Antitumor activity of a human cytotoxic T-cell line (TALL-104) in brain tumor xenografts¹

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Malignant glioma in adults and primitive neuroectodermal tumors/medulloblastomas in children are the most common malignant primary brain tumors that either respond poorly to current treatment or tend to recur. Adoptive therapy with TALL-104 cells—an IL-2-dependent, major histocompatibility complex nonrestricted, cytotoxic T-cell line—has demonstrated significant antitumor activity against a broad range of implanted or spontaneously arising tumors. This study investigates distribution of systemically and locally administered TALL-104 cells and their efficacy in effecting survival of a rat model of human brain tumor. In vitro, TALL-104 cells showed significant cytotoxic activity when added to human glioblastoma cell lines U-87 MG, U-251 MG, and A1690; the medulloblastoma cell lines DAOY, D283 Med, and D341 Med; and the epidermoid cancer cell line A431. In brain tumor-bearing rats, the amount of fluorescent dye-labeled TALL-104 cells in brain increased after they were given by intracarotid injection as compared with i.v. cell administration. However, TALL-104 cells rapidly decreased to low levels within 1 h after intracarotid injection. This finding suggests that TALL-104 cells given sys-

temically may not invade brain or tumor tissues, but rather may remain in the vascular system, making this approach less efficient for brain tumor treatment. In a model of athymic rats engrafted with human A431 carcinoma brain tumor, repetitive local administration of TALL-104 cells directly into the tumor bed resulted in a significant increase in survival time compared with control animals. Therefore, local therapy with TALL-104 cells may be a novel and highly effective treatment approach for malignant brain tumors. *Neuro-Oncology* 2, 103-113, 2000 (Posted to *Neuro-Oncology* [serial online], Doc. 99-51, March 22, 2000. URL <neuro-oncology.mc.duke.edu>)

Anaplastic astrocytoma and glioblastoma, the most common primary brain tumors in adults, are quite refractory to current therapies, with median survival ranging from 19 to 67 weeks (Kornblith and Walker, 1988). Local tumor recurrence also constitutes a significant problem with primitive neuroectodermal tumors/medulloblastomas, the most common childhood brain tumor. Despite 5-year survival rates that exceed 80% in some studies (Packer et al., 1991), nearly half of all patients with primitive neuroectodermal tumors/medulloblastomas will eventually die of tumor progression. Treatment failure in patients with brain tumors is a multifactorial process involving intrinsic resistance of these tumors to radiation therapy and chemotherapy, development of acquired treatment resistance, and limitations of drug delivery to the tumor site due to poor tumor vascularization and/or blood-brain barrier restrictions (Ozols et al., 1988; Phillips, 1991). Local recurrence of a brain tumor represents the most common feature of treatment failure (Silverman and Simpson, 1982). Hence, identifying new therapeutic agents or strategies with high intrinsic activity against brain tumors remains a challenge.

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³Abbreviations used are as follows: i.a., intraarterial; IL-2, interleukin-2; IMDM, Iscove's modified Dulbecco's medium.

⁴Santoli, D., and Lange, B. (1999) Personal communication.

Recent development of a novel cytotoxic T-cell therapy named TALL-104 holds promise for future management of cancer patients, especially after remission induction. TALL-104 is an IL-2³-dependent, human leukemic T-cell line (CD3⁺/TCR $\alpha\beta$ ⁺, CD8⁺, CD4⁻, CD56⁺, CD16⁻), which displays major histocompatibility complex nonrestricted, cytotoxic, and cytostatic activities against tumor cells across several species without affecting normal tissue (Cesano and Santoli, 1992a; Cesano et al., 1993, 1996a, 1996b; O'Connor et al., 1991; Visonneau et al., 1997a, 1999a). TALL-104 cells induce necrotic cell death via secretory pathways involving perforin and serine esterases and can kill tumor targets apoptotically or through release of cytostatic/cytotoxic mediators such as TNF- α , TNF- β , IFN- γ , or TGF- β (Cesano and Santoli, 1992a, 1992b; Cesano et al., 1993, 1996b, 1996c, 1998). The nature and specificity of target antigen(s) recognized by TALL-104 cells are still under investigation. Nonproliferating, irradiated TALL-104 cells display strong antitumor effects in various in vivo allogeneic and xenograft models, even in the absence of exogenously administered IL-2 (Cesano et al., 1994, 1995, 1996a, 1996b, 1998; Visonneau et al., 1997a, 1997b, 1999a, 1999b), thus avoiding adverse effects associated with IL-2 therapy (Rosenberg et al., 1988; Whittington and Faulds, 1993). In addition, TALL-104 cells can be expanded in culture indefinitely, therefore providing an unlimited source of effector cells with stable tumoricidal activity.

