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ORIGINAL ARTICLE

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Gene therapy with cytokine-transfected xenogenic cells (Vero-IL-2) in patients with metastatic solid tumors: mechanism(s) of elimination of the transgene-carrying cells

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Abstract Eleven patients with advanced cancer were treated in a clinical gene therapy trial by repeated intratumoral injections with different doses of xenogenic fibroblasts secreting high amounts of human interleukin-2 (Vero-IL2). Treatments in a total of 14 courses were well tolerated and resulted in clinical responses and measurable biological effects. Together with increases in serum interleukin-2 (IL-2), modifications of the V-β T cell receptor repertoire and induction of intratumoral T-cell infiltration were observed. When the intratumoral expression of endogenous cytokine genes and the persistence of the IL-2 transgene at the application site and in peripheral blood were investigated, rapid disappearance of the transgene at the application site appeared to be the most prominent biological effect. Tests detecting a single Vero-IL2 cell against a background of 10^5 non-transfected cells were not able to demonstrate significant expression of exogenous IL-2 (i.e. the transgene or transgene-carrying cells) in tumor biopsies or blood at different times. Therefore, further studies were performed to evaluate the mechanism(s) involved in the rapid disappearance of xenogenic carrier cells in more detail. We show here that significant in vitro cytotoxicity against transgene-carrying Vero cells can be observed in peripheral blood of all the patients before treatment as well as in healthy controls. "Cold" target inhibition shows that significant killing of Vero-IL2 cells is mediated by natural killer (NK) cells. This was confirmed by showing that established $CD3^-/CD16^+/CD56^+$ peripheral blood NK cell clones kill both K562 and Vero-IL2 target cells. The failure of other mechanisms (complement, antibody-dependent cell cytotoxicity or cytotoxic T lymphocytes) to destroy xenogenic, histoincompatible Vero cells in vitro suggests that NK cells also might be responsible for the killing of Vero-IL2 in vivo and for the failure to detect the transgene at the application site. These results might also be of importance for some aspects of the current discussion of xenotransplantation.

Key words Gene therapy · Cytokine · NK · Xenogenic

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Introduction

The notion that cytokines such as interferons and interleukins can enhance immunogenicity and induce responses to experimental as well as spontaneous tumors in animals has led to increasing investigational efforts and later their clinical use [30, 36, 43]. Clinical trials were started with systemic applications of interferons and interleukin-2 (IL-2) [3, 13]. Systemic high-dose IL-2 applications resulted in durable tumor responses but were associated with substantial systemic toxicity (e.g. capillary leak syndrome) [30]. Numerous studies have subsequently shown that weakly immunogenic tumor cells can be altered to become targets for specific immune rejection by transfection with cytokine genes [1, 2, 4–7, 9, 14–16, 25, 26, 38, 40, 41, 45]. Alternatively, it has been demonstrated that cytokines can also be protective against tumor growth if produced by normal autologous fibroblasts or by cells allo- or xenogenic with respect to the tumor and the host animal [11, 12, 29, 31–36, 43]. On the basis of compelling experimental results in animal models [31, 35, 36] we recently performed a clinical gene therapy study in patients with advanced solid tumors,

using xenogenic fibroblasts secreting human IL-2 (Vero-IL2) [32]. Repeated intratumoral treatment (three times per week) of 11 patients with advanced cancer (a total of 14 treatment courses were performed) with different doses (5×10^5, 5×10^6, or 5×10^7) of xenogenic Vero-IL2 was well tolerated. Toxicity consisted of transient fever in 1 patient and mild itching and erythema in 2 others [32]. One patient with soft-tissue sarcoma showed a more than 90% and more than 50% reduction of the volume of two distant, non-injected metastases, lasting for 34+ and 26 months respectively. In 4 other patients, including a melanoma patient who developed marked vitiligo, the disease was stabilized for 3–9 months (Table 1). Together with increases of serum IL-2, modifications of the V-β T cell receptor repertoire and induction of intratumoral T cell infiltration were observed [32]. However, since recombinant IL-2 (rIL-2) is of human origin, enzyme-linked immunosorbent assays (ELISA) did not allow us to discriminate between exogenous (produced by Vero-IL2) and therapy-induced endogenous IL-2 values in the patients' sera [32]. Investigation of the intratumoral expression of endogenous cytokine genes and determining the persistence of the IL-2 transgene at the application site and in peripheral blood showed that rapid disappearance of the transgene/transgene-carrying cells at the application site (and in blood) was the most prominent biological effect. Therefore, we studied in more detail the mechanism(s) underlying this rapid disappearance.

Materials and methods

Dose, preparation, and administration of Vero-IL2

Green monkey kidney fibroblast Vero cells (ATCC CCL 81) were transfected with pTG5324 human IL-2 expression vector [31]. Clinical Vero-IL2 stock was prepared (Transgène, Strasbourg, France), and 3.6×10^7 cells in 1 ml freezing medium (Dulbecco's modified Eagle medium, DMEM; 2 mM L-glutamine; 1% nonessential amino acids; 0.1% human serum albumin; 5% dimethylsulfoxide, Transgène, Strasbourg, France) were stored in Cryotubes in liquid nitrogen [31]. Cells were thawed at 37 °C in a water bath, transferred to ice, and diluted with vehicle medium (Transgène, Strasbourg, France) to final nominal concentrations of 5×10^5, 5×10^6 or 5×10^7 cells in 1.0 ml or 1.4 ml for the different dose levels. The exact number of viable cells was determined by Trypan blue dye exclusion, counting the cells before administration to the patients. Patients were hospitalized for at least 5 days and received three injections of 5×10^5 (dose level 1), 5×10^6 (dose level 2), or 5×10^7 (dose level 3) of Vero-IL2 cells on days 1, 3 and 5 respectively, under ultrasound or computed tomography guidance. IL-2 production by Vero-IL2 from each batch was determined in supernatants (diluted 1:100–1:200) of approximately 9×10^6 cells cultured in 15 ml DMEM medium (Gibco BRL, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; Seromed, Biochrom KG, Berlin, Germany) in 75-cm^3 tissue-culture flasks (Falcon 3024; Becton Dickinson, Meylan Cedex, France) for 4–24 h by a commercial IL-2 ELISA kit (R&D Systems Europe Ltd, Abingdon, UK). The amount of IL-2 produced by 1×10^6 cells in 24 h was corrected to the exact number of viable cells. The median amount of IL-2 produced by 10^6 Vero-IL2 cells within 24 h was 229 ng/ml (range 106–515 ng/ml). For cytotoxicity assays (see below) Vero-IL2 cells were routinely passaged in DMEM medium/10% FCS.

Table 1 Clinical characteristics of patients. *MR* mixed response, *SD* stable disease, *PD* progressive disease, *NA* not applicable, *CT* computed tomography, *Dose* number of Vero-IL2 cells injected, *m* male, *f* female, *surg* surgery, *chemo* chemotherapy (number of different protocols), *immun* immunotherapy, *horm* hormonal treatment, *radio* radiotherapy

