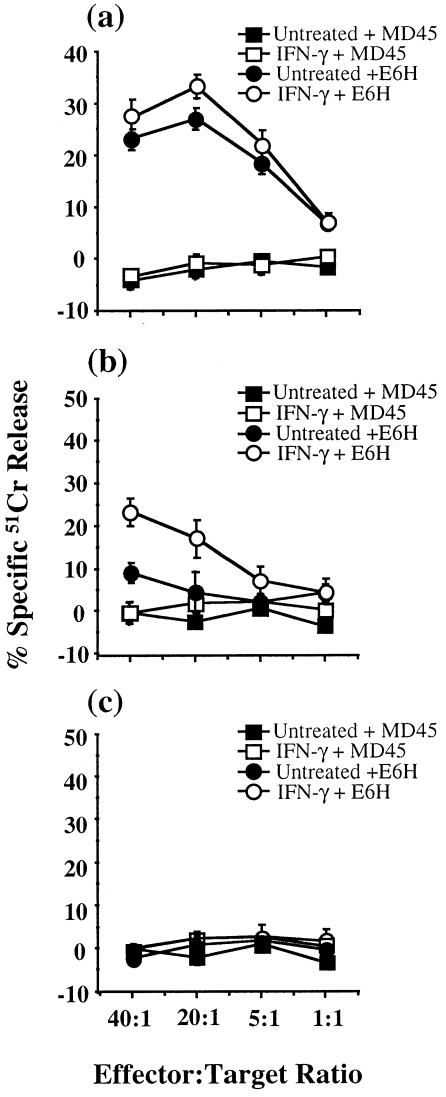

Fig. 7a–c Interferon-γ (IFN-γ) increases sensitivity of tumour targets to FasL-mediated lysis. Regulation of target cell sensitivity to E6H-mediated lysis by IFN-γ was assessed by a 16-h ^{51}Cr-release assay on untreated and IFN-γ-treated (1000 U/ml, 24 h) tumour cell lines. Prior exposure to IFN-γ rendered T47D (**a**) and BT-20 (**b**) more susceptible to E6H-mediated lysis than were the untreated targets. IFN-γ did not increase the sensitivity of MCF-7 to E6H-mediated lysis (**c**). Parental MD45 cells, untreated or IFN-γ-treated, were unable to lyse the tumour cell lines. Results are expressed as specific ^{51}Cr release ± SE(%) for triplicate samples and are representative of two experiments performed

their ability to bind and lyse human tumour cells. This property may be an advantage for redirected CTL in terms of lysing tumour cells with down-regulated MHC class I expression [4, 13].

One potential problem for the use of redirected CTL therapy in a clinical situation is the shedding of membrane-bound target antigen from the surface of tumour cells that may block redirected CTL-mediated lysis. Indeed, we have previously shown that high levels of soluble carcinoembryonic antigen (CEA), found in the blood of colon carcinoma patients, can partially inhibit CTL scFv-anti-CEA-γ-redirected lysis of colon carcinoma cells [7]. However, a study by Ishida et al. [23] has shown that the level of circulating erbB-2 protein in sera from cancer patients is low, and, although the antigen is over-expressed on cancerous tissue, it is not easily shed. Therefore, the erbB-2 antigen may be a more useful target molecule for redirected-CTL therapy than other tumour-associated antigens such as CEA.

