Immuno-Gene Therapy of Established Prostate Tumors Using Chimeric Receptor-redirected Human Lymphocytes

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ABSTRACT

Targeted adoptive immunotherapy is an attractive option for prostate cancer given its accessible primary location, the presence of specific tissue and tumor antigens, and the acceptability of collateral destruction of healthy prostate tissue. The “T-body” approach, which uses genetically programmed, patient-derived lymphocytes transfected with chimeric receptor genes, combines the effector functions of T lymphocytes and natural killer cells with the ability of antibodies to recognize predefined surface antigens with high specificity and in a non-MHC restricted manner. We evaluated the therapeutic efficacy of anti-erbB2 chimeric receptor-bearing human lymphocytes on human prostate cancer xenografts in a SCID mouse model. Local delivery of erbB2-specific T bodies to well-established s.c. and orthotopic tumors, together with systemic administration of interleukin-2, resulted in retardation of both tumor growth and prostate-specific antigen secretion, prolongation of survival, and complete tumor elimination in a significant number of mice. These preclinical studies demonstrate the therapeutic potential of the T-body approach for locally advanced or recurrent prostate cancer as an adjunct to, or after, conventional therapy.

INTRODUCTION

PC3 is the most common diagnosed malignancy and second leading cause of cancer death in American men (1, 2). Radical prostatectomy and radiation therapy are currently the principal curative treatments for organ-confined disease. However, used alone, both these treatments often fail to eradicate locally advanced tumors (3, 4). Furthermore, even when applied to prostate-confined disease, local and distant failures are not uncommon (5), because many of these tumors are more advanced than they appear clinically. Hence, there is a need for neo-adjuvant treatment modalities to improve the success of local PC control.

PC displays several features that make it a very good candidate for adaptive immunotherapy. Although PC is a visceral tumor, the primary tumor site within the prostate is easy to access and image. Thus, effector immune cells can be readily and accurately injected directly into the tumor, assisted by trans-rectal ultrasonography. In addition, PC expresses a wealth of unique tumor and tissue markers, including PSA, PSMA (6), PSCA (7), PCTA-1 (or galectin-8; Ref. 8), STEAP (9), and members of the ErbB gene family (10). These markers can serve not only for screening, detection, and monitoring of PC, but those expressed on the cell surface may also provide useful targets for active or passive immunotherapy (11, 12). Finally, although many of these PC-associated antigens are also expressed on normal prostate tissue, because the prostate is not a vital organ, collateral immune damage to noncancerous prostate tissue, i.e., “biological prostatectomy,” is not hazardous and may even help to reduce local cancer recurrence. However, active immunization against PC may be of limited efficacy because in many cases, especially in advanced disease, a significant fraction of the PC cells fails to express surface HLA molecules (13). It is therefore appealing to exploit various modes of passive immunotherapy, using specific effectors of the humoral and cellular arms of the immune system.

The “T-body,” also known as the “chimeric-immune receptor,” approach involves redirection of immune effector cells toward desired tumor or viral targets using CRs with antibody-like specificity, linked to T cell triggering domains (14, 15). This approach combines the effector functions of T and natural killer cells with the ability of antibodies to recognize a preselected antigen with high specificity and without MHC restriction. This property is of special importance, because it enables elimination of tumor cells that have lost surface HLA expression (13, 16). Experiments from our laboratory (17, 18) and elsewhere (12, 19–22) demonstrated the efficiency of this approach in vitro using several CRs made of various scFvs of antibodies specific for a variety of tumor-associated antigens.

The differential expression of particular growth factor receptors on cancer cells as compared with normal tissues, their cell surface localization, and their active role in the malignant process makes these molecules a suitable target for tumor immunotherapy (23). Overexpression of erbB2 (HER-2) has been detected in ≤82% of PCs (10). More importantly, erbB2 expression has been shown to increase as androgen dependence decreases, both in vitro (24) and in vivo (25).