Patient	Age (years)	Sex	Dose (cells)	Tumor	Pretreatment (no. protocols)	Route of injection/biopsy (injected metastasis)	Response	Follow-up status (time/months)
ML	66	M	5×10^5	Leiomyosarcoma	Surg, chemo (2), immun	Direct/ultrasound (subcutaneous, elbow)	MR	MR, 12; alive, 24
WR	53	M	5×10^5	Renal cell carcinoma	Surg, chemo (2), immun, horm	Direct/ultrasound (subcutaneous, back)	SD	SD, 3; died, 7
GK	52	F	5×10^5	Colon carcinoma	Surg, chemo (3)	Ultrasound/ultrasound (liver)	PD	Died, 5
MB	64	F	5×10^6	Melanoma	Surg, immun, chemo (3)	CT/CT (retroperitoneum)	SD[c]	SD, 9; progression
JS	65	F	5×10^6	Renal cell carcinoma	Surg, chemo (2)	CT/CT (retroperitoneum)	SD	SD, 7; died, 8
MG	66	M	5×10^6	Bladder carcinoma	Surg, chemo (2), immun	Ultrasound/ultrasound (liver)	SD	SD, 9; alive, 24
HRS	54	M	5×10^7	Carcinoma of salivary gland	Surg, chemo (2)	Direct/ultrasound (subcutaneous, neck)	PD	Died, 5
ED	20	F	5×10^7	Synovial sarcoma	Surg, chemo, immun, radio	CT/CT (intrathoracial)	PD	Died, 4
CK	56	F	5×10^7	Leiomyosarcoma	Surg, chemo	CT/CT (retroperitoneum)	NA	Died, 1
GW	43	F	5×10^6	Melanoma	Surg	Ultrasound	PD	Died, 5
AF	62	M	5×10^6	Chondroma of sacrum	Surg, chemo, radio (2)	Ultrasound	PD	Died, 4
(MB)[a]	64	F	5×10^6	Melanoma	See above	See above	PD	Alive, 9; died, 25
(ML)[a]	66	M	5×10^6	Leiomyosarcoma	See above, radio	See above	SD	Alive, 29
(ML)[b]	66	M	5×10^6	Leiomyosarcoma	See above	See above	PD	Alive, 34

[a] Patients MB progressed after 9 month, was treated with a second cycle of Vero-IL2 cells and died after massive further progression (25)
[b] Patient ML showed further progression (24), was treated with a second cycle of Vero-IL2 cells and a third cycle of treatment was added after developing small lung metastases (29)
[c] Patient showed marked vitiligo (*Rochlitz et al.* [32])

Cell lines, blood cells, and tumor biopsies

K562 erythroleukemia (ATCC CCL-243), Daudi Burkitt's lymphoma (ATCC CRL-7917), AsPC1 pancreatic carcinoma (ATCC CRL-1682), and mouse P815 mastocytoma cells (kindly provided by C. Roth, Paris, France) were routinely passaged in RPMI-1640 medium (Gibco BRL, Basel, Switzerland) supplemented with 10% FCS at 37 °C in a humified atmosphere of 5% CO_2. Peripheral blood mononuclear leukocytes (PBL) were obtained from the blood of tumor patients and healthy control persons by Ficoll/Paque (Pharmacia Biotech AB, Uppsala, Sweden) gradient centrifugation on days 1, 3, 5 and 15 of the treatment. Cells were suspended in RPMI-1640/10% FCS and counted by Trypan blue dye exclusion. Tumor biopsies obtained on days 1, 5, and 15 and each comprising about 1×10^7 PBL were snap-frozen in Eppendorf tubes on solid CO_2 and stored at −70 °C. Natural killer (NK) cell clones GDK 4, 31, 55, 59, and 64 were kindly provided by G. De Libero (Experimental Immunology, Department of Research, Kantonsspital Basel, Switzerland). Individual clones were established and cloned from peripheral blood leukocytes after fluorescence-activated cell sorting (FACS) of $CD16^+$ lymphocytes and propagated as described [8]. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) using the Lysis II software for data processing as recently described [20]. To characterize NK cell clones the following monoclonal antibodies (mAb) were used: CD3 (OKT-3), CD16 (BL-LGL/1, Sigma-Aldrich Chemie, Buchs, Switzerland), and CD56 (B159, Pharmingen, San Diego, Calif., USA). Binding specificity was determined using IgG1 (MOPC-21) and IgG2a (UPC-10) isotype control mAb (Sigma-Aldrich Chemie, Buchs, Switzerland).

Preparation of RNA and cDNA from clinical samples

RNA was prepared according to the RNAzol method of Chomczynski [10] from snap-frozen tumor biopsies and PBL, using a commercial RNA extraction kit (Socochim, Lausanne, Switzerland). Single-strand cDNA was synthesized from 1–5 µg total RNA by the Superscript RNase H/reverse transcriptase kit (Gibco BRL, Basel, Switzerland).

Vector detection and endogenous cytokines in tumor specimens and peripheral blood

To determine persistance of Vero-IL2 or of the plasmid vector pTG5324, used to transfect Vero cells with human IL-2 [31], in the tumor patients, specific reverse transcription/polymerase chain reaction (RT-PCR) amplification of the pTG5324-IL2 construct was performed in a final volume of 100 µl containing 10 mmol TRIS/HCl (pH 8.1), 1.5 mmol $MgCl_2$, 50 mmol KCl, 0.1 µg gelatin, 2.5 mmol each dNTP, 50 pmol primers IL–2 TGP5′: TAC CGC ATG CAA CTC CTG TCT TGT, and IL-2 TGP3′: GCC AGA AGT CAG ATG CTC AAG, and 2.5 U AmpliTaq Gold polymerase (Perkin-Elmer, Rotkreuz, Switzerland) together with defined amounts of cDNA prepared from the tumor biopsies and PBL. The primers were designed to include plasmid sequences to avoid amplification of endogenous IL-2. The amplification was performed in a Perkin-Elmer Cetus DNA thermal cycler (catalog no. N801-0150) and the following cycle profiles: 94 °C 10 min, 62 °C 40 s, and 72 °C 1 min for 1 cycle and 94 °C 40 s, 62 °C 40 s, and 72 °C 1 min for 34 cycles. For control of the pTG5324-IL-2 construct, detection cDNA preparations from different numbers (5×10^6 to 5×10) of Vero-IL2 cells against a background of non-transfected cells (K562) was used. Intratumoral expression of endogenous cytokines [IL-2, IL-4, IL-6, IL-10, tumor necrosis factor α (TNFα), and interferon γ (IFNγ)] was analyzed by quantitative RT-PCR, as recently described [32], using cDNA samples prepared from the biopsies. The following primer pairs [27] were used: β-actin (ACT-S2: 5′-GGG AAA TCG TGC GTG ACA TT-3′, ACT-AS2: 5′-GGA GTT GAA GGT AGT TTC GTG-3′), IL-2 (IL-2RNA5: 5′-ATG TAC AGG ATG CAA CTC CTG TCT T-3′, IL-2RNA3: 5′-GTC AGT GTT GAG ATG ATG CTT TGA C-3′), IL-4 (IL-4RNA5: 5′-ATG GGT CTC ACC TCC CAA CTG CT-3′, IL-4RNA3: 5′-CGA ACA CTT TGA ATA TTT CTC TCT CAT-3′), IL-6 (IL-6RNA5: 5′-ATG AAC TCC TTC TCC ACA AGC GC-3′, IL-6RNA3: 5′-GAA GAG CCC TCA GGC TGG ACT G-3′), IL-10 (IL-10RNA5: 5′-AAG GCA TGC ACA GCT CAG CAC T-3′, IL-10RNA3: 5′-TCC TAG AGT CTA TAG AGT CGC CA-3′), TNFα (TFNARNA5: 5′-ATG AGC ACT GAA AGC ATG ATC CGG-3′, TNFARNA3: 5′-GCA ATG ATC CCA AAG TAG ACC TGC CC-3′), and IFNγ (IFNGRNA5: 5′-ATG AAA TAT ACA AGT TAT ATC TTG GCT TT-3′, IFNGRNA3: 5′-GAT GCT CTT GCA CCT CGA AAC AGC AT-3′) (Pharmacia Biotech AB, Uppsala, Sweden). cDNA preparations from phytohemagglutinin(PHA)-stimulated PBL from healthy donors were used as a control for interleukin gene expression. RT-PCR samples were analyzed by HPLC [42] using a DEAE-NPR column (no. N930-2656, Perkin-Elmer, Rotkreuz, Switzerland). Statistical analysis was performed by the Wilcoxon signed-rank test.

