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ORIGINAL ARTICLE

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Tumor-specific targeting of a cell line with natural killer cell activity by asialoglycoprotein receptor gene transfer

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Abstract Targeting of immunological effector cells to tumor cells could be an efficient strategy of adoptive immunotherapy. The success of this strategy depends on the specificity of the effector cells and their availability in sufficient numbers. The aim of this study was to target the human natural killer cell line YT specifically to tumor cells. The cell line was modified by transfection with the cDNA of the human asialoglycoprotein receptor (ASGPR). This C-type lectin recognizes carbohydrates containing terminal galactosyl (Gal) residues, including the β1-Gal bearing Thomsen-Friedenreich (TF) antigen, which is found on tumor cells. Binding assays revealed that the ASGPR-gene-transfected YT cell line binds significantly higher to tested target tumor cell lines than the mock-transfected control cells. Cytolytic activity against the tumor cell lines Raji, Jurkat and the TF-positive KG1 subline was increased. Genetic modification of YT cells could provide a useful tool for tumor targeting in immunotherapy.

Keywords Asialoglycoprotein receptor · Gene transfer · Tumor targeting · Natural killer cells

Introduction

A promising strategy for tumor treatment could be the adoptive immunotherapy with lymphocytes recognizing tumor cells. The success of this procedure is dependent upon the specificity of the transferred immune cells, their number and their ability to reach the target cells. Various immune cells have been tested for this purpose. Lymphokine-activated killer cells, tumor-infiltrating lymphocytes [6, 46] and in vitro antigen sensitized cytotoxic T lymphocytes [18, 24, 41] have been shown to mediate antitumor responses in vitro and in vivo. A recent strategy is based on directing cytotoxic T cells to tumor cells by gene transfer of receptors consisting of single-chain antibodies fused to the signaling domain of the Fc receptor γ or the T cell receptor ζ chain; thus combining the specificity of antibody-based recognition of tumor antigens with the antitumor activity of T cells [12, 13, 19, 27, 29, 33]. However, primary T cells are hardly transfectable with classical methods. Only retroviral systems have been shown to infect effectively activated T cells or TILs [14, 27, 33]. Another potent immune effector cell type are natural killer (NK) cells [9, 43]. They do not express individual antigen-specific receptors and are able to lyse a broad spectrum of virus-infected or tumor cells in an MHC-unrestricted manner. Their cytotoxicity is regulated by activatory and inhibitory NK cell receptors [2, 28]. No successful transfection has been described for primary NK cells. Therefore, some research groups have used NK cell lines instead of primary NK cells. Nagashima et al. [30] described the stable retroviral transduction of the NK cell lines NK92 and YT, respectively, with the interleukin-2 (IL-2) gene, and Tam et al. [38] transfected NK-92 with the IL-2 gene by particle-mediated gene transfer. Liu et al. [25] transfected a YT-1 cell subclone with the CD18 gene by electroporation to restore its lytic function, and Tran et al. [40] reported the retroviral transduction of NK cell clone NK3.3 with the gene of a chimeric zeta receptor.

In the study described here, we examined the possibility of targeting the human cell line YT, which mediates NK cell properties [47], to tumor cells by gene transfer of the human asialoglycoprotein receptor (ASGPR). The cell line YT has several advantages, as it exhibits cytokine-independent growth [47] and is transfectable by electroporation [25]. The ASGPR is a hepatic Ca^{2+}-dependent C-type lectin containing two chains, H1 and H2 [36], which form a heteromultimeric receptor complex [5, 16]. Both chains have a carbohydrate

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recognition domain recognizing terminal galactosyl (Gal) and N-acetylgalactosamine (GalNAc) residues on glycoproteins [26]. An involvement of this hepatic lectin in the development of liver metastasis has been suggested for tumors expressing glycans carrying terminal β1-galactosyl (β1-Gal) residues [4, 10, 11, 31, 32]. D-galactose, arabinoglycan and asialoglycoproteins can inhibit the adhesion between β1-Gal-bearing tumor cells and hepatocytes in vitro [32] and the development of liver metastasis in vivo [4]. It has been demonstrated that patients with stage III colorectal or gastric carcinoma perioperatively treated with D-galactose developed less liver metastasis [20, 22, 42]. β1-Gal is also the terminal part of the Thomsen-Friedenreich (TF) antigen (Galβ1–3GalNAc-), a well-described marker on fetal and malignant epithelia, which is masked on normal tissue by sialinic acid or fucose [7]. Its expression on colon and gastric carcinomas is correlated with an increased appearance of liver metastasis [8, 10] and can be blocked in mice by a TF-specific antibody [35] or by glycoliposomes carrying multiple TF disaccharides [37]. We investigated the targeting of the YT cell line by ASGPR gene transfer to tumor cells expressing terminal β1-Gal residues on their surface, which could be a useful tool in adoptive immunotherapy.