Adoptive immunotherapy using lethally irradiated (nonproliferative) TALL-104 cells might be an effective approach for cancer therapy, since long-term complete or partial remissions have been observed in dogs with spontaneously arising tumors that received this cell treatment (Cesano et al., 1996a; Visonneau 1997a, 1999a, 1999b). Two recently conducted phase I clinical trials in women with advanced breast cancer and in children with leukemia or solid tumors have demonstrated that irradiated TALL-104 can be repeatedly given systemically at high doses (up to 10⁸ cells/kg) without severe side effects.⁴ As postulated for lymphokine-activated killer cell therapy, the efficacy of TALL-104 cells in cancer therapy will greatly depend not only on their cytolytic potential, but also on their ability to migrate to and persist at tumor site. Recent biodistribution studies in mice and dogs have demonstrated significant homing of systemically (i.v., i.p.) administered TALL-104 cells in spleen, liver, and kidney, as well as at primary tumor sites and in metastases (Cesano et al., 1999a, 1999b; Visonneau et al., 1997b). In studies using healthy (non-tumor bearing) mice, TALL-104 cells given systemically were hardly detectable in the brain (Cesano et al., 1999a; Visonneau et al., 1999a). Therefore, in trying to develop effective approaches to treat brain tumors using TALL-104 cells, determining the most efficient route of administration of these cells is crucial. In a previous study, significant antitumor activity was shown by irradiated TALL-104 cells against a human glioblastoma flank xenograft. In that setting, small tumor load and high effector-target ratio were likely a favorable key for tumor regression (Cesano et al., 1995).

In the present study, we evaluated the biodistribution of TALL-104 cells in rats bearing intracranial xenografts of rat or human brain tumors, comparing three routes of administration: intracarotid, i.v., and intratumoral. Numbers of TALL-104 cells in the brain were higher after intracarotid administration, but rapidly decreased to low cell levels. However, repeated local administration of TALL-104 cells directly into the tumor bed increased survival significantly in a metastatic brain tumor model, suggesting that local administration of TALL-104 cells represents the most favorable route of delivery in future clinical applications.

Materials and Methods

Cell Lines

TALL-104 cells were maintained at 37°C in 10% CO₂ in IMDM (Gibco-BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.) and 100 units/ml of recombinant human IL-2 (Chiron, Emeryville, Calif.). The cell line repeatedly tested negative for *mycoplasma* contamination using a commercial polymerase chain reaction kit (American Tissue Culture Center, Rockville, Md.).

The human medulloblastoma cell lines DAOY and D283 Med, the glioma cell line U-87 MG, the epidermoid cancer cell line A431, the leukemia cell line K562, and the rat glioma cell line C6 were purchased from the American Tissue Culture Center. The medulloblastoma cell line D341 Med and the glioma cell line U-251 MG were kindly supplied by Henry Friedman (Duke University, Durham, N.C.), the glioma cell line A1690 was supplied by C. Sher (Children's Hospital of Philadelphia, Philadelphia, Penn.), and the rat glioma cell lines 9L and F98 were furnished by Henry Brem (Johns Hopkins University, Baltimore, Md.). DAOY, D283 Med, D341 Med, U-251 MG, and A1690 cells were grown in Richter's zinc-option medium (Gibco-BRL); U-87 MG and A431 were grown in Dulbecco's modified Eagle's medium (Gibco-BRL); and K562, 9L, C6, and F98 were in IMDM, all supplemented with 10% fetal bovine serum. Cultures were maintained under aseptic conditions and incubated at 37°C in humidified 5% CO₂.

Cytotoxicity Assays

TALL-104 cells were tested at 4–6 concentrations in 18-h ⁵¹Cr-release assays against a fixed number (10⁴/well) of ⁵¹Cr-labeled brain tumor cells in suspension. The highly sensitive cell line K562 was used as control tumor target. The percentage of specific ⁵¹Cr release was calculated from the mean of two replicates (Cesano and Santoli, 1992a; Cesano et al., 1993; O'Connor et al., 1991).

Proliferation Assays

Irradiated (40 Gy) TALL-104 cells were incubated at 4–5 concentrations with a fixed number (10⁴/well) of brain tumor cells. After 48 h, the cultures were pulsed with 1 μ Ci/well [³H]thymidine (2 Ci/mmol; Amersham, Arling-

ton Heights, Ill.) for 18 h and harvested on fiber glass papers. Isotope incorporation was measured in a β -counter (Packard Instruments, Downers Grove, Ill.). The percentage of [^3H]thymidine uptake inhibition was calculated according to the formula: $M - E/M$, where M is cpm from triplicate cultures of tumor cells alone and E is cpm from triplicate cultures of cocultured TALL-104 and tumor cells.

Animals

Male Fischer rats, 4–6 weeks old and weighing approximately 250 g, were purchased from Charles River Laboratories (Wilmington, Mass.), and athymic nude rats were supplied by the National Cancer Institute (Fredericks, Md.). Both animals were maintained in the Laboratory Animal Facility of the Joseph Stokes, Jr., Research Institute of the Children's Hospital of Philadelphia. Nude rats were housed in filter-top cages in a separate facility and maintained in an aseptic environment with laminar flow filtered ventilation. The methods and procedures used in this study were in compliance with an Institutional Animal Care and Use Committee approval protocol (IACUC NO: 1997-4-135).

Intracranial Brain Tumor Xenograft Animal Models

Two animal models were used in this study to investigate TALL-104 cell biodistribution: a syngeneic model of 9L glioblastoma in Fischer rats; and a metastatic human xenograft model of the epidermoid carcinoma cell line A431 in athymic nude rats. 9L and A431 cells grown in vitro cell cultures were harvested in log-phase and resuspended in equal amounts of serum-free medium and 1% methylcellulose. Either 1×10^5 or 10^6 cells in 5 μl was implanted into the right caudate nucleus of anesthetized rats using a Kopf stereotactic instrument (David Kopf Instruments, Tujunga, Calif.) at the coordinates 1 mm anterior to the bregma, 2 mm lateral right, and 3 mm below the surface of the dura.

