Rhabdomyosarcoma Lysis by T Cells Expressing a Human Autoantibody-Based Chimeric Receptor Targeting the Fetal Acetylcholine Receptor

Stefan Gattenlöhrer, Alexander Marx, Birgit Markfort, Sibylle Pscherer, Silke Landmeier, Heribert Juergens, Hans-Konrad Müller-Hermelink, Ian Matthews, David Beeson, Angela Vincent, and Claudia Rossig

Abstract

Rhabdomyosarcomas are the most frequent malignant soft tissue tumors of childhood; however, because current multimodality treatments fail to improve the poor survival rate of children with metastatic rhabdomyosarcoma, new treatments are required. We previously identified the γ-subunit of the fetal acetylcholine receptor (fAChR) as a specific cell surface target in rhabdomyosarcoma. Here, we engineered human T lymphocytes to express chimeric receptors composed of the antigen-binding domain of a human anti-fAChR antibody joined to the signaling domain of the human T-cell receptor ζ-chain. The interaction of fAChRγ-transduced T cells with fAChR-positive rhabdomyosarcoma cell lines, but not with fAChR-negative control cells, induced T-cell activation characterized by strong secretion of IFN-γ and delayed lysis of tumor cells. Importantly, we found that in six of six rhabdomyosarcoma patients, chemotherapy increased fAChR expression on residual tumor cells in vivo. Our observations suggest that these fully human chimeric fAChRγ-transduced T cells, which should be well tolerated by the patient, have potential use in vivo both as a primary treatment for rhabdomyosarcoma and as a complementary approach to eradi cate residual tumor cells after chemotherapy. (Cancer Res 2006; 66(1): 24-28)

Introduction

Rhabdomyosarcomas are the most common soft tissue sarcomas of childhood. Despite intensified, multimodality treatments, the overall survival for high-risk populations has remained at 5% to 20% over the last decades (1, 2). Therapeutic targeting of cancers with adoptively transferred cytotoxic T cells recognizing tumor-specific antigens has had encouraging success in lymphomas (3) and melanoma (4). T cells may be a therapeutic option for rhabdomyosarcoma, and the PAX3-FKHR fusion protein in alveolar rhabdomyosarcoma (5) or MAGE antigens (6) are potential targets. However, rhabdomyosarcomas express only low levels of MHC molecules and resist the MHC-restricted recognition by T cells (6). To bypass immune resistance, T cells have been engineered to express tumor-specific, chimeric receptors (chRec) for antigen recognition (7). In these recombinant receptors, an antibody-derived, single-chain scFv fragment is linked to the cytoplasmic signaling domain of the TCRζ chain. ChRec gene–modified T cells exhibit specific, MHC-independent in vitro lysis and cytokine secretion in response to antigen-expressing target cells and were immunoprotective in murine tumor xenograft models (7). A critical requirement is the presence of a tumor-specific target and availability of a specific antibody. A candidate target in rhabdomyosarcoma is the fetal type of the nicotinic acetylcholine receptor (fAChR; ref. 8). During development of the neuromuscular junction, a change from the fetal type (α2γζδ) to the adult type (α2δζδ) of the AChR occurs, with replacement of the γ-subunit by the δ-subunit (9). After birth, expression of fAChR is almost completely lost from mature muscle but is maintained in thymic myoid cells, some extracorpuscular muscle fibers, and denervated muscle (8, 10). In addition, fAChRs are highly expressed in rhabdomyosarcoma, distinguishing them from normal muscle (8, 10). Moreover, cloned human Fab antibodies specific for fAChR are available (11). Here, we created chimeric T cells with specificity for fAChR and show that they can mediate specific T-cell lysis of rhabdo myosarcoma cells.