Cytotoxicity assay and "cold"-target inhibition

Killer cell activities of PBL from tumor patients and healthy controls against K562 (NK), Daudi (lymphokine-activated killing, LAK), AsPC1 (antibody-dependent cell cytotoxicity, ADCC) and Vero-IL-2 cells were estimated by the ^{51}Cr-release (Amersham, Little Chalfont, UK) technique [21]. ADCC was performed with a polyclonal anti-(carcinoembryonic antigen) (anti-CEA; CD66) antibody (Dako Diagnostics AG, Zug, Switzerland). CD66 expression on AsPC1 cells has been determined by FACScan analysis as described earlier [17, 20]. ADCC activity against xenogenic Vero-IL2 cells was tested by adding 50 µl heat-inactivated, autologous serum to the tests. Samples containing 5000 ^{51}Cr-labelled target cells in 100 µl RPMI-1640/10% FCS were incubated in the presence of 50, 10, 2 or 0.4 times their number of effector cells at 37 °C for 4 h. Aliquots of the radioactive supernatants were then measured in a γ-counter and cytotoxicity was calculated by the formula:

$$\text{Specific release (\%)} = \frac{^{51}\text{Cr(experimental)} - {}^{51}\text{Cr(spontaneous)}}{^{51}\text{Cr(maximal)} - {}^{51}\text{Cr(spontaneous)}} \times 100$$

where the spontaneous ^{51}Cr release was determined without adding effector cells and maximal release was obtained by detergent lysis of target cells. Cold-target inhibition was performed by adding a 100, 50, or 25 times excess of unlabelled ("cold") inhibitor target cells to each well and comparing ^{51}Cr release in the presence and absence of inhibitors. As a control, unrelated mouse cells (P815) were added in the same amount to each well, giving similar results to those obtained without inhibitor cells. Inhibition was calculated according to the formula:

$$\text{Inhibition (\%)} = \left[1 - \left(\frac{\text{inhibitor}}{\text{control}}\right)\right] \times 100$$

Statistical analysis was performed by a two-sided, paired *t*-test.

Results

Intratumoral expression of endogenous cytokines

Tumor biopsies of the injection sites were performed and snap-frozen on days 1 and 5, before injection of Vero-IL2 cells, and on day 15. Expression of endogenous cytokine genes (IL-2, IL-4, IL-6, IL-10, TNFα, and IFNγ) was assayed by RT-PCR/HPLC analysis. Despite the wide variability of individual intratumoral cytokine

expression, an obvious but non-significant ($P > 0.1$) enhancement of IL-2, IL-10, and TNFα expression was observed in tumor biopsies from separate patients (Fig. 1, dotted lines) as well as in the mean of all patients (Fig. 1, solid line) on day 5 as compared to biopsies obtained before (day 1) or 1 week after (day 15) treatment. Expression of IL-6 and IFNγ remained nearly unchanged or tended to decrease during therapy ($P > 0.3$). In contrast, expression of endogenous IL4 could not be detected at all in the tumor biopsies but was easily found in control cDNA from PHA-stimulated PBL (data not shown).

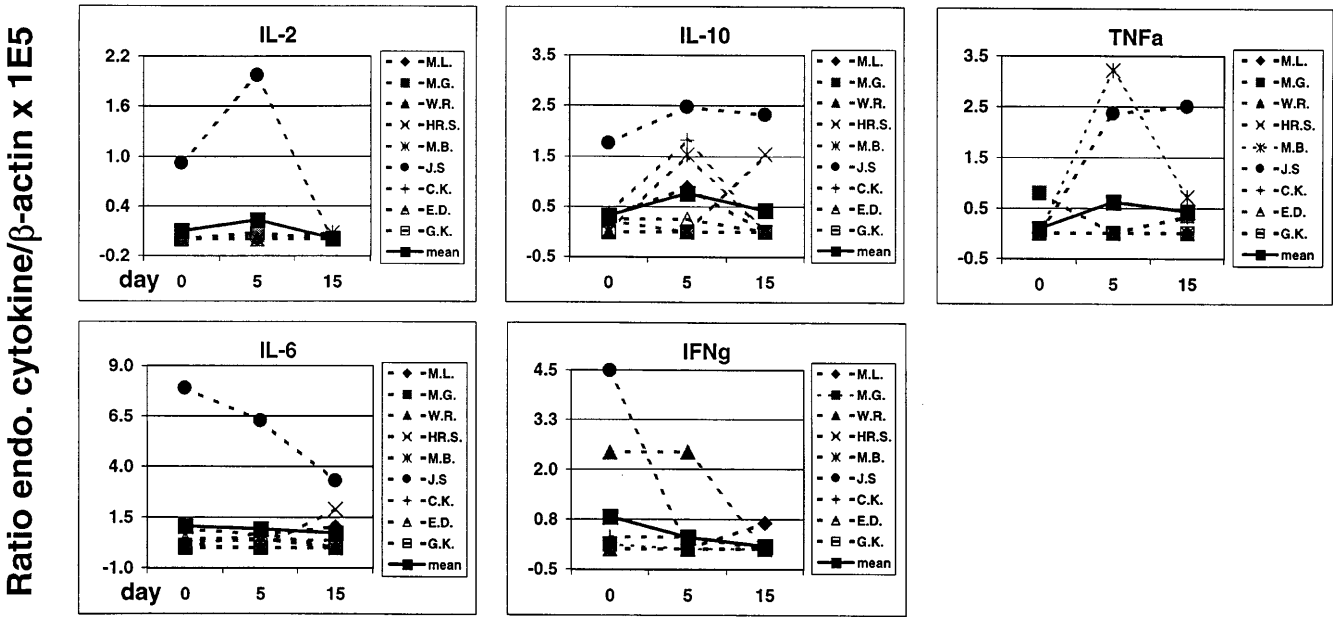
Determination of exogenous IL-2 transgene expression

Using another set of IL-2 primer pairs including plasmid sequences, to avoid amplification of endogenous IL-2, we tried to detect exogenous IL-2 transgene expression in blood and tumor biopsies obtained at different times after Vero-IL2 application. In this case RT-PCR/HPLC analysis failed to detect any expression of the transgene (pTG5324) used to transfect Vero cells with human IL-2, even in those patients who had received the highest cell dosage (5×10^7) intratumorally (data not shown). To verify the sensitivity of transgene detection we mixed various numbers of Vero-IL2 cells ($0–5 \times 10^6$) into a background of 5×10^6 unrelated cells and determined the ratios of exogenous IL-2/β-actin. As shown in Fig. 2, the presence of a single Vero-IL2 cell could be easily demonstrated against a background of 10^5 non-transfected cells.

Killer cell activity in patients and healthy controls

In order to investigate mechanism(s) possibly involved in the disappearance of transgene-carrying xenogenic cells we analyzed different killer cell activities. Beside NK (K652) and LAK (Daudi) killing, we investigated a possible induction of killer cells against Vero-IL2 cells during gene therapy (Fig. 3). NK cell activity of heavily pretreated patients was found to be within the normal range (E:T = 50:1; 12%–67% specific release) and comparable to that of healthy controls (3%–56%). LAK killing was observed only in 2 patients (22% and 10%) and 1 control donor (12%). Unexpectedly, significant cytotoxicity against transgene-carrying Vero-IL2 cells could be observed in the peripheral blood of untreated patients (1 representative patient is shown in Fig. 3) as well as that of healthy controls (not shown). Furthermore, the local therapy [Vero-IL2 producing approx. 229 ng IL-2 $(10^6 \text{ cells})^{-1}$ ml^{-1} in 24 h] did not significantly modify peripheral NK, and LAK, or Vero-IL2 killing (Fig. 3), even in those patients who had received the highest Vero-IL2 dose (5×10^7 cells) intratumorally.