Material and methods

Cell lines

The parental human natural killer cell line YT was kindly provided by J. Yodoi (Institute for Virus Research, Kyoto, Japan) [47]. The acute myelogenous leukemia (AML) cell line KG1 was kindly provided by U. Karsten (Max-Delbrück-Center, Berlin, Germany), the Burkitt's lymphoma cell line Raji and the T cell line Jurkat were kindly provided by J. Kopp (Robert-Roessle-Klinik, Berlin, Germany). These cell lines were cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and 50 μg/ml gentamicin (all from Gibco/BRL, Germany). YT cells were cultured with a maximum cell concentration of 2×10^5 cells per ml. HepG2 cells, obtained from American Type Culture Collection (ATCC, Rockville, Md.), and the human colorectal carcinoma cell line SW480, which was kindly provided by W. Kemmner (Max-Delbrück-Center, Berlin, Germany), were cultured in DMEM (Gibco/BRL) with 10% (v/v) FCS and 50 μg/ml gentamicin.

Cloning

The plasmids containing the cDNA of the ASGPR subunits were kindly provided by M. Spiess (Biozentrum, University of Basel, Switzerland). The plasmid pGA1, containing H1 cDNA, was digested with the enzymes *Eco*RI and *Hin*dIII (Fermentas, Germany) and pGA2, containing H2b cDNA, was digested using *Eco*RI. H2b is a subspecies of H2, lacking 5 amino acids in the extracellular domain immediately following the membrane-spanning region compared to H2a, which is rapidly degraded in the ER [1, 23]. The plasmids pREP4 and pREP8 (Invitrogen, Netherlands) were digested with *Xho*I (Fermentas), and the overhanging 5′-ends were filled in using Klenow fragment (Fermentas). The H1 cDNA fragment was then cloned into pREP8 and the H2 cDNA fragment into pREP4, respectively, using the Rapid-Ligation-Kit (Roche, Germany).

Transfection

An aliquot of 10^7 YT cells was transfected with pREP8-H1 or pREP4-H2 by electroporation using the GenePulser II with Capacitance Extender II (BioRad, Germany) at 250 V and 975 μF. The cells were cultured after electroporation for 48 h before the selection with 2 mM L-histidinol (Sigma, Germany) for pREP8-H1 or 250 μg/ml hygromycin B (Roche) for pREP4-H2 was started. Dead cells were removed after 1 week of selection by density gradient centrifugation using Lymphoprep (1.077 g/ml at 20 °C; Nycomed, Denmark). After 1–2 weeks the cells were transfected with the plasmid containing the cDNA of the second ASGPR chain, under the described conditions, and the selection was performed with 2 mM L-histidinol and 250 μg/ml hygromycin B.

Flow cytometry

ASGPR expression was analyzed using polyclonal anti-H1 or anti-H2 rabbit sera specific for the C-terminal extracellular part of the ASGPR-H1 or -H2 chain (kindly provided by M. Spiess, Biozentrum, University of Basel, Switzerland). Their usage for flow cytometry was tested against the human hepatoma cell line HepG2 that expresses both ASGPR chains on its surface [5]. The cells were stained with these sera, diluted 1:100 in FACS buffer [phosphate buffered saline (PBS, Gibco/BRL), 0.5% (w/v) bovine serum albumin (BSA, Sigma), 0.1% (w/v) NaN_3 (Serva, Germany)], followed by a DTAF-labeled anti-rabbit IgG(H + L) $F(ab')_2$ fragment (Sigma). Unspecific rabbit normal serum (Gibco/BRL) was used as negative control. A saturating dilution of hybridoma supernatant of the mAb A78-G/A7 [21] (kindly provided by U. Karsten, Max-Delbrück-Center, Berlin, Germany), followed by an incubation with a FITC-labeled goat anti-mouse IgG(H + L) $F(ab')_2$ fragment (Dianova, Germany) was used to examine the expression of TF antigen on the tumor cell lines. A comparable concentration of the irrelevant murine IgM mAb M-3273 (MOPC10 A) was used as isotype control. Furthermore, FITC-labeled lectin peanut agglutinin (PNA) (Vector Laboratories, Calif.) was used, diluted 1:100 in FACS buffer. The negative control was blocked with 100 mM (w/v) lactose (Sigma). All antibody or lectin incubations were performed for 15 min at 4 °C. A final concentration of 1 μg/ml propidiumiodid (PI, Sigma) was added to all samples to exclude dead cells from the analysis before measurement using a FACScan (Becton Dickinson, Germany).