In this study, we demonstrate the efficacy of in vivo targeting of localized human PC by direct intratumoral administration of erbB2-specific, CR-bearing human lymphocytes using two different human PC xenograft models in SCID mice. We show here that significant and specific tumor growth retardation, decrease in serum PSA levels, and prolonged survival could be achieved exclusively in mice treated with the erbB2-specific lymphocytes. These results demonstrate the feasibility of this new approach toward the adoptive immunotherapy of localized PC.

MATERIALS AND METHODS

PC Xenografts. As a representative of human PC, the androgen-dependent CWR22 human adenocarcinoma PC xenograft (a generous gift from Prof. Pretlow) was used. This xenograft secretes PSA into the sera of tumor-bearing mice and overexpresses the erbB2 growth factor receptor (26). WISH-PC14 is a novel human prostatic adenocarcinoma xenograft that we have recently established4 from a late recurrent primary tumor after definitive radiation therapy. This xenograft represents a major clinical problem in PC management: local disease recurrence after radiation therapy. The xenograft overexpresses erbB2, erbB3, and erbB4 and secretes PSA into the sera of tumor-

4 J. H. Pinthus and Z. Eshhar, unpublished observations.
RESULTS

Expression of CRs in Human PBLs. We and others have developed previously a procedure that enables reproducible and efficient functional expression of the CR genes in normal T lymphocytes (Refs. 12, 28, and references therein). Thus, a few days after activation and transduction with retrovector containing a bicistronic tripartite CR bearing mice. Xenografts were maintained by serial passages in androgen-supplemented SCID mice.

Antibodies and Cell Lines. The N29 anti-ErbB2 mAb (27) was used as a source of the scFv for construction of the CR. Rabbit anti-N29 idiotype antibodies were made in our laboratory. SP6 (used as control scFv) is an anti-TNP mAb (28), and 20.5 is an anti-Sp6 idiotype mAb. FITC-labeled antimonosource IgG antibodies and rhodamine-pycroythrin-conjugated mouse antihuman CD4-, CD8-, and CD56-specific mAbs were purchased from Coulter Immunology (Coulter Corp., Hialeh, FL).

CR Genes and Their Expression in Human Lymphocytes. The CR genes used in this study were of the tripartite configuration in which the scFv was linked through the nonligand part of CD28 to the intracellular part of the FcγR γ chain (28). CRs derived from two scFvs were used in this study, an ErbB2 specific (28) made from the N29 mAb and, as a control, a TNP specific one (28) made from the Sp6 mAb. The CR cDNA construct was cloned into the pBullet vector followed by an IRES and the GFP gene (28). Transduction of anti-CD3 plus CD28 mAb-activated PBLs from healthy donors was performed with virus-containing supernatants in the presence of IL-2 on Retronectin (FN-Takara Shuzo Ltd., Otsu, Japan) as described previously (28).

Treatment of PC Xenografts in SCID Mice. All animal experiments were performed in compliance with the guide of care and use of laboratory animals. Male (c-b-17/cr-scid-bg) SCID, 7–10-week-old mice (specific pathogen-free colony of The Weizmann Institute of Science) were transplanted s.c. with 90-day slow release testosterone pellets (12.5 mg/pellet; Innovative Research of America, Sarasota, FL). Xenograft tissue or cells were transplanted s.c. together with Matrigel (Becton Dickinson, Bedford, MA) as described previously (29). Mice bearing established tumors, with similar tumor load, as determined by both tumor volume and PSA secretion, were randomized to different experimental groups. In experiments in which continuous systemic administration of IL-2 was used, alzet mini-osmotic pumps (Duract Corp., Cupertino, CA) were transplanted i.p. 24–72 h before the first intratumoral treatment. Each pump was loaded with recombinant human IL-2 (R&D Systems, Inc., Minneapolis, MN) and set to continuously release 105 IU/h.