Intraarterial Injections

Animals were anesthetized with i.p. ketamine/xylazine/EtOH (25 mg/ml ketamine, 2.5 mg/ml xylazine, 0.14 ml/ml 100% EtOH given at 3 ml/kg) and placed in supine position with all limbs restrained. A 2-cm longitudinal midline skin incision was made from the level of the hyoid bone to the sternal notch. The s.c. tissue was sharply divided, and the left sternocleidomastoid and paratracheal muscles were retracted laterally and medially, respectively. The course of the left common carotid and the bifurcation of the external and internal carotid were exposed and the lateral left one third of the hyoid bone was removed. The pterygopalatine branch of the internal carotid, and the occipital and the superior thyroid arteries were bipolarized, and the external carotid was cannulated in retrograde fashion to the bifurcation of the common carotid using PE-10 polyethylene tubing. The cannula was then secured with silk suture to completely occlude flow in the external carotid so that all injections would enter the distal common carotid and then, subse-

quently, the internal carotid artery only. After cells were injected into the internal carotid artery, the cannula was removed, the external carotid was ligated proximally, and the skin incision was closed with surgical clips. Animals were allowed to recover before killing them at various time points (Schuster et al., 1993).

Intravenous Injections

Intravenous injections were made in the left femoral vein through a femoral cutdown, and the skin was closed with surgical clips.

Labeling of TALL-104 Cells

TALL-104 cells were stained with the fluorescent, DNA-binding dye bisbenzimidazole (Hoechst 33342; Sigma, St. Louis, Mo.) by incubating 5×10^7 cells for 30 min at 37°C in 1 ml serum-free IMDM with 6 μg bisbenzimidazole dye, washing them twice in IMDM, and resuspending them in 500 μl IMDM for i.a. and i.v. injection. The fluorescence was stable for several weeks if protected from light (Brenan and Parish, 1984).

Distribution Studies of TALL-104 Cells after Intracarotid and Intravenous Injection

Twelve days after intracranial tumor implantation (1×10^6 cells/5 μl), Fischer rats with 9L glioblastoma or athymic nude rats with A341 epidermoid tumors were injected with 5×10^7 fluorescent dye-labeled TALL-104 cells in 500 μl IMDM into the left femoral vein or the left internal carotid. Animals were killed 5 min, 10 min, 1 h, 3 h, or 24 h after injection. Brain, lungs, spleen, and liver were snap-frozen in Tissue Tek OCT^R freezing medium (Sakura, Torrance, Calif.); tissues were cut in 18- μm sections with a cryostat. Hematoxylin and eosin-staining was performed. Fluorescent dye-labeled TALL-104 cells were detected under a UV microscope with DAPI-filter at 365 and 435 nm (Leica DNRBE, Leica). Quantitative analyses were performed at 200 $\times$ magnification: The means of counted TALL-104 cells were given from 5 views.

Intracranial Tumor Treatment by Intratumoral TALL-104 Cell Injections

Groups of athymic rats bearing intracranial A431 xenograft tumors received single or multiple treatments of 1×10^7 TALL-104 cells in 5 μl serum-free IMDM by stereotactic injection into the tumor area. For single treatment on day 3, or multiple treatments on days 3, 7, and 10, intracranial tumors were induced with 1×10^5 A431 cells. For single treatment on day 10, intracranial tumors were induced with 1×10^6 A431 cells. Control animals for the appropriate days received the same number of A431 cells, but were not treated with TALL-104 cells. Animals were examined for evidence of neurologic abnormality, weight loss, or death 2–3 times each week for the first 2 weeks, and daily thereafter. Intracranial tumors were confirmed by visual inspection of the brain at the time of death. Response was assessed as the difference between median survival times for controls and that

Table 1. In vitro cytotoxic activity of TALL-104 cells against human and rodent tumor cell lines

Cell lines	Effector-target ratio					
	50:1 (%)	25:1 (%)	12.5:1 (%)	6.25:1 (%)	3.125:1 (%)	1.06:1 (%)
Human PNET/medulloblastoma						
DAOY	84	84	86	73	77	52
D283 Med	62	60	63	61	53	46
D341 Med	61	61	45	51	38	36
Human glioblastoma						
U251 MG	80	69	70	45	ND	ND
U-87 MG	100	98	91	81	73	68
A1690	71	72	56	71	50	51
Epidermoid carcinoma						
A431	ND	ND	93	99	100	88
Rat glioblastoma						
9L	12	10	8	4	ND	ND
F98	22	21	18	11	ND	ND
C6	22	11	8	4	ND	ND
Human leukemia cell line (control)						
K562	79	74	64	64	ND	ND

Values represent the percentage of specific ^{51}Cr release.

Abbreviations: ND, not determined.

of TALL-104-treated animals. Statistical significance in median survival between groups was estimated by log-rank test ($P < 0.05$, statistically significant).