Fig. 1 Detection of endogenous cytokines in tumor biopsy samples. Intratumoral expression of endogenous cytokine genes during Vero-IL2 gene therapy was determined by the RT-PCR/HPLC method as described [42]. Values of endogenous cytokines were standardized against the housekeeping gene for β-actin. Data are shown as individual (– – –) and mean values (——) of the cytokine/β-actin ratio from all patients. Expression of interleukin-2 (IL-2), IL-10, and tumor necrosis factor α (*TNFα*) was increased on day 5 (not significant $P > 0.1$), whereas IL-6, and interferon γ (*IFNγ*) expression was not altered significantly. IL-4 expression (not shown) was not detected at all in tumor biopsies. cDNA preparations from phytohemagglutinin-stimulated peripheral blood lymphocytes (PBL) from healthy donors were used as positive controls. Statistical analysis was performed by the Wilcoxon signed-rank test (*P* values for cytokines day 1/day 5 are IL-2 = 0.500, IL-10 = 0.062, TNFα = 0.187, IL-6 = 0.312, and IFNγ = 0.312. IL-4 not done)



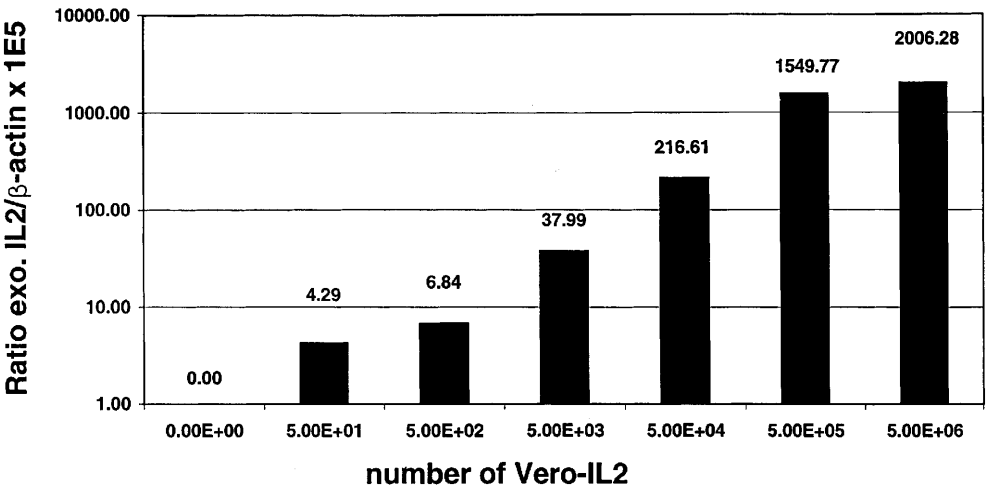


Fig. 2 Detection sensitivity of exogenous IL-2 in cell samples. Expression of the plasmid vector pTG5324, used to transfect Vero cells with human IL-2, was determined by the RT-PCR/HPLC method as described [42]. Values of exogenous IL-2 were standardized against the housekeeping gene β-actin. Sensitivity was determined by mixing Vero-IL2 (0–5×10^6) with non-transfected K562 cells (5×10^6–0). Expression of pTG5324 from at least one Vero-IL2 cell has been detected against a background of 10^5 unrelated cells

Killer cell activity after systemic IL-2 administration

To determine whether the killing of Vero-IL2 cells might also be influenced by IL-2 administration, we compared it to NK, and LAK cytotoxicity after systemic high-dose IL-2 administration in 2 patients suffering from renal cell carcinoma. In contrast to local Vero-IL2 therapy, i.v. application of systemic IL-2 (6×10^6 U) rapidly enhanced both NK cytotoxicity and the killing of Vero-IL-2 cells and induced significant LAK cytotoxicity in a time-dependent (8 h > 4 h > 0 h) manner (Fig. 4). In addition, 24 h in vitro cultivation of PBL from patients or healthy donors with supernatants collected from Vero-IL2 cultures (approx. 2 μg IL-2/ml) similarly enhanced NK, LAK and anti-Vero-IL2 cytotoxicity. Alternatively, in vitro cocultivation of PBL with 1×10^6 mitomycin-treated Vero-IL2 cells for 24 h or 48 h also resulted in a time-dependent stimulation of various killer cells [21] (and data not shown).

Characterization of Vero-IL2 killer cells

The cells responsible for killing Vero-IL2 killer cells were characterized in more detail by three types of experiment. To exclude the possibility that natural antibodies in patients' or healthy donors' sera were responsible for Vero-IL2 killing, we tested autologous sera and PBL in complement-dependent and ADCC assays respectively. Incubating Vero-IL2 cells with fresh autologous plasma did not result in complement-dependent lysis of the cells (data not shown). Addition of heat-inactivated autologous serum to PBL and ^{51}Cr-labelled Vero-IL2 cells did not induce significant antibody-dependent cytotoxicity (effector:target ratio = 50:1). By contrast, PBL from patients and healthy controls (E:T = 50:1; 10:1) displayed significant ADCC killing (as a positive control) of anti-CEA-antibody-coated AsPC1 tumor cells (data not shown).

Both NK and Vero-killing cells exist constitutively in the blood of untreated patients or healthy donors. To investigate their relationship, we compared their "target specificity" by cold-target inhibition assays in 7 healthy donors. As shown in Fig. 5 (left-hand diagrams), a dose-dependent inhibition of NK activity or the killing of Vero cells was induced by adding unlabelled K562 or Vero-IL2 cells (and vice versa) in 100-, 50-, or 25-fold excess to 5×10^3 ^{51}Cr-labelled K562 and Vero-IL2 cells respectively (E:T = 50:1). This significant inhibition (Fig. 5, right-hand diagrams) was not seen when no cells (50 μl RPMI-1640 medium) or a 50-fold excess of unlabelled, irrelevant (P815 mastocytoma) cells were added (Fig. 5, right-hand diagrams). In two experiments, addition of unlabelled Daudi cells (LAK target) inhibited neither NK activity nor Vero cell killing significantly (data not shown).

In a further step we investigated killer cell activities of five established, $CD3^-/CD16^+/CD56^+$ (Fig. 6A, flow cytometry histogram) NK cell clones on different target cells. As shown in Fig. 6B (upper diagram) all five NK cell clones exhibited a very similar, dose-dependent (E:T = 0.2:1–30:1) killing of K562 cells. Three of these NK cell clones killed both K562 and Vero-IL2 cells to a similar extent whereas two other clones only displayed marginal killing of Vero-IL2 cells (Fig. 6B: lower diagram).

Discussion

Preclinical studies in various mouse tumor models have established that administration into syngeneic hosts of tumor cells engineered to secrete IL-2 can stimulate both specific and non-specific antitumor immune responses [5–7, 14–16, 25, 26]. These experiments inspired a series of clinical studies using IL-2-transfected autologous or allogenic tumor cells in patients with different malignancies [37].