The tumor-bearing mice were treated with the CR-bearing human lymphocytes or medium alone (HBSS). The CR-bearing lymphocytes (10⁷ lymphocytes in 150 µl of HBSS) were directly injected into the tumor, daily for 3–4 consecutive days. Tumor size (length, width, and height) was measured by caliper, and the tumor volume was calculated (30). Survival of mice was defined as the time until the tumor reached 10% of the mouse’s weight. Past this point, mice were euthanized, and their data were not further included in the experiment. When >50% of the mice in a certain group scored dead, we stopped recording the results of this group. In the orthotopic tumor model, mice were orthotopically transplanted with 1.5 × 10⁶ WISH-Pc14 cells into the dorsal prostate as described previously (30). Two to 3 weeks thereafter, mice with well-established tumors (as indicated by serum PSA) were treated with a single intratumoral administration of the CR-bearing lymphocytes. The surgical procedures of both orthotopic tumor implantation, intratumoral injection, and the i.p. miniosmotic pump implantation required laparotomy, which was performed under ketamin plus xylazine general anesthesia (127.5 and 4.5 mg/kg, respectively). The prostate tumor area was exposed and injected with 5 × 10⁷ erbB2 or TNP-specific lymphocytes, suspended in 150 µl of HBSS containing 50 units/ml IL-2.

Statistical Analysis. Statistical analysis was performed using JMP statistical software (SAS Institute, Inc., Cary, NC). Tumor volume and serum PSA level data were analyzed by Dunnett’s test to analyze the effect of the different treatments. The therapeutic effect was further verified using the Fit model to test the effect of the different treatments along the experimental period.

When indicated, comparisons between two different individual treatments (e.g., anti-erbB2 plus IL-2 versus anti-TNP plus IL-2) were performed using a one-way ANOVA test.

Expression of CRs in Human PBLs. We and others have developed previously a procedure that enables reproducible and efficient functional expression of the CR genes in normal T lymphocytes (Refs. 12, 28, and references therein). Thus, a few days after activation and transduction with retrovector containing a bicistronic tripartite CR (28), we could routinely achieve 40–80% expression of both GFP and the CR in normal lymphocytes. Most (90–95%) of the GFPP expressing cells coexpressed the CR on their surface (data not shown) as measured by anti-idiotypic antibody to the scFv; hence, we could use GFP as a marker for CR expression and to follow the fate of the programmed cells in vivo.

Phenotypically, the transducted PBLs consisted of 25–30% CD4+, 65–70% CD8+, and 10–15% CD56-positive cells (data not shown). This shift toward the CD8+ subtype, versus the phenotype of normal PBLs, was caused by the activation process, which used anti-CD3 and CD28 antibodies, and culture conditions, because the same distribution of surface markers was seen on activated untransducted lymphocytes and GFP-negative transfectants. The CR-bearing cells could be specifically stimulated to proliferate and release cytokines (IL-2, IFN-γ, and tumor necrosis factor-α) in response to their relevant antigen, either immobilized to plastic (28) or overexpressed on tumor cell lines and PC xenografts (data not shown).

Suppression of Growth of PC Xenografts by Intratumoral Administration of erbB2-specific, CR-expressing Lymphocytes. To study the therapeutic potential of the CR-expressing lymphocytes (T-bodies) on existing primary PC tumors, we administered human PBLs, transduced with the tripartite CR, directly into established s.c. CWR22 prostate adenocarcinoma xenografts in SCID mice (Fig. 1). The mice used had a relatively large tumor burden, as was also evident by high levels of PSA in their sera (mean 44 ng/ml). The treatment protocol consisted of five consecutive intratumoral injections (adopted from Alteneschmidt et al.; Ref. 31), each of 10⁷ CR-bearing lymphocytes [specific to erbB2 or TNP (control) or the same volume of HBSS buffer]. Of these three groups, only the mice treated with erbB2-specific T bodies showed delayed and reduced tumor growth (Fig. 1A) and PSA secretion (Fig. 1B). The mean survival time of this group was 10.5 weeks, which was double the mean survival time of the control groups, which was 5.5 weeks (data not shown). The progression of tumors that were treated with anti-TNP, CR-bearing lymphocytes was similar to that of tumors that were treated with HBSS. Therefore, the antitumor response was not caused by an alloregeneic response of the human T cells against the tumor.
Although significant survival advantages were seen, the treatment protocol described above did not result in a complete eradication or cure of the tumor. To improve the potency of the T bodies and optimize the treatment protocol, the effect of simultaneous systemic administration of IL-2, which was shown previously to enhance effector function of adoptively transferred immune cells in mouse models (32, 33), was tested (Fig. 2). In this experiment, only three consecutive daily injections of $10^7$ CR-bearing lymphocytes were administered to the s.c. CWR22 tumors (mean volume 70 mm$^3$). As seen in Fig. 2, the continuous systemic administration of IL-2 significantly enhanced the antitumor effect of anti-erbB2, CR-bearing lymphocytes. The suppression of tumor growth was significantly higher ($P < 0.014$) in the presence of IL-2 (Fig. 2A), whereas no significant difference between these two groups on PSA secretion ($P < 0.20$; Fig. 2B) was observed. In the absence of specific CR-bearing receptor cells, IL-2 alone did not exhibit an antitumor effect.