Results

In Vitro Susceptibility of Human and Rodent Brain Tumor Cell Lines to TALL-104 Cell-Mediated Cytotoxicity

TALL-104 cells displayed significant cytotoxic activity as measured by the ^{51}Cr -release assay against all 7 tested human cancer cell lines (Table 1). The greatest cytotoxic effect at lowest effector-to-target ratio tested (E:T ratio 3.125:1) was seen against the human epidermoid cancer cell line A431. [^3H]thymidine uptake assays demonstrated a nearly total inhibition of proliferation in all human tumor cell lines by TALL-104 cells (Table 2). These findings indicate that A431 is extremely sensitive to the cytotoxic effects of TALL-104 cells. By contrast, we found little or no cytotoxic effects of TALL-104 cells in any of the rat glioma cell lines tested (9L, C6, and F98) (Tables 1 and 2). Therefore, A431 was selected for subsequent use as a model of metastatic human brain tumor.

Specific Labeling of TALL-104 Cells for Biodistribution Studies

TALL-104 cells were labeled with fluorescent vital bis-benzimide dye at a concentration of 6 μg added to 5×10^7 TALL-104 cells/ml medium. Fluorescence-activated cell sorting analyses demonstrated a labeling efficiency of nearly 100% (data not shown). Importantly, fluorescent labeling at that concentration did not impair TALL-104 cell ability to kill K562 tumor cells, as measured in short-

term ^{51}Cr -release assay, or affect cell viability as assessed by a trypan blue exclusion test (data not shown).

Biodistribution of TALL-104 Cells in Intracranial Brain Tumor–Xenograft Models after Intracarotid and Intravenous Administration

Kinetics of distribution of fluorescent dye-stained TALL-104 cells in normal brain tissue and brain tumors during the first 24 h after TALL-104 cell injection was similar in TALL-104-sensitive (A431) and –resistant (9L) tumor models. Therefore, to evaluate i.v. and i.a. TALL-104 cell distribution in brain, we combined data accumulated from these tumor models. After i.v. injection of the fluorescent dye-labeled TALL-104 cells, <1 cell per $200\times$ view was detectable in brain or brain tumor. By contrast, after intracarotid (i.a.) administration, significant amounts of TALL-104 cells were detected in brain tissue, with a peak in cell count 10 min after cell injection (41.6 cells/ $200\times$ magnification view) (Fig. 1A). Nevertheless, cells injected into the carotid artery rapidly decreased to baseline within 1 h. In brain tumor, the maximum TALL-104 cell count was also detectable 10 min after i.a. injection (2.8 cells per $200\times$ magnification view), but was undetectable in brain tumor 1, 3, and 20 h after i.a. administration.

Based on previous investigations (Cesano et al., 1999a, 1999b; Visonneau et al., 1997b), we additionally looked at TALL-104 distribution in systemic organs, such as lungs, spleen, and liver. There was no significant difference in the distribution pattern and amount of TALL-104 cells in these extracranial organs after intracarotid or i.v. cell administration. This result was expected, as first-pass distribution kinetics would be different only for brain but not for extraneural organs after intracarotid injection. Therefore, these values are combined in Fig. 1B. The highest labeled cell counts were observed in lungs within the

Table 2. In vitro inhibition of proliferation of brain tumor cell lines by irradiated TALL-104 cells

Cell lines	Effector-target ratio				
	50:1 (%)	25:1 (%)	12.5:1 (%)	6.25:1 (%)	3.125:1 (%)
Human PNET/medulloblastoma					
DAOY	98	98	97	95	88
Human glioblastoma					
U251 MG	96	94	95	94	94
U-87 MG	97	93	95	93	84
A1690	97	91	95	91	88
Rat glioblastoma					
9L	0	0	0	0	ND
F98	0	0	0	0	ND
C6	0	0	0	0	ND
Human leukemia cell line (control)					
K562	98	98	99	99	ND

Values represent the percentage inhibition of [3 H]thymidine uptake.

Abbreviations: ND, not determined.

first 10 min after i.v. or intracarotid injection (238 cells/200 $\times$ magnification view) followed by a rapid clearance within the next hour. TALL-104 cell numbers in spleen and liver rose within 24 h to 12.7 and 12 cells/view.

Fig. 2 illustrates the distribution of TALL-104 cells in brain tumor and normal brain tissue, compared with systemic organs and blood at different time points after intracarotid injection.

Motility of TALL-104 Cells after Intracranial/ Intratumoral Injection

Although TALL-104 cell accumulation in brain and brain tumor was high 10 min after intracarotid administration of the cells, their subsequent rapid clearance suggested that this route of administration was unlikely to yield efficient tumoricidal effects. Therefore, we investigated

Fig. 1. Biodistribution of nonirradiated TALL-104 cells after i.v. and intracarotid injection in brain tumor-bearing athymic or Fischer rats. Fluorescent bisbenzamide dye (Hoechst 33342)-labeled TALL-104 cells were injected into the left femoral vein (i.v.) or the left internal carotid artery (i.a.). Organs were harvested from rats that were killed 5 min ($n = 1$), 10 min ($n = 1$), 1 h ($n = 5$), 3 h ($n = 9$), and 24 h ($n = 3$) after injection. Cell counts represent means of fluorescent TALL-104 cells out of 5 views at original magnification $\times 200$. A. Distribution of TALL-104 cells in brain tissue and brain tumor after intracarotid (i.a.) injections. After i.v. injection, TALL-104 cells were <1 cell per view at all time points tested and were therefore not included in the graph. B. Kinetics of TALL-104 cell distribution in lungs, spleen, and liver during a 20-h observation period.