Cell sorting

The human AML cell line KG1 contains two subpopulations, which differ in their expression of the TF antigen [21]. A sample of 10^7 KG1 cells was incubated with the TF-specific mAb A78-G/A7, followed by incubation with a MicroBead-labeled goat anti-mouse IgM antibody. The cell sorting was performed using the VarioMACS apparatus and BS columns according to the manufacturer's instructions. MACS was also used to increase the population of ASGPR-transfected YT cells expressing both receptor chains. ASGPR-transfected YT cells were stained with anti-H2 rabbit serum, followed by a MicroBead-labeled anti-rabbit IgG(H + L) antibody, and enriched using MS^+ columns and a MiniMACS separator (all from Miltenyi Biotec, Germany). Sterile degassed MACS buffer, consisting of PBS supplemented with 0.5% (w/v) BSA and 2 mM EDTA (Serva), was used for the staining and sorting procedure.

Flow cytometric binding assay

An aliquot of 10^7 ASGPR- or mock-transfected YT cells was labeled with 2 ml 2 μM PKH26 (Sigma), a red fluorescent membrane-inserting dye, for 5 min at RT, as described by the manufacturer. The tumor cell lines Raji, Jurkat, SW480 and the TF^+ and TF^- KG1 sublines were labeled with the green fluo-

rescent membrane-linking dye D275 (3,3′-dioctadecycloxacarbocyanine perchlorate; Molecular Probes). An aliquot of 10^7 target cells was incubated with 10 μg/ml D275 in 2 ml RPMI-1640 supplemented with 1% (v/v) FCS for 30 min at 37 °C. The labeled cells were washed 4 times with RPMI-1640 containing 10% (v/v) FCS. For each sample, 2.5×10^5 effector cells and 2.5×10^5 target cells were resuspended in PBS supplemented with 2 mM $CaCl_2$ (Sigma), 0.5% (w/v) BSA, 0.1% (w/v) NaN_3, and 1 μg/ml PI were transferred into 0.6 ml tubes (Greiner, Germany). All samples were performed as duplicates. The tubes were vortexed and incubated for at least 30 min at RT. The binding assay was analyzed using a FACScan (Becton Dickinson). Before measurement, the tubes were strongly vortexed for 3 s. The percentage of the green (FL1-H) and red (FL2-H) double fluorescent population, referring to total counts (debris and dead cells excluded), was evaluated. For blocking experiments a final concentration of 100 mM D-galactose (Sigma) or 5 mM EDTA (Serva) was added to the samples.

Cytotoxicity assay

The cytotoxicity was examined with standard 4 h ^{51}Cr-release assays. Briefly, target cell lines Raji, Jurkat, SW480, KG1-TF$^+$ and KG1-TF$^-$ were labeled using [^{51}Cr]-sodium chromate (NEN, Germany). The labeled target cells were washed at least thrice with RPMI-1640 supplemented with 10% (v/v) FCS. ASGPR-transfected YT cells and the mock-transfected controls were used as effector cells in this assay. A combination of 1.5×10^5 effector cells and 3×10^3 [^{51}Cr]-labelled target cells per well were transferred into U-bottom 96-well plates (TPP, Switzerland), in Iscove's modified Dulbecco's Medium (IMDM, Gibco/BRL) supplemented with 10% (v/v) FCS, corresponding to an effector to target (E:T) ratio of 50:1. Further E:T ratios were performed by half-dilution steps of the effector cells. Spontaneous and maximum [^{51}Cr] release were determined by incubating target cells in medium or in 1% (v/v) Triton-X100 (Sigma), respectively. All samples were performed in triplicate. After 4-h incubation at 37 °C and 5% CO_2, 50 μl of supernatant from each well was transferred to a 96-well LumaPlate (Packard Bioscience, Germany). Dried and sealed plates were measured in the β-scintillation counter TopCount (Packard). The percent lysis was calculated as follows:

$$\text{lysis [\%]} = \frac{\text{lysis [cpm]} - \text{spontaneous lysis [cpm]}}{\text{100\% lysis [cpm]} - \text{spontaneous lysis [cpm]}} \times 100\%$$