Tumor cells that escaped this immunotherapy protocol were analyzed for expression of the N29 epitope recognized by the CR. These cells were found to express reduced levels of the N29 epitope (data not shown). Interestingly, when stained with L96, another anti-erbB2 antibody directed at a different epitope, no treatment-related drop of erbB2 expression was observed. N29 recognizes a carbohydrate-associated epitope. These results may therefore suggest the selection by the anti-erbB2 treatment of tumor cells whose N29 epitope was not accessible to the redirected lymphocytes. If such an escape appears to be a problem, treatment could combine CR against several different epitopes or antigens.

The CR Approach Is Amenable to a Variety of PC Tumors. The antitumor effects of the transduced lymphocytes could also be demonstrated in another, freshly isolated human PC xenograft, WISH-PC14, an adenocarcinoma line derived in our laboratory$^6$ from locally recurrent PC postdefinitive radiation therapy. The WISH-PC14 xenograft secretes PSA into the SCID mouse serum. As can be seen in Fig. 3, intratumoral treatment of locally advanced, established s.c. xenograft (mean pretreatment serum PSA of 100 ng/ml) with the erbB2-specific T bodies with or without concurrent systemic administration of IL-2 significantly reduced the mean tumor volume ($P < 0.001$; Fig. 3A and $P < 0.011$; Fig. 3C) versus the controls, TNP-specific lymphocytes, and HBSS-treated groups. Here too, a significant ($P < 0.036$) additive effect of systemic administration of IL-2 on the specific T-body effect was noted. Statistically significant drops in serum PSA levels were measured only in the group treated erbB2-specific T bodies and concurrent systemic administration of IL-2 ($P < 0.001$; Fig. 3B). Without systemic coadministration of IL-2 (Fig. 3D), the erbB2-specific T bodies have no effect over the controls. IL-2 was therefore used in all our additional experiments. A significant reduction in the mean tumor volume ($P < 0.016$; Fig. 3E) and serum PSA levels ($P < 0.0075$; Fig. 3F) over the control groups was demonstrated in a similar experiment. Moreover, 55% (five of nine) of mice treated with the erbB2-specific lymphocytes and IL-2 were tumor free for $>3$ months after the initiation of treatment.

Histologically, tumors that were treated by direct administration of erbB2-specific, CR-bearing lymphocytes show massive destruction accompanied by a large degree of lymphocytic infiltration (Fig. 4C). These results were further demonstrated by specific antihuman CD45RO immunostaining (Fig. 4F). Tumors of control mice, treated with either HBSS or TNP-specific human lymphocytes together with systemic administration of IL-2, were conserved with no evidence of lymphocytic infiltration. Interestingly, in tumors that received lymphocytes of irrelevant specificity, the human lymphocytes are restricted to the borders of the tumor nodules but did not invade the tumor (Figs. 4, B and E).