Fig. 2. Distribution of TALL-104 cells in tumor and different organs of athymic rats after intracarotid injection. UV microscopic images of rat tissues were obtained 10 min after injection. A. Normal brain tissue. B. A431 brain tumor. White arrows indicate two fluorescent dye-labeled TALL-104 cells. C. Whole-blood smear showing circulating TALL-104 cells. D. Lungs. E. Subsequent reticuloendothelial accumulation of TALL-104 cells at 3 h after intracarotid injection in spleen. These are pseudocolor images for the purpose of contrast. Original magnification $\times 200$.

the distribution and motility of TALL-104 cells injected directly into the tumor as an alternative approach.

As shown in Fig. 3A and B, 3 h after intratumoral injection of 1×10^7 TALL-104 cells in 5 μ l serum-free medium into A431 brain tumors, the TALL-104 cells were detectable along the injection tract and at the injection site. Brains sectioned 3 days after TALL-104 cell injection demonstrated persistence of TALL-104 cells in the injected tumor area (Fig. 3C). Tumor regions distant from the injection site were not infiltrated by TALL-104 cells (Fig. 3D). One rat had a large A431 tumor of the caudate nucleus that invaded the lateral ventricle. In this animal, TALL-104 cells were found in the ventricle adjacent to the tumor, but not in the contralateral ventricle system or brain hemispheres.

Survival of Rats Receiving Intratumoral Treatment with TALL-104 Cells

Survival time of nude rats bearing intracranial A431 xenografts after single and multiple intratumoral injections of TALL-104 cells are illustrated in Fig 4A–C. Single treatment with TALL-104 cells at a stage of progressed disease did not produce survival advantage (Fig. 4A). Intracranial injection of 10^6 A431 cells yielded very large tumors, and untreated animals died within 13 to 18 days. Although one animal showed extended survival after single intratumoral injection of 10^7 TALL-104 cells on day 10, at a progressed stage of disease, no significant increase in survival was observed in this experimental group ($P = 0.99$; Fig. 4A). By contrast, prolonged survival over controls was observed

Fig. 3. Coronal sections of brains from A431 brain tumor-bearing athymic rats electively killed 3 h and 3 days after intratumoral injection of 10^7 fluorescent dye-labeled TALL-104 cells. A. TALL-104 cells lining the injection track of normal rat brain (white arrows) (3 h). B. TALL-104 cells accumulated at the base of injection site within A431 tumor (3 h). This image depicts brain tumor and TALL-104 cells inferior to and continuing to the left of panel A. C. TALL-104 cells retained within A431 tumor at 3 days after injection. D. Tumor regions distant from the injection site and not infiltrated by TALL-104 cells (3 days). The white line represents the border of brain and brain tumor. These are pseudocolor images for the purpose of contrast. Original magnification $\times 200$.

when treatment was initiated earlier, when tumor burden was lower (Fig. 4B and C). Control animals developed severe neurological symptoms or died within 26 days after injection of 10^5 tumor cells. While a single intracranial/intratumoral administration of 10^7 TALL-104 cells on day 3 after tumor challenge resulted in a prolonged survival ($P = 0.15$; Fig. 4B), multiple administrations of 1×10^7 TALL-104 cells on days 3, 7, and 10 conferred a statistically significant survival advantage ($P = 0.029$; Fig. 4C).

Discussion

Strategies for the development of new brain tumor treatments emphasize at least three considerations to achieve local tumor control: demonstration of tumor-specific cytotoxicity; absence of cross-resistance with other effective therapies; and effective delivery of therapeutic agents across blood-tumor or blood-brain barriers. Currently, chemotherapeutic agents in anaplastic astrocytomas and glioblastomas rarely achieve local tumor control, and reported response rates are $<30\%$ for most agents (Kornblith and Walker, 1988). For childhood primitive neu-

roectodermal tumors/medulloblastomas, response rates to conventional chemotherapeutic agents are considerably higher; however, 30%–50% of patients with this tumor will have local tumor recurrence and eventually die from their disease (Packer et al., 1991). Accordingly, targeted therapy based on selective recognition of cell-surface antigens or receptors represents an alternative strategy that deserves further consideration.

Immunologic approaches may represent an important new avenue to the problem of local tumor control. Among the various immunologic approaches, therapeutic strategies based on natural killer and lymphokine-activated killer cells have certain distinct advantages, one of the most important being that their antitumor activity does not require prior effective immunization with a specific tumor-derived antigen. TALL-104 cell therapy is a new adoptive therapy approach with selective cytotoxic activity against a broad range of human tumors with no cytotoxic activity against normal cells. TALL-104 cells do not need exogenous IL-2 administration to be effective in vivo. Therefore, the serious side effects observed with systemic IL-2 administration for LAK cell therapy are not observed with this form of adoptive immunother-

Fig. 4. Survival of A431 brain tumor-bearing athymic rats treated with TALL-104 cells (10^7). A. Survival of rats on day 10. There was no significant survival advantage with a single intracranial/intratumoral injection of TALL-104 cells on day 10 ($n = 5$; $P = 0.99$). B. Survival of rats on day 3. There was a survival advantage after single intracranial/intratumoral injection of TALL-104 cells on day 3 ($n = 7$) after xenograft implantation (10^5 A431 cells; $P = 0.15$), compared with controls. C. Survival of rats treated repeatedly on days 3, 7, and 10. There was a significant survival advantage after multiple intratumoral injections of TALL-104 cells on days 3, 7, and 10 after xenograft implantation ($n = 7$), compared with controls ($n = 3$; $P = 0.029$).