Materials and Methods

Cell lines and antibodies. Cell lines were provided by Ewa Koscielniak (Olghospital, Stuttgart, Germany; rhabdomyosarcoma: FL-OH, Ax-OH-1), Paul H. Sorensen (University of British Columbia, Vancouver, British Columbia, Canada; rhabdomyosarcoma: Birch), Gary P. Nolan (Massachusetts Institute of Technology, Cambridge, MA; Phoenix-fco), and American Type Culture Collection (Manassas, VA; rhabdomyosarcoma: TE-671, A-204; leiomyosarcoma: SK-LMS-1). The DNA encoding adult AChR (α1, β, δ, γ) and fetal AChR (α1, β, δ, γ) was generated as described (9). Murine monoclonal antibody B8 (MIB8) binds to the γ-subunit of human AChR as shown previously (12). Santa Cruz Biotechnology (Heidelberg, Germany) provided the rabbit polyclonal antiserum (SC-1453) against the fAChR γ-subunit.

Cloning of chRec genes. The human recombinant Fab fragment, Fab 35, specific for the extracellular domain of the fetal AChR γ-chain, was isolated by phage display technology from a pregnant woman whose fetus was affected by in utero myasthenia gravis (11). The variable Fab domains Vh and Vk were assembled as scFv fragment by PCR. To generate the fAChR γ-chRec gene, the DNA encoding Vh and Vk were amplified by PCR and ligated into pHSV (provided by Zelig Eshhar, Rehovot, Israel) in frame with the DNA coding for the human IgG1 hinge domain and transmembrane and intracellular domain of the human TCR ζ-chain. The chimeric gene was subcloned into the retroviral vector SFG (13). Expression of the chRec in SFG-transduced cells was proved by reverse transcription-PCR (RT-PCR). The negative control CD19 chRec was cloned as reported previously (14).

Note: S. Gattenlöhrer and A. Marx share first authorship.

Requests for reprints: Stefan Gattenlöhrer, Institute of Pathology, University of Wuerzburg, Josef-Schneider-Str. 2, D-97080 Wuerzburg, Germany; Department of Pediatric Hematology and Oncology, University Children’s Hospital Münster, Münster, Germany; and Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom.
Production of recombinant retrovirus. Fresh retroviral supernatants from transiently transfected Phoenix-eco cells were used to infect the packaging cell line PG-13 in the presence of polybrene (8 μg/mL) for 48 hours at 32°C. After overnight incubation at 37°C in fresh culture medium, infected cells were subjected to a second identical round of infection. Supernatants were generated on the resulting bulk producer cell lines by incubation for 24 hours at 32°C and used to transduce T cells.

Isolation and transduction of lymphocytes. Fresh peripheral blood mononuclear cells from five healthy donors were prestimulated with CD3- and CD28-specific antibodies and transduced as described (15). Bulk-transduced T cells containing >98% CD3+ T cells (coexpressing CD8 in mononuclear cells from five healthy donors were prestimulated with CD3- and CD28 antibodies in 24-well plates with fAChR~–transduced tumor cells at a stimulator-to-effector ratio of 3:1 in the presence of rhIL-2. After 5 days, transduced (CFSE–) and nontransduced (CFSE+; CD3–) T cells were analyzed for IFN-γ production by ELISPOT. Isolated lymphocytes were transduced with chRec, combined with primer 3-17 years, mean = 10.7 years) with either an alveolar (T1; y) or embryonal (T2; h) histology and grown on histologic slides or in cell culture flasks were subjected to single-agent chemotherapy for 3 days using either doxorubicin (250 nmol/L), etoposide (5 nmol/L), or cisplatinum (140 nmol/L). TE-671 cells on glass slides were fixed with acetone before immunofluorescence analysis. For RT-PCR and flow cytometry studies, TE-671 cells were released from cell culture flasks by trypsinization.

Immunohistochemistry and immunofluorescence studies. Immunohistochemistry and immunofluorescence studies on fresh frozen sections and rhabdomyosarcoma cells grown on histologic slides, respectively, followed standard protocols. Percentages of MIB8-immunoreactive cells were calculated from tumor cells counted in at least 10 high-power fields (at >400 magnification).