Results

Expression of ASGPR

Both ASGPR chains could be detected on the surface of HepG2 cells by flow cytometry, using polyclonal rabbit anti-H1 and -H2 sera compared to the unspecific rabbit normal serum (Fig. 1A, B). The H1 chain was detected on up to 80% of the YT cells after electroporation with pREP8-H1 and selection with L-histidinol (Fig. 1C), whereas no surface expression of H2 was found on pREP4-H2 transfected YT cells after selection with hygromycin B (Fig. 1D) compared to mock (pREP8 or pREP4) transfected controls. After transfection with the plasmid containing the second ASGPR chain, independent of the order in which both plasmids were transfected, 10–40% of the YT cells expressed both ASGPR chains on their cell surface. ASGPR-transfected YT cells expressing both receptor chains were enriched by cell sorting against the H2 chain. After enrichment, 92% of the cells expressed the H1 chain, as determined by flow cytometry analysis using anti-H1 serum (Fig. 1E); 86% of the cells expressed the H2 chain, as determined using anti-H2 serum (Fig. 1F).

Expression of terminal β1-Gal residues on tumor cell lines

The human ASGPR recognizes terminal β1-Gal-bearing carbohydrates. We used the TF-specific mAb A78-G/A7 and the lectin PNA to determine the expression of carbohydrates including ligands of the ASGPR on several tumor cell lines (Fig. 2). The parental KG1 cell line was sorted into two sublines with a different TF expression using A78-G/A7. The TF$^-$ phenotype of the KG1-TF cells was not stable during long-term culture and repeated sorting was necessary. The cell lines Raji, Jurkat, SW480 and both KG1 sublines expressed carbohydrate ligands recognized by PNA. The expression of the PNA ligands was higher on the cell lines Raji and KG1-TF$^+$ compared to Jurkat, SW480 and the TF$^-$ KG1 subline. The TF antigen was strongly expressed on Raji, SW480 and KG1-TF$^+$ cells. Jurkat cells expressed only a little amount of the TF antigen. Although Raji showed a similar pattern of TF antigen expression to KG1, it could not be sorted into two sublines with distinct TF$^+$ and TF$^-$ phenotypes.

Binding assay

A flow cytometric binding assay was used to analyze tumor cell binding by ASGPR-transfected YT cells (Fig. 3). In the presence of 2 mM $CaCl_2$ ASGPR-transfected YT cells bound significantly to Jurkat, SW480 and KG1-TF$^+$. The TF$^-$ subline of KG1 was bound 4 times less than TF$^+$ KG1 cells by ASGPR-transfected YT cells. Mock-transfected YT cells did not bind to Jurkat, SW480, TF$^+$ and TF$^-$ KG1 cells. The tumor cell binding of ASGPR-transfected YT cells was inhibited completely by the addition of 5 mM EDTA or 100 mM D-galactose, respectively.