apy. Two recently completed phase I trials in patients with advanced tumors, including children with malignant brain tumors, revealed no significant toxicity associated with multiple TALL-104 cell administration by i.v. with up to 10^8 cells/kg.⁴

An antitumor effect of TALL-104 cells against U-87 MG glioblastoma, *in vitro* and *in vivo* in s.c. flank xenograft tumors, has been demonstrated (Cesano et al., 1995). Moreover, biodistribution studies in mice and dogs have shown that systemically administered TALL-104 cells accumulate at the site of primary and metastatic tumor. In these models, no significant accumulation of TALL-104 cells in normal brain tissue after i.v., i.p., or s.c./intratumoral administration was detected. In fact, the activity detected in brain after systemic administration of TALL-104 cells was always lower than blood activity at any time point studied (Cesano et al., 1999a, 1999b; Visonneau et al., 1997b). These observations suggest that blood-to-brain transfer of TALL-104 cells is impeded by an intact blood-brain barrier.

The blood-brain barrier and blood-tumor barrier may be abnormal within or adjacent to brain tumors, and significant heterogeneity in permeability and blood flow have been reported (Blasberg and Groothuis, 1986; Groothuis et al., 1991; Vick et al., 1977). Intraarterial (intracarotid) administration of therapeutic agents may represent a method to improve selective therapy of brain tumors by increasing local drug delivery while, at the same time, reducing the overall systemic dose, thereby minimizing systemic toxicity (Bullard et al., 1985; Kurpad et al., 1995, 1997; Schuster et al., 1993). The relatively localized growth pattern of malignant gliomas and their proximity to the major blood vessels (the carotid and vertebral arteries) represent specific advantages for i.a. drug delivery (Theron et al., 1986).

To evaluate TALL-104 cell distribution in normal brain and brain tumors after i.v. or intracarotid injection, we used a 9L gliosarcoma cell line that is syngeneic to immunocompetent Fischer rats. It should be noted, however, that all rodent tumor cell lines assayed in this study, including 9L gliosarcoma, are relatively insensitive to TALL-104 cells, as demonstrated by *in vitro* cytotoxicity assays. To anticipate the possibility that sensitivity to TALL-104 cytotoxic effects might be an important factor in the selective accumulation of TALL-104 cells in brain tumors, we used the human A431 epidermoid carcinoma cell line with highest sensitivity at all tested effector-to-target ratios to the *in vitro* lytic activity of TALL-104 cells to develop a model of metastatic brain tumor in athymic rats.

In these experiments, TALL-104 cells were labeled with a fluorescent bisbenzamide dye (Hoechst 33342) that does not affect cell viability and/or functional activity. This approach avoids the limitations of radiolabeling methods as well as problems associated with primary antibody cross-reactivity (for example, CD3) between TALL-104 cells and normal rodent brain or lymphocytes when immunohistochemical detection methods are used. Results of TALL-104 cell distribution in extracranial organs, such as lungs, spleen, and liver, in the present study are comparable to those performed with ⁵¹Cr- or ¹¹¹In-labeled TALL-104 cells (Cesano et al., 1996a,

1999a, 1999b), indicating that bisbenzamide cell labeling represents a reliable method for cellular biodistribution studies. Our results further suggest that blood flow and perfusion are the initial distribution factors, followed by cell-cell interactions responsible for the tumor localization of these major histocompatibility complex non-restricted effectors, such as vascular adhesion mechanisms, receptor molecules, and chemotactic factors.

Accumulation of TALL-104 cells in normal brain tissue and brain tumor appeared to be determined by the route of administration. We found no significant accumulation of TALL-104 cells in normal brain tissue nor in brain tumor after i.v. injection of maximum feasible concentrations and volumes of cells. These results were concordant with previous findings in extracranial tumor models (Cesano et al., 1999a; Visonneau et al., 1997b). TALL-104 cells were detected in normal brain and brain tumors 10 min after intracarotid injection. However, the subsequent rapid decrease in detectable TALL-104 cells at 1 h suggests that the earlier time point (10 min) likely represents a brief retention of TALL-104 cells within the cerebral vascular compartment without subsequently crossing the normal blood-brain barrier or even the highly permeable blood-tumor barrier of TALL-104-sensitive (A431) or resistant (9L) tumors. Previous studies reported that TALL-104 cells continue to accumulate in primary or metastatic tumor sites from 48 to 72 h after i.v. administration. However, in those experiments, significant accumulation of TALL-104 cells was identified in systemic solid tumors by 24 h, which is significantly different from our results with brain tumor models. While we cannot exclude the possibility that slow release of TALL-104 cells from reticuloendothelial compartments may permit continued low-level delivery of TALL-104 to brain tumors, we consider this to be unlikely, and evaluation of these late time points is precluded by the limitations of the intracarotid procedure in experimental animals.

Using an intratumoral route of TALL-104 cell administration, we evaluated TALL-104 antitumor efficacy in nude rats bearing human A431 brain tumors. Our *in vivo* distribution studies demonstrated an extremely low accumulation of TALL-104 cells in A431 brain tumors after i.v. or intracarotid administration, corresponding to a $\geq 1:1000$ effector-to-target ratio, which is insufficient for cytotoxic activity *in vitro*. Consistent with National Institutes of Health and institutional guidelines for rational use of laboratory animals, we considered these routes of administration to be ineffective and did not conduct survival studies to evaluate the therapeutic efficacy of TALL-104 after i.v. or intracarotid injection in laboratory animals. To optimize effector-target ratios for survival studies, TALL-104 cells were stereotactically injected into the tumor bed of A431 brain tumors.