The encouraging results of our controlled adjuvant trial, treating dogs and cats with spontaneous sarcomas and melanomas using IL-2 transfected xenogenic Vero fibroblasts [31], led to the implementation of a clinical

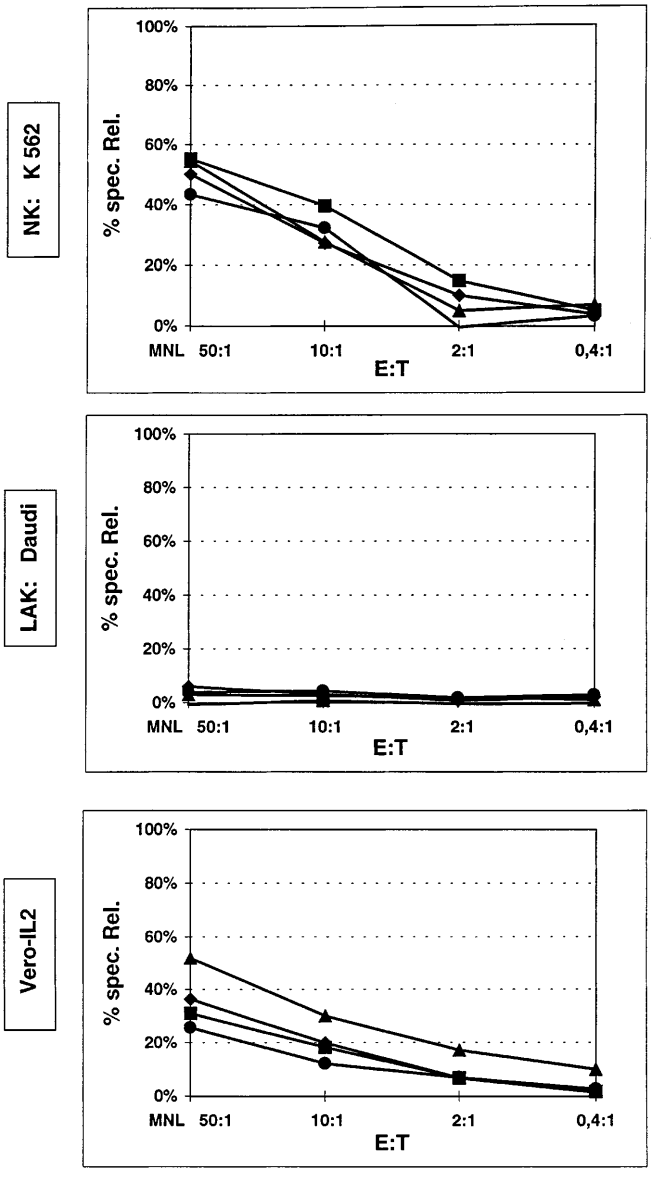


Fig. 3 Profile of natural killer (*NK*) and lymphocyte-activated killer (*LAK*) cytotoxicity, and killing of Vero cells during Vero-IL2 gene therapy. Data show NK and LAK-activity and killing of Vero cells on day 1 (◆, pretreatment), day 3 (■), day 5 (▲), and day 15 (●) of intratumoral Vero-IL2 gene therapy of 1 (1/11) representative patient at different effector:target ratios (50:1, 10:1, 2:1, and 0.4:1). Two patients (and 1 control) also displayed LAK cytotoxicity (data not shown). The local therapy [approx. 229 ng IL-2 (10^6 cells)$^{-1}$ ml^{-1} in 24 h] did not change the different peripheral killer cell activities significantly. PBL from healthy control donors (9) showed similar cytotoxic activities against K562, Daudi and Vero-IL2 cells as did patients' cells (data not shown)

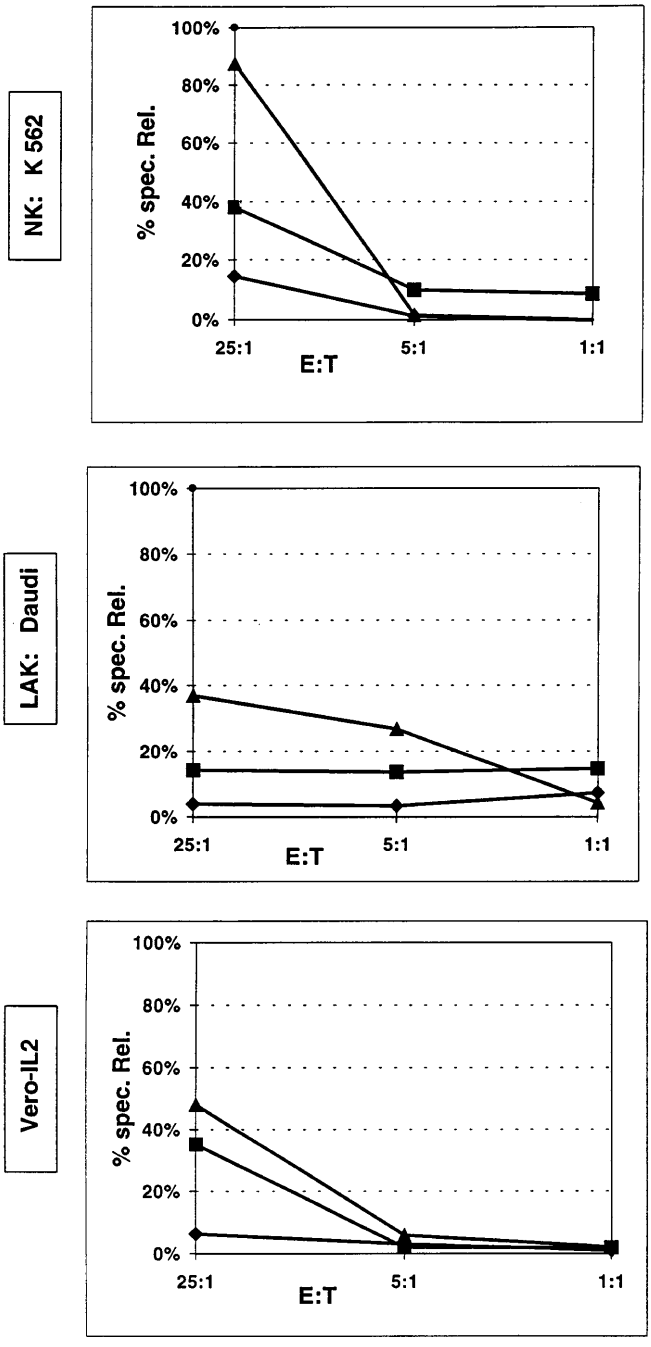


Fig. 4 Enhancement of NK and LAK cytotoxicity and killing of Vero cells during systemic IL-2 therapy. Peripheral blood NK activity, and killing of Vero cells were rapidly enhanced, and LAK activity was rapidly induced in 2 renal carcinoma patients within 4 h (■), and 8 h (▲) compared to killing before (◆) systemic administration of high-dose IL-2 (6×10^6 U i.v.). Data show killer cell activities of 1 patient at different effector:target ratios (25:1, 5:1, and 1:1). Killer cells of the 2 patients showed similar enhancement but started from different baseline values

phase I gene therapy trial in patients with advanced solid tumors. Our study demonstrated that intratumoral application of Vero-IL2 cells in 11 patients (14 treatment courses) with advanced solid malignancies was safe, accompanied by little toxicity and showed signs of biological and clinical activity [32].

Observations of intratumoral T cell infiltration and significant but transient enhancement of serum IL-2 [32] have led us to investigate the induction of endogenous cytokine secretion and expression of the exogenous IL-2-transgene after administration of the xenogenic transfectants. A weak (not significant, $P > 0.1$) intratumoral induction of endogenous IL-2, IL-10, and