Cytotoxicity

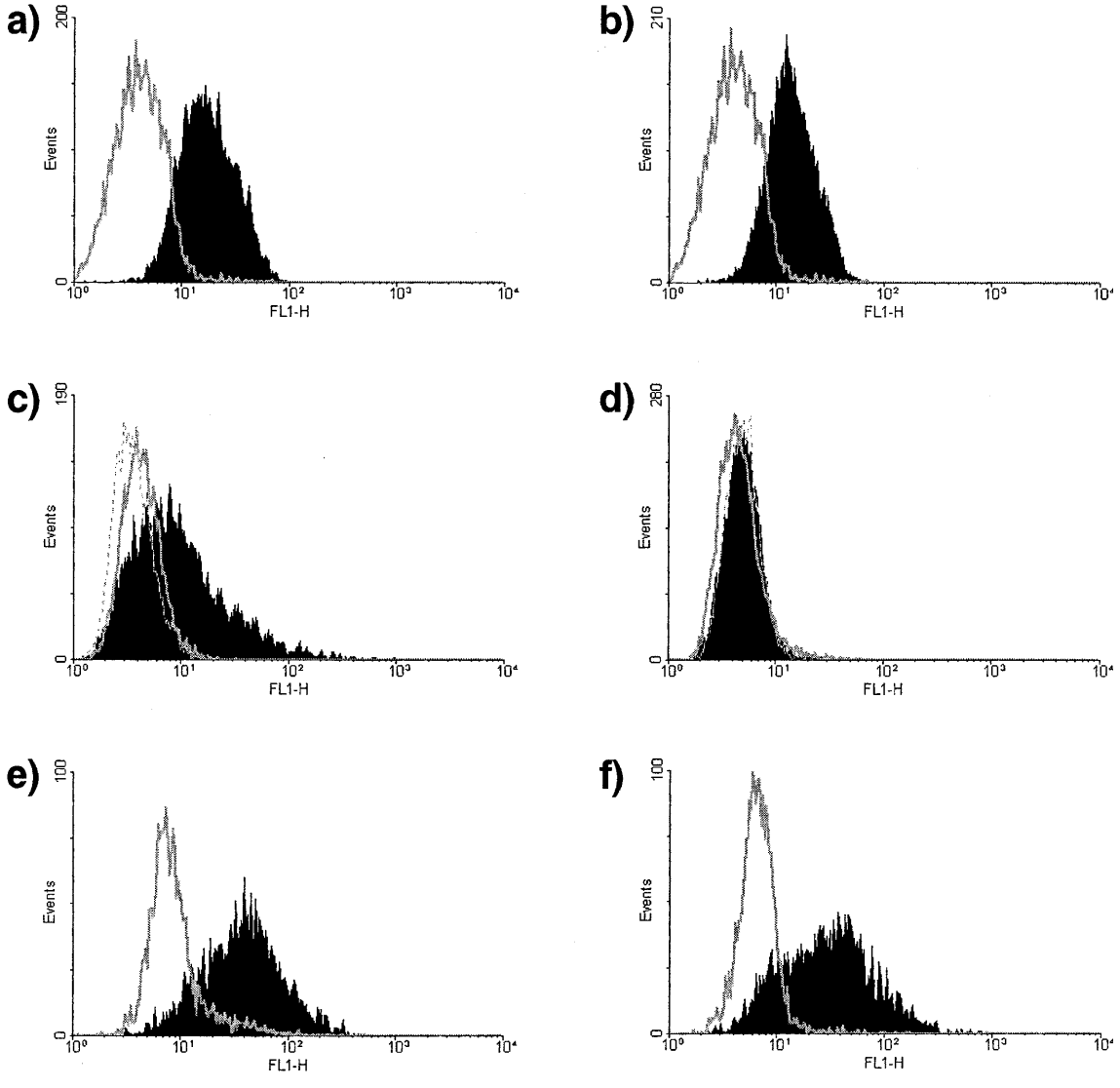
The lysis of the cell lines Raji and Jurkat by ASGPR-transfected YT cells was 10% higher compared to mock-transfected YT cells (Fig. 4A, B). ASGPR- and mock-transfected YT cells did not lyse the human colon carcinoma cell line SW480 (Fig. 4C). Lysis of the AML line KG1 (Fig. 3D) was achieved only with ASGPR-transfected YT against the TF$^+$ KG1 subline, whereas the TF$^-$ KG1 subline was killed neither by ASGPR-transfected nor by mock transfected YT (Fig. 4D).