Repeated injections of TALL-104 cells started at an early stage of tumor challenge induced significant prolongation of survival in athymic rats compared with control animals or animals treated at an advanced tumor stage. However, no long-term survivors (>50 days) were observed. TALL-104 cells persisted at the injection site in the tumor and did not infiltrate tumor surrounding brain tissue or the contralateral hemispheres. In one animal with a large tumor of the

caudate nucleus invading the lateral ventricle, TALL-104 cells were also found in the adjacent ventricle system. Discrepancy in high susceptibility of tumor cells in vitro compared with the demonstrated efficacy in vivo may reflect the low ratio of cytotoxic TALL-104 cells to tumor cells and the short doubling time and rapid growth of this xenograft. Additionally, our survival results may underestimate the true response rate of intracranial tumors to TALL-104 cells because of the method of administration. Although stereotactic intracranial injection of the cells with the use of the same coordinates as those for tumor injection increases the probability that the killer cells will be delivered within the tumor bed itself, it is possible that some injections missed the tumor or were delivered directly into the lateral ventricle.

In summary, we demonstrated the efficacy of local treatment with TALL-104 cells in the therapy of intracranial human brain tumor xenografts in athymic rats. Repeated intracranial/intratumoral delivery of

TALL-104 cells at an early stage of tumor challenge substantially increased survival rates. Therefore, local therapy is superior to systemic administration in this experimental model. Significant cytotoxic activity against different brain tumor cell lines in vitro prolonged survival in rats bearing a human metastatic brain tumor. Low toxic effects and high tumor specificity suggest the potential for this adjuvant, adoptive therapy approach in the treatment of patients with high-grade CNS malignancies.

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References

- Blasberg, R.G., and Groothuis, D.R. (1986) Chemotherapy of brain tumors: Physiological and pharmacokinetic considerations. *Semin. Oncol.* **13**, 70–82.
- Brenan, M., and Parish, C.R. (1984) Intracellular fluorescent labelling of cells for analysis of lymphocyte migration. *J. Immunol. Methods* **74**, 31–38.
- Bullard, D.E., Bigner, S.H., and Bigner, D.D. (1985) Comparison of intravenous versus intracarotid therapy with 1,3-bis(2-chloroethyl)-1-nitrosourea in a rat brain tumor model. *Cancer Res.* **45**, 5240–5245.
- Cesano, A., and Santoli, D. (1992a) Two unique human leukemic T-cell lines endowed with a stable cytotoxic function and a different spectrum of target reactivity analysis and modulation of their lytic mechanisms. *In Vitro Cell. Dev. Biol.* **28A**, 648–656.
- Cesano, A., and Santoli, D. (1992b) Inducible expression of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α , and interferon- γ in two human cytotoxic leukemic T cell lines. *In Vitro Cell. Dev. Biol.* **28A**, 657–662.
- Cesano, A., Visonneau, S., Clark, S.C., and Santoli, D. (1993) Cellular and molecular mechanisms of activation of MHC non-restricted cytotoxic cells by IL-12. *J. Immunol.* **151**, 2943–2957.
- Cesano, A., Visonneau, S., Cioe, L., Clark, S.C., Rovera, G., and Santoli, D. (1994) Reversal of acute myelogenous leukemia in humanized SCID mice using a novel adoptive transfer approach. *J. Clin. Invest.* **94**, 1076–1084.
- Cesano, A., Visonneau, S., and Santoli, D. (1995) Treatment of experimental glioblastoma with a human major histocompatibility complex nonrestricted cytotoxic T cell line. *Cancer Res.* **55**, 96–101.
- Cesano, A., Visonneau, S., Jeglum, K.A., Owen, J., Wilkinson, K., Carner, K., Reese, L., and Santoli, D. (1996a) Phase I clinical trial with a human major histocompatibility complex nonrestricted cytotoxic T-cell line (TALL-104) in dogs with advanced tumors. *Cancer Res.* **56**, 3021–3029.
- Cesano, A., Visonneau, S., Pasquini, S., Rovera, G., and Santoli, D. (1996b) Antitumor efficacy of a human major histocompatibility complex nonrestricted cytotoxic T-cell line (TALL-104) in immunocompetent mice bearing syngeneic leukemia. *Cancer Res.* **56**, 4444–4452.
- Cesano, A., Pierson, G., Visonneau, S., Migliaccio, A.R., and Santoli, D. (1996c) Use of a lethally irradiated major histocompatibility complex nonrestricted cytotoxic T-cell line for effective purging of marrows containing lysis-sensitive or -resistant leukemic targets. *Blood* **87**, 393–403.
- Cesano, A., Visonneau, S., Deaglio, S., Malavasi, F., and Santoli, D. (1998) Role of CD38 and its ligand in the regulation of MHC-nonrestricted cytotoxic T cells. *J. Immunol.* **160**, 1106–1115.
- Cesano, A., Visonneau, S., Tran, T., and Santoli, D. (1999a) Biodistribution of human MHC non-restricted TALL-104 killer cells in healthy and tumor bearing mice. *Int. J. Oncol.* **14**, 245–251.
- Cesano, A., Wortman, J.A., Pourdehnad, M., Visonneau, S., Mozley, D., Bhatnagar, A., Alavi, A., and Santoli, D. (1999b) Effects of development of host immunity on the biodistribution of xenogeneic MHC non-restricted cytotoxic T cells: Implications for adoptive cell therapy of cancer. *Int. J. Oncol.* **14**, 233–244.
- Groothuis, D.R., Vriesendorp, F.J., Kupfer, B., Wamke, P.C., Lapin, G.D., Kuruvilla, A., Vick, N.A., Mikhael, M.A., and Patlak, C.S. (1991) Quantitative measurements of capillary transport in human brain tumors by computed tomography. *Ann. Neurol.* **30**, 581–588.
- Kornblith, P.L., and Walker, M. (1988) Chemotherapy for malignant gliomas. *J. Neurosurg.* **68**, 1–17.
- Kurpad, S.N., Friedman, H.S., Archer, G.E., McLendon, R.E., Petros, W.M., Fuchs, H.E., Guaspari, A., and Bigner, D.D. (1995) Intraarterial administration of melphalan for treatment of intracranial human glioma xenografts in athymic rats. *Cancer Res.* **55**, 3803–3809.
- Kurpad, S.N., Dolan, M.E., McLendon, R.E., Archer, G.E., Moschel, R.C., Pegg, A.E., Bigner, D.D., and Friedman, H.S. (1997) Intraarterial O⁶-benzylguanine enables the specific therapy of nitrosourea-resistant intracranial human glioma xenografts in athymic rats with 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Chemother. Pharmacol.* **39**, 307–316.
- O'Connor, R., Cesano, A., Lange, B., Finan, J., Nowell, P.C., Clark, S.C., Raimondi, S.C., Rovera, G., and Santoli, D. (1991) Growth factor requirements of childhood acute T-lymphoblastic leukemia: Correlation between presence of chromosomal abnormalities and ability to grow permanently in vitro. *Blood* **77**, 1534–1545.
- Ozols, R.F., Masuda, H., and Hamilton, T.C. (1988) Mechanisms of cross-resistance between radiation and antineoplastic drugs. *NCI Monogr.* **6**, 159–165.
- Packer, R.J., Sutton, L.N., Goldwein, J.W., Perilongo, G., Bunin, G., Ryan, J., Cohen, B.H., D'Angio, G., Kramer, E.D., Zimmerman, R.A., Rorke, L.B., Evans, A.E., and Schut, L. (1991) Improved survival with the use of adju-