Previous work from Groner's laboratory [1, 2, 32] has shown that naive T lymphocytes retrovirally transduced with the single-chain antibody directed against the human erbB-2 receptor could specifically recognize and lyse erbB-2-expressing tumour targets. However, the exact mechanism of target cell lysis used by these redirected CTL was not determined in these studies. We have clearly demonstrated in this study that target cell lysis employed by MD45-scFv-anti-erbB-2-γ involved a FasL-mediated pathway to cell death. The possible involvement of the granule exocytosis pathway is unlikely, since previous investigations have demonstrated the absence of perforin in MD45 [16]. However, in general this pathway could be used potentially by redirected CTL to mediate a similar cytolytic effect [28]. It is important to note that Fas-sensitive Jurkat cells that lacked erbB-2 expression were insensitive to MD45-scFv-anti-erbB-2-γ-mediated lysis, highlighting the importance of effector cell recognition of the erbB-2 tumour-associated antigen. This interaction between erbB-2 and the chimeric receptor may have been important at two levels: (i) as an adhesive interaction that approximated the effector and target cell and (ii) the induction of MD45-scFv-anti-erbB-2-γ cytolytic capacity through receptor ligation. Overall, this study suggests that the dual requirement of tumour-associated antigen and functional Fas on target cells may provide an extra degree of specificity for the tumour over normal tissue, which is of concern in the absence of tumour-specific antigens for most malignancies.

One possible escape mechanism used by some tumours is the down-regulation of Fas expression and/or the partial loss of Fas function [37]. However, the ability to induce the sensitivity of some tumours to Fas-mediated apoptosis by IFN-γ treatment suggests that some tumour cells can modulate their Fas pathway but do not lose their components of the Fas death pathway through mutation [25]. We have previously shown that IFN-γ treatment can increase the expression of both Fas and CEA on some colon carcinoma cell lines, which may account for their increased sensitivity to Fas-mediated lysis by the MD45 transfectant clones [7]. However, other reports have demonstrated that Fas expression is not always indicative of function [34, 35] and that IFN-γ can induce target cell Fas sensitivity by the induction of numerous pro-apoptotic stimuli that have been shown to play a direct role in the Fas death pathway [25, 27, 33]. Induction of one or several of these apoptosis-related genes may account for the enhanced sensitivity of T47D and BT-20 breast carcinoma cells to MD45-scFv-anti-erbB-2-γ-mediated lysis following IFN-γ treatment,

since their cell-surface expression of Fas and erbB-2 were unaltered. The issue of how IFN-γ regulates Fas and other components of the Fas death pathway remains complex and requires further investigation. Nevertheless, regulation of the Fas pathway by IFN-γ treatment may potentially provide a means to enhance the therapeutic application of redirected CTL, particularly against Fas^- or low-expressing Fas^+ tumour target cells.

Overall, this study has demonstrated that the FasL-dependent pathway of lysis can be used effectively by redirected CTL, even against tumour cells that lack the appropriate MHC or significant constitutive Fas expression. It remains to be determined, however, whether these findings can be extended to include other effector lines or primary lymphocytes. Study of the cytolytic pathways used by redirected CL has provided scope for identifying potential means for manipulating these pathways, possibly through combined cytokine treatment, to enhance the lytic activity of gene-modified lymphocytes against tumour targets and thereby optimise the efficacy of this approach as a therapy for cancer.