Results

FACHR−transduced T cells produce IFN-γ in response to FACHR-expressing tumor cells. To show specific recognition of FACHR-expressing rhabdomyosarcoma cells by chR-transduced T cells, we measured IFN-γ secretion after 72 hours of coculture. ChRec composed of the scFv domains of the anti-FACHR Fab 35 in V~g~V~h~ orientation fused to the signaling moiety of the anti-CD19 scFv (Fig. 1B) secreted much less IFN-γ. Thus, it seems that the interaction between FACHR~−~ and FACHR triggers a functional and specific T-cell response associated with IFN-γ secretion.

Increasing FACHR density on rhabdomyosarcoma cells confers higher susceptibility to FACHR-mediated immediate cytotoxicity. Although FACHR~−~ T cells exhibited FACHR-specific IFN-γ production (Fig. 1A), they failed to mediate significant lysis of these rhabdomyosarcoma cell lines within the 4-hour incubation period typically used in 51Cr release assays (Fig. 2A). Therefore, we transiently transfected TE-671 cells with fetal AChR (α~h~β, γ; henceforth called TE-671/~γ~), which led to higher affinity for the surface FACHR expression level (Fig. 2B). Indeed, TE-671/~γ~ cells were consistently lysed better than native TE-671 cells (Fig. 2A) or TE-671 cells transduced with adult AChR (α~h~β, γ; henceforth called TE-671/~e~). Furthermore, the native FACHR~high~
rhabdomyosarcoma cell line Ax-OH-1 was even better lysed than TE-671/γ cells (Fig. 2A). Moreover, secretion of granzyme B, an important mediator of immediate T-cell cytolysis, was lower in cocultures with fAChR<sub>low</sub> (TE-671; TE-671/κ; Birch; FL-OH-1) compared with fAChR<sub>high</sub> (TE-671/γ; Ax-OH-1) rhabdomyosarcoma cell lines (Fig. 2C).

fAChR<sub>C</sub>-expressing T cells efficiently lyse fAChR-expressing tumor cells during long-term coculture. The above findings showed resistance of low AChR–expressing rhabdomyosarcoma

---

**Figure 2.** Short-term cytotoxicity of chRec<sup>+</sup> (fAChR<sub>C</sub>) and nontransduced (NT; negative control) T cells toward different fAChR-positive and fAChR-negative rhabdomyosarcoma cell lines. A, cytolysis of fAChR<sub>high</sub> rhabdomyosarcoma cell line Ax-OH-1 (spontaneously fAChR<sub>high</sub>) and TE-671/γ (fAChR<sub>high</sub> due to transfection) by fAChR<sub>C</sub> T cells was compared with cytolysis of nontransduced T cells and T cells transfected with the fAChR<sub>C</sub>-chain in a 4-hour <sup>51</sup>Cr release assay. *, P < 0.01. One representative experiment of three. Points, percentage of the maximum <sup>51</sup>Cr release following Triton X-100 treatment. E:T, ratio of effector (E) T cells to target (T) cell lines. B, expression of fAChRs on rhabdomyosarcoma cell lines TE-671, TE-671/κ, TE-671/γ, and Ax-OH-1 by flow cytometry analysis following staining with anti-fAChR antibody MIB8. C, granzyme B release assay with fAChR-positive rhabdomyosarcoma (TE-671, TE-671/κ, TE-671/γ, Ax-OH-1, FL-OH-1, and Birch) and fAChR-negative control (SK-LMS-1) cell lines. Granzyme B secretion is given as the number of GrB-positive spots per well using ELISPOT technology. One representative result of four experiments.