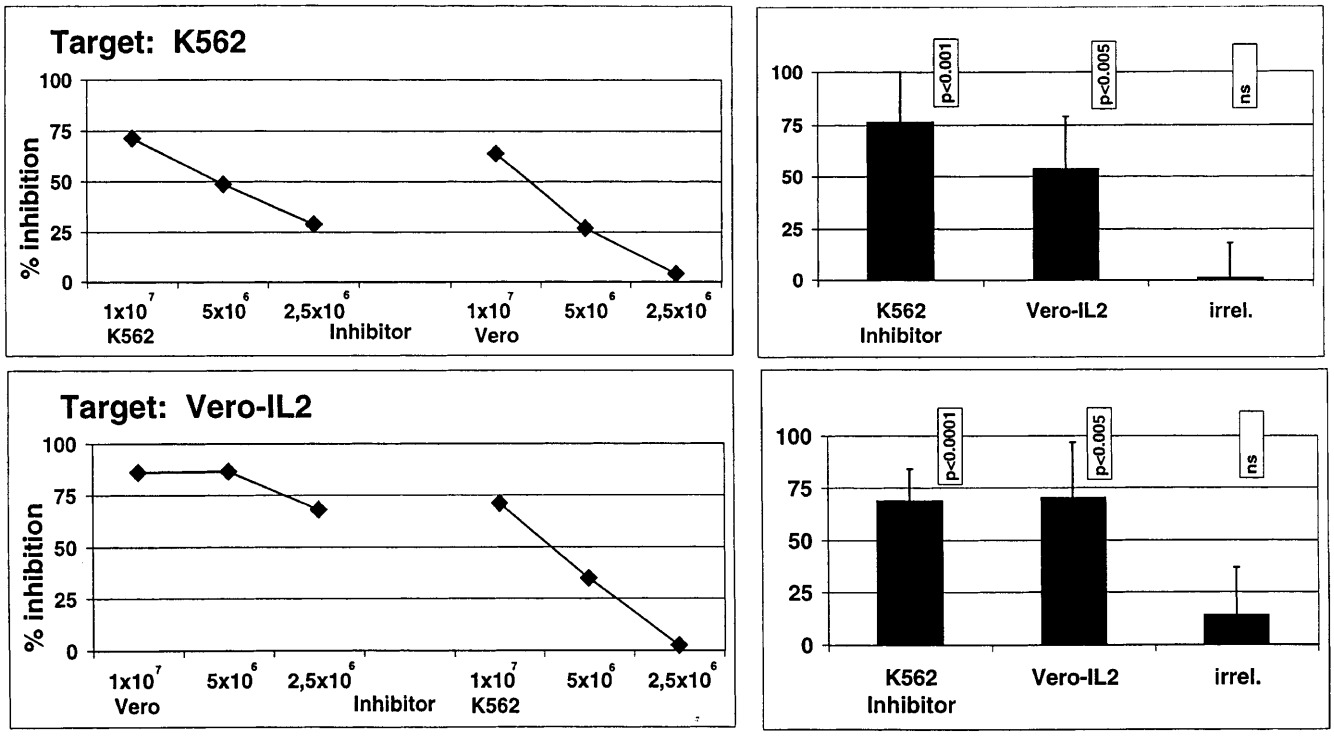


Fig. 5 "Cold"-target inhibition analysis of NK activity and the killing of Vero cells. Inhibition of peripheral blood NK activity and Vero cell killing was compared in 7 healthy donors. *Left* dose-dependent inhibition of NK activity (*upper*) or killing of Vero cells (*lower*) for 1 representative donor (E:T = 50:1) follow in addition of unlabelled K562 or Vero-IL2 (and vice versa) in 100-, 50-, or 25-fold excess to 1×10^5/ml ^{51}Cr-labelled K562 and Vero-IL2 cells respectively. *Right* mean values and standard deviation of inhibition of NK activity (*upper*) or Vero cell killing (*lower*) for 7 healthy donors following addition of no cells (50 µl RPMI medium) or a 50-fold excess of unlabelled K562 ($P < 0.001$ and < 0.005), Vero-IL2 ($P < 0.0001$ and < 0.005) and irrelevant (P815 mastocytoma) cells ($P > 0.5$ and > 0.1), respectively, as inhibitors (*ns* not significant). Statistical analysis was performed by a two-sided, paired *t*-test

TNFα (but not of IL-6 or IFNγ) gene expression was observed in the patients but did not correlate to the Vero-IL2 dosage (Fig. 1, Table 1). In contrast to endogenous cytokines, we could not detect the IL-2 transgene in the tumor biopsies at different times. The rapid disappearance of the transgene/transgene-carrying cells at the application site confirms our findings in cats and dogs where the transgene could be detected in biopsies by RT-PCR for 1 h after injection and then rapidly disappeared [31]. The more sensitive RT-PCR/HPLC detection ($< 1/10^5$ cells) of the exogenous IL-2 transgene used here (Fig. 2), however, should allow Vero-IL2 cells to be detected, at least when applied at the highest dosage (5×10^7 cells). The failure of our test indicates that the cells were rapidly and completely removed from the application site. The fact that we could not detect Vero cells or the transgene in the blood excludes a simple "dilution" or "wash away" from the tumor tissue, and indicates that the rapid disappearance rather results from cell destruction.

We have shown here that spontaneous killing of Vero-IL2 by NK cells is a probable mechanism that can lead to destruction of the cells and, therefore, might be responsible for the rapid disappearance of the transgene at the application site in tumor patients. We did not examine whether Vero cells themselves or the IL-2 transfectants are the targets of spontaneous killing but it is arguable that in vitro killing results from recognition of the monkey fibroblasts irrespective of the cytokine gene transfection. The low amounts of IL-2 produced by transfected target cells (5000/well) are not able to induce a relevant lymphokine-activated cytotoxicity in untreated blood leukocytes within 4 h [24] and the killing of Vero-IL2 could not be inhibited by LAK (Daudi) target cells. The killing of Vero cells (by LAK cells) can be enhanced by a 24-h cocultivation with Vero-IL2 (1×10^6/well) in vitro [21] (and data not shown) or by high-dose IL-2 in vivo (Fig. 4); however, this was not the case in the patients (Fig. 3).

There is no direct evidence that NK cells also destroy the Vero-IL2 cells in vivo but the idea is supported by our immunohistological finding of a weak but significant NK cell infiltration already detected in most of the untreated tumor biopsies [32]. Furthermore, an additional induction of anti-xenogenic CTL [18] in our patients can be excluded since anti-Vero-IL2 cytotoxicity was not significantly enhanced during the intratumoral cytokine therapy (Fig. 3). Anti-xenogenic antibody-dependent cellular cytotoxicity [23, 44] or complement-dependent lysis also do not appear to be involved in Vero-IL2 killing (not shown), although at least 2 of the patients developed ELISA-detectable titers of antibodies against Vero-IL2 [32]. Our data confirm that NK cell cytotox-