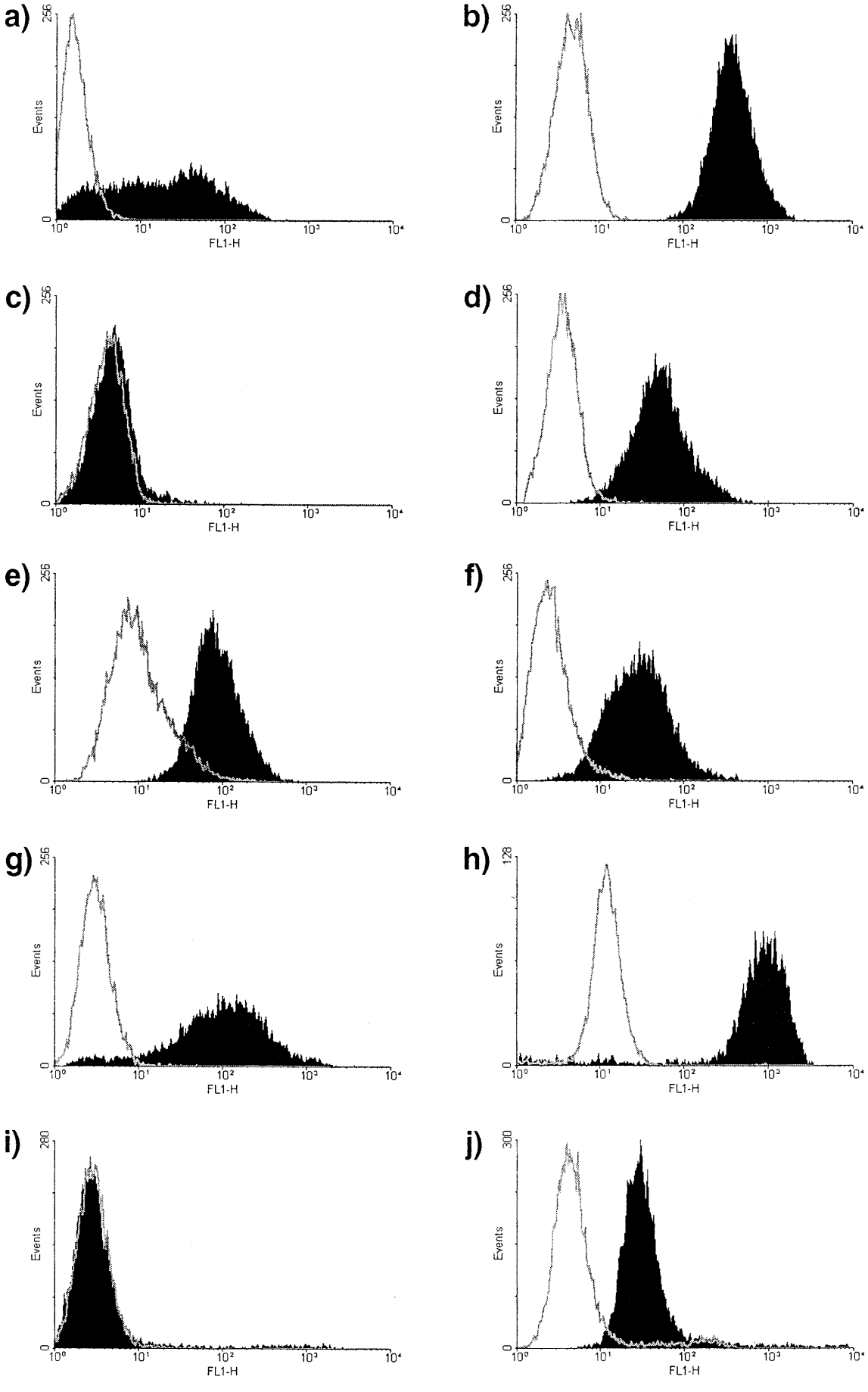
Discussion

Adoptive immunotherapy depends on the availability of sufficient numbers of immunological effector cells with specificity for tumor cells. Moreover, the individual transfection of tumor patients' cells might be problematic for practical application in large clinical trials. Therefore, it would be helpful to employ an established effector cell line that has unlimited availability and could be genetically modified just once for any purpose. In this study, we modified the human NK cell line YT by ASGPR gene transfer to obtain these properties. This cytokine-independent growing effector cell line was successfully transfected with both ASGPR chains, H1 and H2, by electroporation, without the need of viral vectors. Both ASGPR chains were expressed on the surface of YT cells after transfection. The H1 chain was able to reach the cell surface of YT cells independently of the H2 chain, whereas the surface expression of H2 was dependent on the presence of H1, as previously shown in ASGPR-transfected NIH3T3 fibroblasts [15, 34]. The expression of ASGPR ligands on different

Fig. 1A–F FACS analysis of ASGPR expression. The human hepatoma cell line HepG2 was stained with the polyclonal rabbit anti-H1 serum (**A**) and anti-H2 serum (**B**) (*filled*). Unspecific rabbit normal serum was used as negative control (*solid gray line*). YT cells transfected with pREP8-H1 (**C**) were stained with polyclonal rabbit anti-H1 serum (*filled*) and pREP4-H2 transfected YT cells (**D**) were stained with rabbit anti-H2 serum (*filled*). As controls, mock-transfected YT cells were stained analogously (*solid gray line*) and ASGPR-transfected YT cells were stained with rabbit antiserum against the other ASGPR chain (*dotted line*). YT cells transfected with both ASGPR chains and enriched by cell sorting against the H2 chain were analyzed using rabbit anti-H1 serum (**E**) and anti-H2 serum (**F**) (*filled*). Mock-transfected YT cells are the controls (*solid gray line*)

Fig. 2 FACS analysis of the expression of β1-Gal residues using the TF-specific mAb A78-G/A7 (**A**, **C**, **E**, **G**, **I**) and PNA (**B**, **D**, **F**, **H**, **J**). Expression of β1-Gal residues on tumor cell lines Raji (**A**, **B**), Jurkat (**C**, **D**), SW480 (**E**, **F**) and the TF^+ KG1 subline (**G**, **H**) and TF^- KG1 subline (**I**, **J**). Isotype mAb (**A**, **C**, **E**, **G**, **I**) or PNA blocked with lactose (**B**, **D**, **F**, **H**, **J**) were used as controls (*solid gray line*)

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tumor cell lines was analyzed using the mAb A78-G/A7 and the lectin PNA, both recognizing Galβ1-3GalNAc-residues [3, 7, 21]. A soluble ASGPR is only described for the H2 chain (H2a) [39, 45]. However, a single subunit of the ASGPR would not be useful for FACS analysis due to very low affinity for its ligands ($< 10^{-3}$ M) [26, 34]. Only the multimeric transmembrane receptor complex binds with a high affinity to ligands bearing multiple terminal β1-Gal residues (10^{-7}–10^{-9} M) [26, 34].