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References

1. Altenschmidt U, Kahl R, Moritz D, Schnierie BS, Gerstmayer B, Wels W, Groner B (1996) Cytolysis of tumor cells expressing the Neu/erbB-2, erbB-3 and erbB-4 receptors by genetically targeted naive T lymphocytes. Clin Can Res 2:1001
2. Altenschmidt U, Kludt E, Groner B (1997) Adoptive immunotherapy of in vitro-targeted, activated T lymphocytes results in total tumor regression. J Immunol 159:5509
3. Aruffo A, Seed B (1987) Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc Natl Acad Sci USA 84:8573
4. Blottiere HM, Zennadi R, Gregoire M, Aillet G, Denis MG, Meflah K, Le Pendu J (1993) Analysis of the relationship between stage of differentiation and NK/LAK susceptibility of colon carcinoma cells. Int J Cancer 53:409
5. Brocker T, Riedinger M, Karjalainen K (1996) Redirecting the complete T cell receptor/CD3 signalling machinery towards native antigen via modified T cell receptor. Eur J Immunol 26:1770
6. Conzelmann A, Corthesy P, Cianfriglia M, Silvia A, Nabholz M (1982) Hybrids between rat lymphoma and mouse T cells with inducible cytolyic activity. Nature 298:170
7. Darcy PK, Kershaw MH, Trapani JA, Smyth MJ (1998) Cytotoxic T lymphocyte expression of a single chain anti-carcinoembryonic antigen antibody. Redirected Fas ligand-mediated lysis of colon carcinoma. Eur J Immunol 28:1663
8. Drebin JA, Link VC, Greene MI (1988) Monoclonal antibodies specific for the neu oncogene product directly mediate anti-tumor effects in vivo. Oncogene 2:387
9. Eischen CM, Schilling JD, Lynch DH, Krammer PH, Leibson PJ (1996) Fc receptor-induced expression of Fas ligand on activated NK cells facilitates cell-mediated cytotoxicity and subsequent autocrine NK cell apoptosis. J Immunol 156:2693
10. Eshhar Z, Waks T, Gross G, Schindler DG (1993) Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc Natl Acad Sci USA 90:720
11. Foster BJ, Kern JA (1997) HER2-targeted gene transfer. Hum Gene Ther 8:719
12. Fujimori K, Covell DG, Fletcher JE, Weinstein JN (1989) Modeling analysis of the global and microscopic distribution of immunoglobulin G, F(ab′)2 and Fab in tumors. Cancer Res 49:5656
13. Garrido MA, Perez P, Titus JA, Valdayo MJ, Winkler DF, Barbieri SA, Wunderlich JR, Segal DM (1990) Targeted cytotoxic cells in human peripheral blood lymphocytes. J Immunol 144:2891
14. Gillott DJ, Nouri AM, Compton SJ, Oliver RT (1993) Accurate and rapid assessment of MHC antigen upregulation following cytokine stimulation. J Immunol Methods 165:231
15. Hege KM, Cooze KS, Finer MH, Zsebo K, Roberts MR (1996) Systemic T cell-independent tumour immunity and transplantation of universal receptor-modified bone marrow into SCID mice. J Exp Med 184:2261
16. Helgason CD, Prendergast JA, Berke G, Bleackley RC (1992) Peritoneal exudate lymphocyte and mixed lymphocyte culture hybridoma are cytolytic in the absence of cytotoxic cell proteinases and perforin. Eur J Immunol 22:3187
17. Henkart PA (1994) Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity 1:343
18. Hudziak RM, Lewis GD, Kinget M, Fendly BM, Shepard HM, Ullrich A (1989) p185HER2 monoclonal antibody has anti-proliferative effects in vitro and sensitized human breast tumor cells to tumor necrosis factor. Mol Cell Biol 9:1165
19. Hwu P, Shafer GE, Treisman J, Schindler DG, Gross G, Cowherd R, Rosenberg SA, Eshhar Z (1993) Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. J Exp Med 178:361
20. Hwu P, Yang JC, Cowherd R, Treisman J, Shafer GE, Eshhar Z, Rosenberg SA (1995) In vivo anti-tumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. Cancer Res 55:3369
21. Hynes NE (1993) Amplification and over-expression of the *erbB*-2 gene in human tumors: its involvement in tumor development, significance as a prognostic factor and potential as a target for cancer therapy. Semin Cancer Biol 4:19
22. Hynes NE, Stern DF (1994) The biology of the *erbB*-2/neu/HER-2 and its role in cancer. Biochim Biophys Acta 1198:165
23. Ishida T, Tsujisaki M, Hinoda Y, Imai K, Yachi A (1994) Establishment and characterization of mouse-human chimeric monoclonal antibody to *erbB*-2 product. Jpn J Cancer Res 85:172
24. Jain RK (1989) Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. J Natl Cancer Inst 81:570
25. Keane MM, Ettenberg SA, Lowrey GA, Russell EK, Lipkowitz S (1996) Fas expression and function in normal and malignant breast cell line. Cancer Res 56:4791
26. Kershaw MH, Darcy PK, Trapani JA, Smyth MJ (1996) The use of chimeric human Fc (epsilon) receptor I to redirect cytotoxic T lymphocytes to tumors. J Leukoc Biol 60:721
27. Kershaw MH, Trapani JA, Smyth MJ (1995) Cytotoxic lymphocytes: redirecting the cell-mediated immune response for the therapy of cancer. Ther Immunol 2:173
28. Koshiji M, Adachi Y, Sogo S, Taketani S, Oyaizu N, Than S, Inaba M, Phawa S, Hioki K, Ikehara S (1998) Apoptosis of colorectal adenocarcinoma (COLO 201) by tumor necrosis factor-alpha (TNF-alpha) and/or interferon-gamma (IFN-gamma), resulting from down-modulation of Bcl-2 expression. Clin Exp Immunol 111:211
29. Mobus VJ, Asphal W, Knapstein PG, Kreienberg R (1993) Effects of interferon gamma on the proliferation and modulation of cell-surface structures of human ovarian carcinoma cell lines. J Cancer Res Clin Oncol 120:27