Suppression of Tumor Growth of Orthotopic PC Xenografts. To mimic the potential therapeutic application of the T-body approach in PC patients, we next tested the tumoricidal activity of the CR-bearing lymphocytes, administered into orthotopically explanted WISH-PC14 xenografts. Fig. 5 depicts the results of such an experiment. As shown in the s.c. model, the ErbB2-specific T-bodies, administered into well-established tumors in the prostate, together with systemic IL-2, induced reduction of tumor size and PSA secretion (Fig. 5, A and B). Practically, it should be noted that administration of the engineered lymphocytes requires a complex surgical procedure, and parts of the tumor may not receive sufficient numbers of engineered lymphocytes. This is reflected in the large variation in tumor size in the treated groups in this experiment (Fig. 5A). Histological sections of the treated tumors, removed 2 months after the initiation of treatment, show regions infiltrated with human lymphocytes (Fig. 6, A and B). Interestingly, the lack of lymphocyte and mononuclear cell infiltration in the control TNP T body-treated group (Fig. 6, C and D) provides further evidence that the gross antitumor effect of the T bodies in this experimental model does not result from an allogeneic or xenogeneic immune response but rather from a specific response against erbB2-bearing tumor cells. These findings, together with experiments described above, demonstrate the potential that the T-body approach holds as a neo-adjuvant treatment for localized PC disease.

**DISCUSSION**

The preclinical model described here demonstrates the antitumor potency of genetically engineered human lymphocytes, programmed to express cancer-specific CRs. We show that intratumor administration of erbB2-specific effector lymphocytes impedes PC growth and causes tumor elimination in a significant proportion (≥50% in some experimental settings) of SCID mice bearing human prostate tumors.

The human lymphocytes endowed with erbB2 specificity display...
both CD8⁺ (65–75%), CD4⁺ (15–20%), and CD56⁺ (10%) cells. This is somewhat diverted from the normal PBL population and mostly caused by the in vitro activation and propagation phase, which in the presence of IL-2, gives a growth advantage to the CD8⁺ subpopulation. It has been reported that the presence of CD4⁺, helper T cells is essential for optimal activity of adoptively transferred effector CD8⁺ cells (34, 35) and for their persistence in patients. Most likely, these effects are mediated by lymphokines released by helper CD4⁺ T cells. Indeed, on in vitro activation of the transduced lymphocytes, we could detect secretion of T H 1 lymphokines (IL-2, Fig. 3. The effect of CR-bearing lymphocytes on the freshly established WISH-PC14 human adenocarcinoma of the prostate s.c. xenografts were treated by three consecutive daily intratumoral injections (arrow) of either TNP or erbB2-specific human lymphocytes or medium (HBSS) with (n = 8) or without systemic administration of IL-2 (n = 6–7). A, C, and E, tumor growth. B, D, and F, PSA secretion. Results in E and F are from similar experiments in which the s.c. WISH-PC14 xenografts were treated by four consecutive daily intratumoral injections (arrow) of either TNP or erbB2-specific human lymphocytes or medium (HBSS) with systemically administered IL-2. Results are mean ± SE.

Fig. 4. Histological view of the short-term antitumor effect induced by the anti-erbB2, CR-bearing lymphocytes. WISH-PC14 xenografts treated by either TNP or erbB2-specific human lymphocytes in the presence of IL-2 were removed 5 days after the beginning of the treatment. The normal architecture of the adenocarcinoma is preserved with no evidence of lymphocytic infiltration in both HBSS (A) and TNP-specific human lymphocyte (B) controls. In contrast, massive lymphocyte infiltration and tumor destruction are evident in tumor that was treated by erbB2-specific lymphocytes (C). Immunohistochemical staining with antihuman CD45RO mAb (D–F) shows that the anti-TNP, CR-bearing human lymphocytes are mainly restricted to the borders of the tumor nodules and hardly invade the tumor foci (E), as opposed to the massive lymphocyte infiltration of the tumors treated with anti-erbB2, CR-bearing human lymphocytes (C and F).
Moreover, such activation protects these cells from apoptosis and avoids anergy, functions that are also circumvented by exogenous IL-2 (40).

The effector mechanism by which the CR-bearing lymphocytes cause the arrest of tumor growth and its eventual elimination is most likely a combination of several arms. As has been shown before in in vitro experiments, T bodies can directly kill tumor cells and release proinflammatory cytokines (18, 22, 41) on their interaction with their target cells. Histological preparations made from the PC xenografts, several days or months after the administration of the erbB2-specific T bodies, show intense tumor necrosis and infiltration of human lymphocytes and of murine polymorphonuclear cells (Figs. 4, C and F and 6, A and B). This rejection process is caused by the tumor-specific human lymphocytes (stained by anti-CD45RO). Xenografts injected with the control TNP-specific T bodies show low levels of the human lymphocytes at the margin of the tumor with no apparent inflammatory response from the host SCID mouse. It should be noted here that the antitumor activity of the T bodies might be lower in our SCID mouse model because the strain that we used lacks natural killer cells, which are important players in the process of tumor rejection.