---

**Figure 3.** fAChR<sub>C</sub>-transduced T cells lyse fAChR-positive rhabdomyosarcoma cells during 7 days of coculture. TE-671 cells (A, B, and C) and Ax-OH-1, Birch, and FL-OH-1 rhabdomyosarcoma cells (C) were cultured with nontransduced and fAChR<sub>C</sub>-transduced T cells at T cell-to-tumor cell ratios of 1:1 (A), 5:1 (B), and 1:2.5 (C). Tumor cells were quantified by flow cytometry at 1, 2, 3, and 7 days after intracellular desmin staining. Representative of five experiments.
cells toward fAChR-specific cytolysis in short-term culture. Coincubation of rhabdomyosarcoma targets with fAChR-transduced T cells for 7 days, however, resulted in gradual elimination of tumor cells (Fig. 3A-C). Tumor cell numbers declined after 24 hours of coculture, dropped steeply up to day 3, and were almost undetectable by day 7. fAChR-expressing tumor cells incubated with nontransduced T cells were not lysed during long-term cocultures (Fig. 3A and B) nor were fAChR-negative control SK-LMS-1 cells (not shown).

**fAChR expression in rhabdomyosarcoma after chemotherapy.** It has been reported in a single case of rhabdomyosarcoma that AChR expression was increased after chemotherapy (17). To confirm this finding, we studied biopsies from six individual patients, both before and after chemotherapy, by immunohistochemistry and RT-PCR, using simultaneous amplification of the α- and γ-subunit mRNA (arrowheads α and γ, respectively, in C, lane 1). In the same patient, the residual rhabdomyosarcoma infiltrates in a biopsy after chemotherapy exhibit increased cell size and a relative increase of γ-subunit compared with α-subunit mRNA (F, lane 1). Analogous effects of chemotherapy were observed in a total of six patients with either embryonal (n = 4) or alveolar (n = 2) rhabdomyosarcoma (lanes 1-6 in C and F). Doxorubicin treatment (3 days, 250 nmol/L) has a profound influence on the in vitro morphology, including an increase in cell size (G and I) and fAChR γ-subunit expression (H and J) of embryonal rhabdomyosarcoma cell line TE671. There was up-regulation of fAChR α- and γ-subunit mRNA (arrowheads α and γ, respectively) in TE-671 cells after 3 days of *in vitro* treatment with cisplatinum (Cis, 140 nmol/L), etoposide (Eto, 5 nmol/L), and doxorubicin (Doxo, 250 nmol/L) compared with untreated control cells (ctr), as revealed by semiquantitative RT-PCR (K). H&E staining in (A and D); immunoperoxidase in (B and E); 400). mRNA was detected by semiquantitative RT-PCR (ethidium bromide stained 1.5% agarose gels in C, F, and K; M, molecular size marker; ref. 10). The morphology and fAChR protein expression of TE-671 cells are shown by phase contrast (G and I; 100×) and immunofluorescence (H and J) microscopy (×200; anti-AChR γ-subunit antibody SC-1453, respectively).

**Discussion**

We show that a chimeric T-cell receptor consisting of a recombinant human-derived scFv, specific for human fAChR and fused to the T-cell receptor ζ-chain, specifically recognizes fAChR-expressing rhabdomyosarcoma cells as indicated by short-term IFN-γ secretion and granzyme B release, and cytolysis of >90% of fAChR+ rhabdomyosarcoma cells after prolonged coculture. During short-term cultures, substantial cytolysis occurred only when rhabdomyosarcoma cells expressed higher fAChR levels, either spontaneously or by transfection. Moreover, we found that fAChR expression was increased after chemotherapy in biopsies of residual tumors of rhabdomyosarcoma patients compared with the respective tumors before chemotherapy. Based on these two findings, the fully human fAChR+ transduced T cell has some potential as a future treatment option for rhabdomyosarcoma and could, in addition, be helpful in eradicating residual cells after chemotherapy.

We chose to target the fAChR γ-chain because it is the first antigen shown to be overexpressed on both embryonal and alveolar rhabdomyosarcoma (8, 10). Due to its restricted expression on only a few...
extraocular muscle fibers and thymic myoid cells but not on innervated skeletal muscle (10), immune targeting of fAChR in patients with rhabdomyosarcoma could be tumor selective and the risk of side effects by cross-reactivity with normal tissues should be limited. This is supported by the observation that maternal autoantibodies specific for the fAChR can be present in asymptomatic mothers while causing paralysis in their developing fetuses (18).