30. Moller P, Koretz K, Leithauser F, Bruderlein S, Henne C, Quentmeier A, Krammer PH (1994) Expression of APO-1 (CD95), a member of the neoplastic colon epithilium. Int J Cancer 57:371
31. Moritz D, Groner B (1995) A spacer region between the single chain antibody and the CD3 ζ chain domain of chimeric T cell receptor components is required for efficient ligand binding and signalling activity. Gene Ther 8:539
32. Moritz D, Wels W, Mattern J, Groner B (1994) Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. Proc Natl Acad Sci USA 91:4318
33. Ossina NK, Cannas A, Powers VC, Fitzpatrick PA, Knight JD, Gilbert JR, Shekhtman EM, Tomei LD, Umansky SR, Kiefer MC (1997) Interferon-gamma modulates a p53-independent apoptotic pathway and apoptosis-related gene expression. J Biol Chem 272:16351
34. Owen-Schaub LB, Meterissian S, Ford RJ (1993) Fas/APO-1 expression and function on malignant cells of hematologic and nonhematologic origin. J Immunother 14:234
35. Owen-Schaub LB, Radinsky R, Kruzel E, Berry K, Yonehara S (1994) Anti-Fas on nonhaematopoietic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. Cancer Res 54:1580
36. Renner C, Jun W, Sahin U, Lier R van, Pfreundschuh M (1995) The role of lymphocyte subsets and adhesion molecules in T cell-dependent cytotoxicity mediated by CD3 and CD28 bispecific monoclonal antibodies. Eur J Immunol 25:2027
37. Robertson MJ, Manley TJ, Pichert G, Cameron C, Cochran KJ, Levine H, Ritz J (1995) Functional consequences of APO-1/Fas (CD95) antigen expression by normal and neoplastic hematopoietic cells. Leuk Lymphoma 17:51
38. Rosenberg SA (1992) Karnofsky Memorial Lecture. The immunotherapy and gene therapy of cancer. J Clin Oncol 10:180
39. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med 319:1676
40. Rouvier E, Luciani MF, Golstein P (1993) Fas involvement in Ca^{2+}-independent cell-mediated cytotoxicity. J Exp Med 177:195
41. Stancovski I, Schindler DG, Waks T, Yarden Y, Sela M, Eshhar Z (1993) Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors. J Immunol 151:6577
42. Suda T, Okazaki T, Naito Y, Yokota T, Arai N, Ozaki S, Nakao K, Nagata S (1995) Expression of the Fas ligand in cells of T cell lineage. J Immunol 154:3806
43. Weijtens ME, Willemsen RA, Valerio D, Stam K, Bolhuis RL (1996) Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. J Immunol 157:836
44. Yang Y, Xiang Z, Ertl HC, Wilson JM (1995) Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell mediated elimination of recombinant adenovirus-infected heptocytes in vivo. Proc Natl Acad Sci USA 92:7257