- vant chemotherapy in the treatment of medulloblastoma. *J. Neurosurg.* **74**, 433–440.
- Phillips, P.C. (1991) Antineoplastic drug resistance in brain tumors. *Neurol. Clin.* **9**, 383–404.
- Rosenberg, S.A., Packard, B.S., Aebersold, P.N., Solomon, D., Topalian, S.L., Toy, S.T., Simon, P., Lotze, M.T., Yang, J.C., Seipp, C.A., Simpson, C., Carter, C., Bock, S., Schwartzentruber, D., Wei, J.P., and White, D.E. (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N. Engl. J. Med.* **319**, 1676–1680.
- Schuster, J.M., Friedman, H.S., Archer, G.E., Fuchs, H.E., McLendon, R.E., Colvin, O.M., and Bigner, D.D. (1993) Intraarterial therapy of human glioma xenografts in athymic rats using 4-hydroperoxycyclophosphamide. *Cancer Res.* **53**, 2338–2343.
- Silverman, C.L., and Simpson, J.R. (1982) Cerebellar medulloblastoma: The importance of posterior fossa dose to survival and patterns of failure. *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 1869–1876.
- Theron, J., Villemure, J.G., Worthington, C., and Tyler, J.L. (1986) Superselective intracerebral chemotherapy of malignant tumours with BCNU: Neuroradiological considerations. *Neuroradiology* **28**, 118–125.
- Vick, N.A., Khandekar, J.D., and Bigner, D.D. (1977) Chemotherapy of brain tumors: The "blood-brain barrier" is not a factor. *Arch. Neurol.* **34**, 523–526.
- Visonneau, S., Cesano, A., Tran, T., Jeglum, K.A., and Santoli, D. (1997a) Successful treatment of canine malignant histiocytosis with the human major histocompatibility complex nonrestricted cytotoxic T-cell TALL-104. *Clin. Cancer Res.* **3**, 1789–1797.
- Visonneau, S., Cesano, A., Torosian, M.H., and Santoli, D. (1997b) Cell therapy of a highly invasive human breast carcinoma implanted in immunodeficient (SCID) mice. *Clin. Cancer Res.* **3**, 1491–1500.
- Visonneau, S., Cesano, A., Jeglum, K.A., and Santoli, D. (1999a) Adoptive therapy of canine mammary carcinoma with the human cytotoxic T cell line TALL-104. *Oncol. Rep.* **6**, 1181–1188.
- Visonneau, S., Cesano, A., Jeglum, K.A., and Santoli, D. (1999b) Adjuvant treatment of canine osteosarcoma with the human cytotoxic T-cell line TALL-104. *Clin. Cancer Res.* **5**, 1868–1875.
- Whittington, R., and Faulds, D. (1993) Interleukin-2: A review of its pharmacological properties and therapeutic use in patients with cancer. *Drugs* **46**, 446–514.