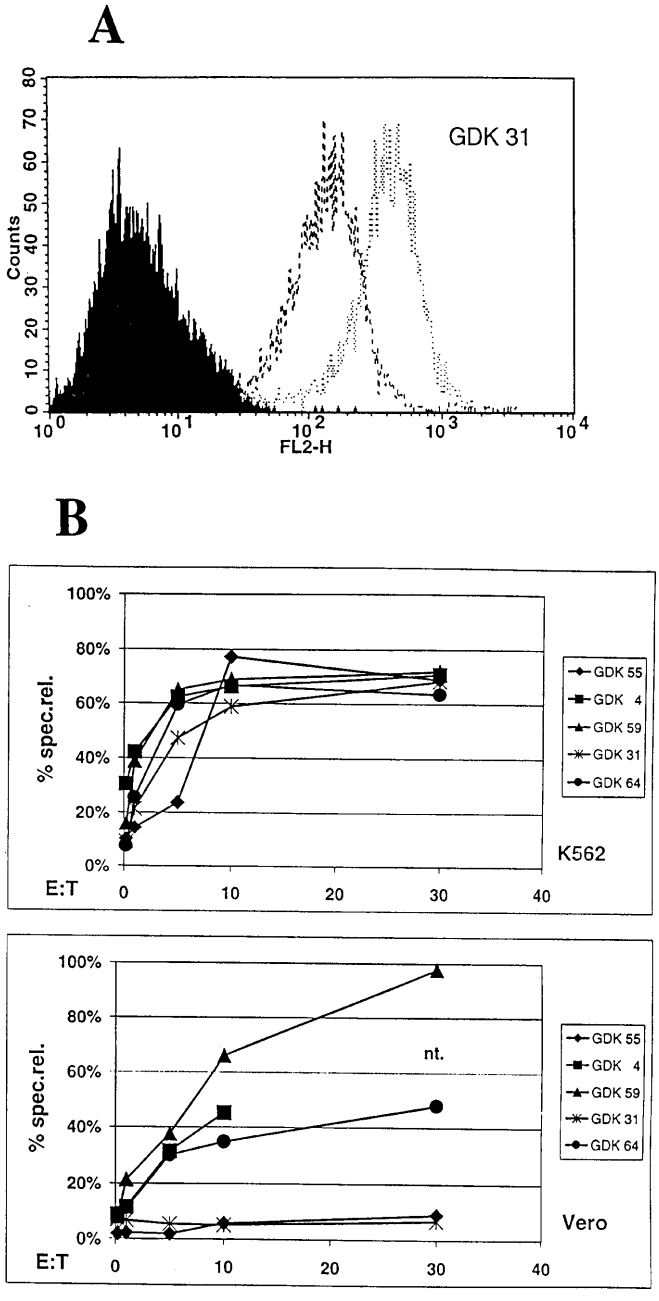


Fig. 6 NK cytotoxicity and killing of Vero cells by established $CD3^-/CD16^+/CD56^+$ NK cell clones. **A** Expression of NK cell markers CD16 and CD56 was determined by flow cytometry using monoclonal antibodies BL-LGL/1 (CD16 ···, Sigma-Aldrich Chemie, Buchs, Switzerland), and B159 (CD56 – – –, Pharmingen, San Diego, Calif., USA) and compared to that of isotype control(s) (*solid histogram*). A representative histogram is shown for one clone (GDK31). NK cell clones did not express CD3 (OKT3) (data not shown). **B** Killing of K562 (*upper diagram*) and Vero-IL2 (*lower diagram*) cells was determined by ^{51}Cr-release assay at different effector:target cell ratios (30:1, 10:1, 5:1, 1:1, and 0.2:1). All five NK cell clones (GDK55, 31, 4, 59, and 64) killed K562 in a dose-dependent manner. Three of these clones (GDK 59, 4, and 64) showed a comparable killing of Vero-IL2 whereas two clones displayed only marginal lysis of these cells

icity [39] might be one of the mechanisms leading to rapid (acute) destruction/rejection of xenogenic primate cell grafts. This mechanism clearly differs from the hyperacute rejection of non-primate xenografts mainly caused by natural antibodies to (α1,3)-galactosyl epitopes, which are not expressed on primate cells or tissues [28].

Despite the rapid intratumoral disappearance of the IL-2 transgene, clear tumor responses have been observed in mice, cats, dogs and tumor patients [31, 32]. In the animal tumor models, this was not a simple additive effect of recombinant human IL-2 (hIL-2) and xenogenic Vero cells, since the separate application of either rIL-2 or unmodified Vero cells did not result in a tumor response [31]. It is of note that the transient production of IL-2 by Vero cells persisted for a much longer period than when recombinant hIL-2 protein was injected (half-life: 12.9 min) [22]. In cats suffering from fibrosarcomas, significant rIL-2 serum levels could be detected for at least 48 h [31]. Since rIL-2 is of human origin, we can not decide whether enhanced serum IL-2 levels in our tumor patients' sera on day 5 of therapy [32] result from exogenous (produced by Vero-IL2) or therapy-induced endogenous IL-2. This prolonged persistance might be an important factor in the antitumor effects of Vero-IL2 therapy [31], although the way in which Vero-IL2 is acting and the kind of effector cells that are activitated in animals or tumor patients remain to be clarified [31, 32].

In conclusion, the antitumor response induced by xenogenic Vero-IL2 cells in experimental tumor models and patients does not seem to be limited by the killing of carrier cells and rapid transgene eradication. This fact tends to enhance the safety of the therapeutic procedure since the rapid elimination of histoincompatible carrier cells leads to only a transient expression of the cytokine, thereby causing fewer side-effects than would a more prolonged in vivo synthesis/persistance of cytokines [30]. Furthermore, it might reduce the potential biological hazard associated with the administration of genetically modified cells. In addition, manipulation of histoincompatible carrier cells permits the possibility of using a single "universal" therapeutic agent that can easily be produced in homogeneous batches under conditions of good manufacturing practice [31]. The safety, feasibility and therapeutic potential of the xenogenic gene transfer strategy justify further evaluation of intratumoral treatment with Vero-IL2. Our results might also be of importance for some aspects of the current discussion of xenotransplantation.