Besides tumor specificity, the successful immunotherapeutic use of chRec-directed T cells requires two more antigen features. First, accessible extracellular domains and, second, stable expression on all or most tumor cells. The fAChR is a transmembrane protein with an extracellular domain that is a proved target for autoantibodies (9). In addition, although primary rhabdomyosarcoma tumor biopsies (8, 10) and rhabdomyosarcoma cell lines (Fig. 2B) show variable fAChR expression, most persisting rhabdomyosarcoma cells following chemotherapy exhibit strong fAChR expression (Fig. 4; ref. 17). Thus, the use of fAChR-specific T cells as an adjuvant to conventional chemotherapy should be considered.

One potential obstacle for the successful therapeutic use of chRec-expressing T cells is the limited survival of adoptively transferred xenogeneic T cells. The antigen-binding domains of most tumor-specific chRecs described to date are derived from murine antibodies, including the recently described immunotoxin that reacts with another target specifically expressed on rhabdomyosarcoma cells (19). Human anti-mouse antibody responses mediate rapid clearance of murine antibodies from the circulation, especially after repeated administration (20), and may thus limit the life span of T cells bearing murine antibody-derived recognition domains. Furthermore, humanization of mouse antibodies by complementarity determining region grafting does not completely abrogate immunogenicity. Our observation that the killing process in vitro requires prolonged contact times between sarcoma targets and effector T cells indicates that extended in vivo survival of chimeric T cells following adoptive transfer may be important for effective treatment of rhabdomyosarcoma. In addition, whereas immunotherapies against leukemias typically follow short-term treatment protocols (21), due to the easy accessibility of tumor cells, efficient T-cell homing to solid tumors may require prolonged survival or repeated application of the chimeric T cells (19, 20). Because the chimeric T-cell receptor described here is fully human, it should show enhanced persistence in vivo.

The molecular mechanisms underlying long-term cytotoxicity of fAChR-directed T cells toward fAChR§ rhabdomyosarcoma cell lines remain unclear. However, the Fas/FasL system seems not to be involved: Although we found virtually no expression of FAS on TE-671 or FL-OH-1 cells by flow cytometry, substantial FAS expression occurred on Birch or Ax-OH-1 cells (not shown). Nevertheless, fAChR-directed T cells killed the various rhabdomyosarcoma cell lines to a similar extent (Fig. 3).

In summary, we show for the first time that fAChR§-transduced T cells are specifically activated by tumor target cells expressing fAChR, and specifically eliminate rhabdomyosarcoma cells from long-term cocultures. Furthermore, we show that fAChR expression on rhabdomyosarcoma can be increased by chemotherapeutic agents in vitro and confirm the previous observation (17) that fAChR hyperexpression is characteristic of rhabdomyosarcoma cells surviving chemotherapy in rhabdomyosarcoma patients. Therefore, our findings open new perspectives for clinical trials to evaluate fAChR§-transduced T cells for potential immunother-apy of rhabdomyosarcoma and as a tool to eradicate chemoresistant residual disease in rhabdomyosarcoma patients.

Acknowledgments

Received 2/18/2005; revised 9/12/2005; accepted 11/11/2005.

Grant support: Bundesministerium für Bildung und Forschung (C. Rossig), Dr. Mildred-Scheel-Stiftung der Deutschen Krebshilfe grant 10-1859 (C. Rossig), and Wilhelm-Sander-Stiftung grant 99.1121 (S. Gattenlöchner and A. Marx), all in Germany.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Malcolm K. Brenner and Cliona A. Rooney for helpful discussions and Erwin Schmitt and Margrit Bonengel for expert technical assistance.

References


Downloaded from cancerres.aacrjournals.org on April 8, 2017. © 2006 American Association for Cancer Research.
Rhabdomyosarcoma Lysis by T Cells Expressing a Human Autoantibody-Based Chimeric Receptor Targeting the Fetal Acetylcholine Receptor

Stefan Gattenlöhner, Alexander Marx, Birgit Markfort, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/1/24

Cited articles
This article cites 21 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/1/24.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/66/1/24.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.