Because the human PBLs used in this study were obtained from healthy donors, unrelated to the patient, the contribution of an allo- geneic response to the antitumor activity cannot be entirely excluded. To evaluate the contribution of such effect, all of the experiments included controls of irrelevant TNP-specific, CR-bearing lymphocytes produced in parallel and under the same conditions as the erbB2-specific ones. Indeed, in some experiments, especially in the presence of IL-2, some antitumor effect was observed by such TNP-specific lymphocytes, which was similar to that of untransduced lymphocytes (data not shown; Fig. 3). Nevertheless, this effect was insignificant in terms of the overall antitumor activity in comparison with the effect obtained by the erbB2-specific allogeneic lymphocytes. That allogeneic response does not play a major role in the antitumor effect observed by the tumor-specific human lymphocytes is also manifested by the histological examination (Figs. 4 and 6), where the control lymphocyte-treated groups showed no effect.

In this model system, we have demonstrated the therapeutic potential of the T-body approach by intratumoral administration of the genetically redirected lymphocytes into primary PC xenografts. This therapeutic model is especially attractive and feasible for localized primary prostate tumor and other localized tumors. PC is a slowly progressing disease that can be detected quite early and monitored in particular risk groups. Therefore, a sufficient number of PBLs for later transfection can be removed and kept frozen at an early phase of the disease. The presence of PC antigens, such as PSA that serve as soluble marker, and, more importantly, prostate-associated surface antigens, such as PSMA (42), PSCA (7), PC antigen 1 (8), or STEAP (9), have been well characterized, and specific antibodies against these antigens have been prepared and can serve as a source of recognition units for PC-specific CRs (12). Most of these surface antigens (including the erbB2, used in the present study) are overexpressed on prostate adenocarcinoma cells. Although some of these antigens (such as PSMA, PSCA, and STEAP) are also expressed on normal prostate tissue or on some other normal tissues (e.g., PSMA), the intratumoral administration of the T bodies favors a local effect and damage only to the prostatic tissue. Biological prostatectomy is an acceptable consequence in this disease and advantageous because it may prevent local recurrence. The erbB2 orphan growth factor receptor chain is overexpressed in 40% of primary PC cases and increased to 80% of the cases with the progression of the disease (43). Taking into account the availability of humanized anti-erbB2 antibodies, which have already received regulatory approval, the option of using erbB2-based, T-body therapy for PC appears feasible.

8 D. Frydman-Morvinsky, A. Bendavid, D. G. Schindler and Z. Eshhar, unpublished data.
Currently, the T-body approach could be applied for PC therapy in several stages of the disease: (a) as a neo-adjuvant treatment of localized PC in cases where either surgery or radiotherapy (as monotherapies) are not recommended because of the locally advanced status of the disease (5); and (b) it may also serve as a second-line salvage therapy for local recurrent PC that resists other treatments. In practice, the T-body approach may also be applied for metastatic disease. For disseminated disease, a systemic application of the T bodies would be required. As yet, we could not achieve any therapeutic effect with systemic administration of T bodies, even with high amounts of the redirected human lymphocytes (data not shown). We doubt whether systemic therapy can be tested in the experimental SCID mouse model, because of species-specific incompatibilities between the human lymphocytes and the mouse environment and/or to the underdeveloped lymphatic system in the SCID mouse. More recent efforts using adoptive transfer of melanoma-specific, tumor-infiltrating lymphocytes into patients proved successful after lymphoablative treatment (44). Using similar strategies and the appropriate infiltration of lymphocytes into patients proved successful after lymphoablative treatment (44). Using similar strategies and the appropriate infiltration of lymphocytes into patients proved successful after lymphoablative treatment (44). Using similar strategies and the appropriate infiltration of lymphocytes into patients proved successful after lymphoablative treatment (44).

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