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References

1. Allione A, Consalvo M, Nanni P, Lollini PL, Cavallo F, Giovarelli M, Forni M, Gulino A, Colombo MP, Dellabona P, *et al* (1994) Immunizing and curative potential of replicating and nonreplicating murine mammary adenocarcinoma cells engineered with interleukin (IL)-2, IL-4, IL-6, IL-7, IL-10, tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factor, and gamma-interferon gene or admixed with conventional adjuvants. Cancer Res 54:6022
2. Belli F, Arienti F, Sule-Suso J, Clemente C, Mascheroni L, Cattelan A, Santantonio C, Gallino GF, Melani C, Rao S, Colombo MP, Maio M, Cascinelli N, Parmiani G, Sanatonio C (1997) Active immunization of metastatic melanoma patients with interleukin-2-transduced allogeneic melanoma cells: evaluation of efficacy and tolerability. (Published erratum appears in Cancer Immunol Immunother 1997 45:119). Cancer Immunol Immunother 44:197
3. Bindon C, Czerniecki M, Ruell P, Edwards A, McCarthy WH, Harris R, Hersey P (1983) Clearance rates and systemic effects of intravenously administered interleukin 2 (IL-2) containing preparations in human subjects. Br J Cancer 47:123
4. Bronte V, Tsung K, Rao JB, Chen PW, Wang M, Rosenberg SA, Restifo NP (1995) IL-2 enhances the function of recombinant poxvirus-based vaccines in the treatment of established pulmonary metastases. J Immunol 154:5282
5. Bubenik J (1989) Local immunotherapy of cancer with interleukin 2 (see Comments). Immunol Lett 21:267
6. Bubenik J, Voitenok NN, Kieler J, Prassolov VS, Chumakov PM, Bubenikova D, Simova J, Jandlova T (1988) Local administration of cells containing an inserted IL-2 gene and producing IL-2 inhibits growth of human tumours in nu/nu mice. Immunol Lett 19:279
7. Bubenik J, Simova J, Jandlova T (1990) Immunotherapy of cancer using local administration of lymphoid cells transformed by IL-2 cDNA and constitutively producing IL-2. Immunol Lett 23:287
8. Carena I, Shamshiev A, Donda A, Colonna M, Libero GD (1997) Major histocompatibility complex class I molecules modulate activation threshold and early signaling of T cell antigen receptor-gamma/delta stimulated by nonpeptidic ligands. J Exp Med 186:1769
9. Cayeux S, Richter G, Becker C, Beck C, Aicher A, Pezzutto A, Dorken B, Blankenstein T (1997) Lack of correlation between rejection of tumor cells co-expressing interleukin-2 and B7.1 and vaccine efficiency. Eur J Immunol 27:1657
10. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156
11. Cohen R, Cabaniols JP, Levraud JP, Pannetier C, Rochlitz CF, Roth C, Kourilsky P (1995) Approaches to anti-tumor immunotherapy in mouse and humans. In: Gachelin G, Ikawa Y, Ishii S, Kourilsky P, Yaniv M (eds) Genes for development, cell growth and infectious diseases. John Libbey Eurotext, Paris, p 227
12. Cressent M, Pidoux E, Cohen R, Modigliani E, Roth C (1995) Interleukin-2 and interleukin-4 display potent antitumour activity on rat medullary thyroid carcinoma cells. Eur J Cancer 31A:2379
13. deKernion JB, Sarna G, Figlin R, Lindner A, Smith RB (1983) The treatment of renal cell carcinoma with human leukocyte alpha-interferon. J Urol 130:1063
14. Fearon ER, Pardoll DM, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Vogelstein B, Frost P (1990) Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell 60:397
15. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, Gilboa E (1990) Retroviral vector-mediated gamma-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. Cancer Res 50:7820
16. Gansbacher B, Zier K, Daniels B, Cronin K, Bannerji R, Gilboa E (1990) Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. J Exp Med 172:1217
17. Grunert F, Daniel S, Nagel G, von Kleist S, Jantscheff P (1995) CD66b, CD66c and carcinoembryonic antigen (CEA) are independently regulated markers in sera of tumor patients. Int J Cancer 63:349
18. Hagihara M, Shimura T, Takebe K, Munkhbat B, Sato T, Tsuchida F, Sato K, Tsuji K (1996) Xenogeneic iso-skin graft and mixed lymphocyte reaction studies using HLA-DP transgenic mice. Transpl Immunol 4:220
19. Jantscheff P, Winkler L, Karawajew L, Kaiser G, Bottger V, Micheel B (1993) Hybrid hybridomas producing bispecific antibodies to CEA and peroxidase isolated by a combination of HAT medium selection and fluorescence activated cell sorting. J Immunol Methods 163:91
20. Jantscheff P, Nagel G, Thompson J, Kleist SV, Embleton MJ, Price MR, Grunert F (1996) A CD66a-specific, activation-dependent epitope detected by recombinant human single chain fragments (scFvs) on CHO transfectants and activated granulocytes. J Leukoc Biol 59:891
21. Jantscheff P, De Libero G, Bürk MR, Carena I, Grunert F, Kammerer R, von Kleist S, Herrmann R, Rochlitz CF (1997) Differential expression of CD66a (biliary glycoprotein) epitopes on activated T-cell subsets. In: (Kishimoto T, Kikutani H, Borne AEG von dem, Goyert SM, Miyasaka M, Moretta L, Okomura K, Shaw S, Springer TA, Sugamura K, Zola H (eds) Leukocyte typing. VI. White cell differentiation antigens. Garland, New York, p 115
22. Konrad MW, Hemstreet G, Hersh EM, Mansell PW, Mertelsmann R, Kolitz JE, Bradley EC (1990) Pharmacokinetics of recombinant interleukin 2 in humans. Cancer Res 50:2009
23. Kumagai-Braesch M, Satake M, Qian Y, Holgersson J, Moller E (1998) Human NK cell and ADCC reactivity against xenogeneic porcine target cells including fetal porcine islet cells. Xenotransplantation 5:132
24. Lange A, Flad HD, Ernst M, Jacak A, Ulmer AJ (1986) Effect of human recombinant interleukin 2 on natural killing of low density Percoll fraction cells. Immunol Lett 12:243
25. Ley V, Roth C, Langlade-Demoyen P, Larsson-Sciard EL, Kourilsky P (1990) A novel approach to the induction of specific cytolytic T cells in vivo. Res Immunol 141:855
26. Ley V, Langlade-Demoyen P, Kourilsky P, Larsson-Sciard EL (1991) Interleukin 2-dependent activation of tumor-specific cytotoxic T lymphocytes in vivo. Eur J Immunol 21:851
27. Luscher U, Filgueira L, Juretic A, Zuber M, Luscher NJ, Heberer M, Spagnoli GC (1994) The pattern of cytokine gene expression in freshly excised human metastatic melanoma suggests a state of reversible anergy of tumor-infiltrating lymphocytes. Int J Cancer 57:612
28. Magee JC, Platt JL (1994) Xenograft rejection–molecular mechanisms and therapeutic implications. Ther Immunol 1:45
29. Mertelsmann R, Lindemann A, Boehm T, Brennscheidt U, Franke B, Kulmburg P, Lahn M, Mackensen A, Rosenthal FM, Veelken H (1995) Pilot study for the evaluation of T-cell-mediated tumor immunotherapy by cytokine gene transfer in patients with malignant tumors. J Mol Med 73:205
30. Panelli MC, Marincola FM (1998) Immunotherapy update: from interleukin-2 to antigen-specific therapy. American Society of Clinical Oncology 34th Annual Meeting ed. In: Perry MC (ed) ASCO Educational. ASCO, Chicago p 467
31. Quintin-Colonna F, Devauchelle P, Fradelizi D, Mourot B, Faure T, Kourilsky P, Roth C, Mehtali M (1996) Gene therapy of spontaneous canine melanoma and feline fibrosarcoma by intratumoral administration of histoincompatible cells expressing human interleukin-2. Gene Ther 3:1104
32. Rochlitz CF, Jantscheff P, Bongartz G, Dietrich PY, Quinquerez AL, Schatz C, Mehtali M, Courtney M, Tartour E, Dorval T, Friedman WH, Herrmann R (1999) Gene therapy study of cytokine-transfected xenogenic cells (Vero-IL2) in patients with metastatic solid tumors. Cancer Gene Ther 6:271–281

33. Rosenthal FM, Cronin K, Bannerji R, Golde DW, Gansbacher B (1994) Augmentation of antitumor immunity by tumor cells transduced with a retroviral vector carrying the interleukin-2 and interferon-gamma cDNAs. Blood 83:1289
34. Rosenthal FM, Zier KS, Gansbacher B (1994) Human tumor vaccines and genetic engineering of tumors with cytokine and histocompatibility genes to enhance immunogenicity. Curr Opin Oncol 6:611
35. Roth C, Mir LM, Cressent M, Quintin-Colonna F, Ley V, Fradelizi D, Kourilsky P (1992) Inhibition of tumor growth by histoincompatible cells expressing interleukin-2. Int Immunol 4:1429
36. Roth C, Rochlitz C, Kourilsky P (1994) Immune response against tumors. Adv Immunol 57:281
37. Roth JA, Cristiano RJ (1997) Gene therapy for cancer: what have we done and where are we going? J Natl Cancer Inst 89:21
38. Russell SJ, Eccles SA, Flemming CL, Johnson CA, Collins MK (1991) Decreased tumorigenicity of a transplantable rat sarcoma following transfer and expression of an IL-2 cDNA. Int J Cancer 47:244
39. Saadi S, Platt JL (1998) Immunology of xenotransplantation. Life Sci 62:365
40. Stoppacciaro A, Paglia P, Lombardi L, Parmiani G, Baroni C, Colombo MP (1997) Genetic modification of a carcinoma with the IL-4 gene increases the influx of dendritic cells relative to other cytokines. Eur J Immunol 27:2375
41. Van Elsas A, Aarnoudse C, Minne CE van der, Spek CW van der, Brouwenstijn N, Osanto S, Schrier PI (1997) Transfection of IL-2 augments CTL response to human melanoma cells in vitro: immunological characterization of a melanoma vaccine. J Immunother 20:343
42. Van Hille B, Lohri A, Reuter J, Herrmann R (1995) Non-radioactive quantification of mdr1 mRNA by polymerase chain reaction amplification coupled with HPLC. Clin Chem 41:1087
43. Viret C, Lindemann A (1997) Tumor immunotherapy by vaccination with cytokine gene transfected cells. Int Rev Immunol 14:193
44. Watier H, Guillaumin JM, Vallee I, Thibault G, Gruel Y, Lebranchu Y, Bardos P (1996) Human NK cell-mediated direct and IgG-dependent cytotoxicity against xenogeneic porcine endothelial cells. Transplant Immunol 4:293
45. Zatloukal K, Schneeberger A, Berger M, Schmidt W, Koszik F, Kutil R, Cotten M, Wagner E, Buschle M, Maass G, et al. (1995) Elicitation of a systemic and protective anti-melanoma immune response by an IL-2-based vaccine. Assessment of critical cellular and molecular parameters. J Immunol 154:3406