After ASGPR gene transfer, YT cells bound significantly stronger to tumor cells expressing the TF antigen. This binding was blocked by the addition of EDTA or galactose, emphasizing a mediation by this galactose-specific C-type lectin. The enhanced binding by ASGPR-transfected YT cells led to an increase of cytotoxicity if the tumor cell lines were lysed already by the parental or mock-transfected YT cell line to a certain level. The ASGPR did not induce cytotoxicity against tumor cell lines that were not lysed by the parental YT cells at all, probably, due to a missing signal chain. A modification of the cytoplasmatic part of one or both ASGPR chains by the signal domain of activatory NK cell receptors that are members of the C-type lectin family, like NKG2C [17] and NKG2D [44], might overcome this problem. Further, it should be considered whether YT

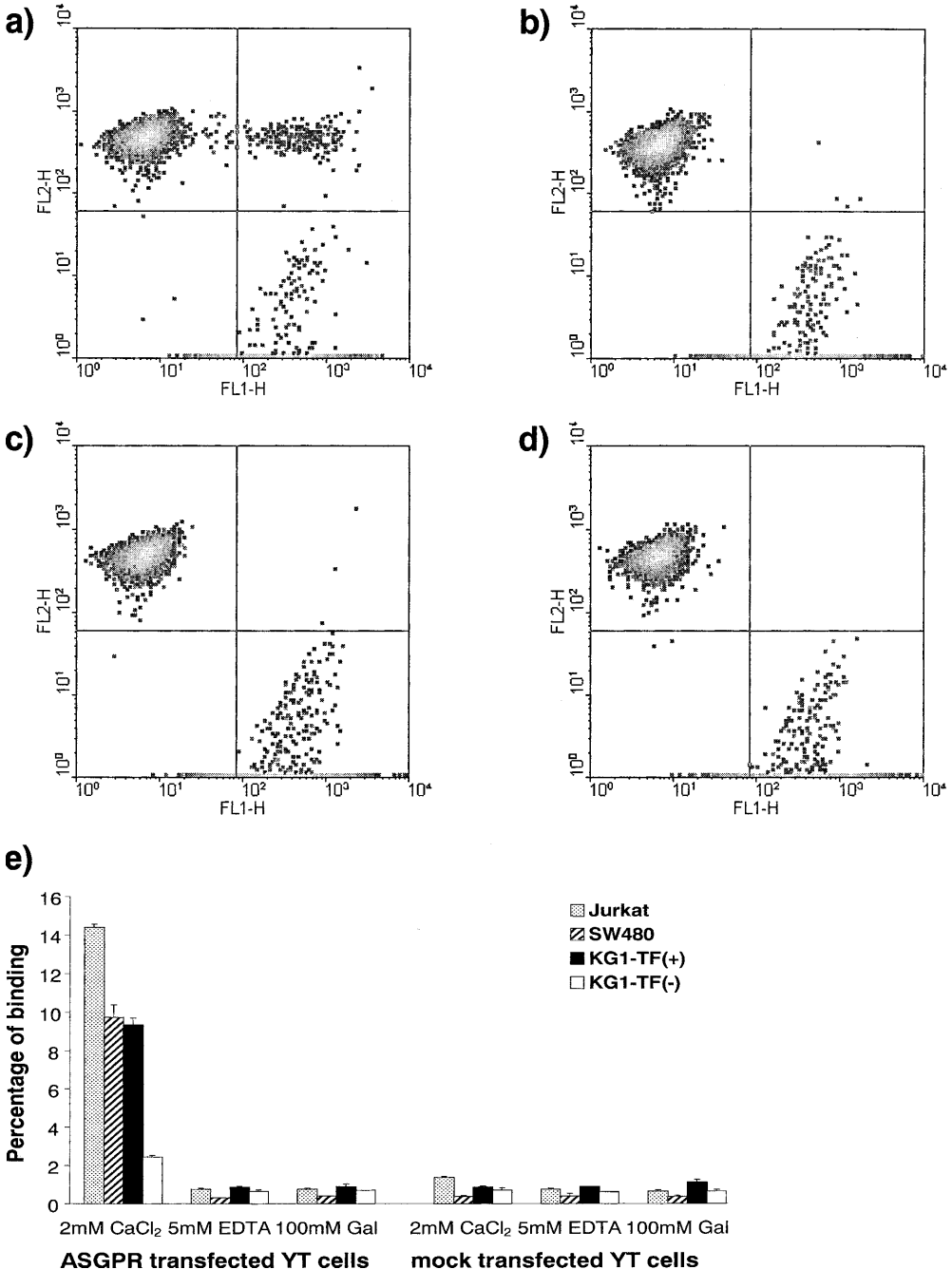

Fig. 3A–E Flow cytometric binding assays. **A** Binding between D275-labeled (*FL1-H*) TF^+ KG1 cells and PKH26-labeled (*FL2-H*) ASGPR transfected YT cells. **B** Binding between D275-labeled TF^+ KG1 cells and PKH26-labeled mock transfected YT cells. **C** Binding between D275-labeled FL1-H) TF^+ KG1 cells and PKH26-labeled ASGPR-transfected YT cells blocked with EDTA, or **D** blocked with galactose. The FL1-H/FL2-H double fluorescent population (*right upper quarter*) represents the tumor cells bound to ASGPR- or mock-transfected YT cells, respectively. **E** Percentages of the binding population are shown in the *vertical bar chart* representing data from one of two similar experiments

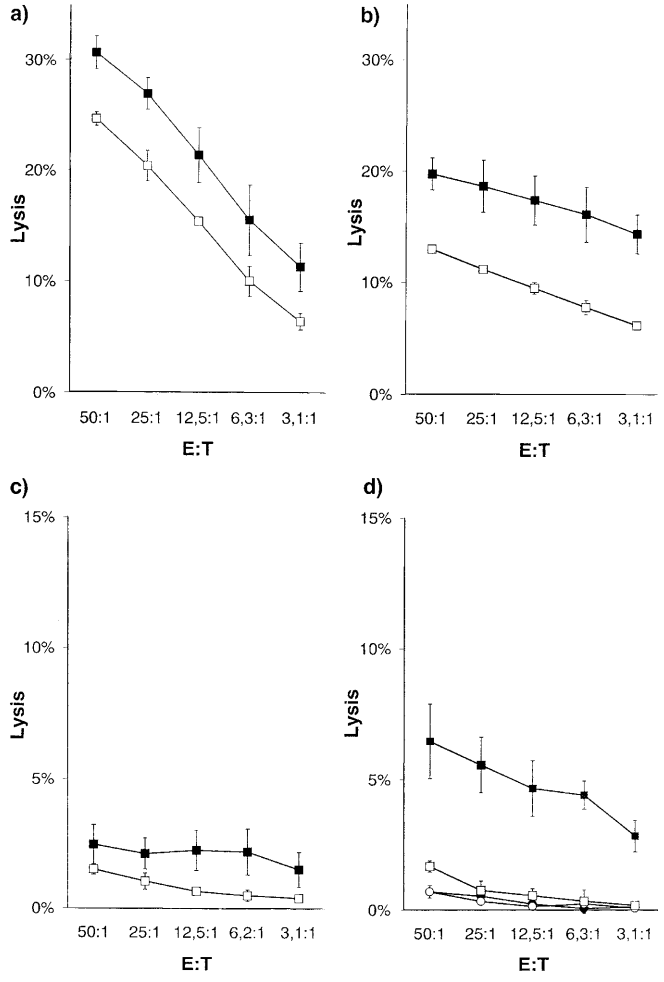

Fig. 4A–D Cytotoxicity assays. YT cells transfected with both ASGPR chains (*closed squares*) and mock-transfected YT (*open squares*) were used as effector cells against the tumor target cell lines Raji (**A**), Jurkat (**B**), SW480 (**C**), or the TF^+ KG1 subline (**D**). **D** also shows the lysis of TF^- KG1 cells by ASGPR-transfected YT cells (*closed circles*) and mock-transfected YT cells (*open circles*). The figure depicts data from one of two similar experiments

used in an allogeneic system gives an advantage compared to the use of autologous strategies.

Taken together, YT cells can be targeted by ASGPR transfer to tumor cells expressing the TF antigen or other ASGPR ligands on their surface. The binding to tumor cells was increased and their lysis could be improved. A human NK cell line, modified by receptor gene transfer, which is cytokine-independent for growth, tumor-specific and available in unlimited amounts, could be an interesting tool for adoptive cancer immunotherapy.

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