Ligand-mediated cytotoxicity of tumor cells: Use of heregulin- ζ chimeras to redirect cytotoxic T lymphocytes

Ashok Muniappan, Babak Banapour, Jane Lebkowski, and Sohel Talib

RPR Gencell, Santa Clara, California 95054.

New specificities may be engrafted onto lymphocytes by the transfer of genes for chimeric receptors that combine antigen recognition and signal-transducing elements. We have engineered and evaluated a new class of chimeric receptors that use the natural ligands of receptors found to be frequently overexpressed by cancer cells. The heregulin molecule, a ligand for Her3 and Her4 receptors when fused with the CD3 ζ -chain, was capable of redirecting T lymphocytes to recognize and respond to cancer cell lines that overexpress these receptors. Thus, CD8⁺ T lymphocytes were isolated from a healthy individual and transduced to express the chimeric heregulin- ζ receptor. These modified effector cells acquired the ability to specifically lyse a breast cancer cell line that overexpresses Her3 and Her4. A new class of chimeric receptors, such as heregulin- ζ , endowing anti-cancer effector cells with the potential to recognize and eliminate tumor targets, are likely to increase the effectiveness of adoptive immunotherapy for the treatment of cancer. **Cancer Gene Therapy (2000) 7, 128–134**

Key words: T lymphocytes; cytotoxicity; signal transduction; T-cell receptors; immunotherapy.

A primary objective of adoptive immunotherapy of cancer is the delivery of tumor-specific lymphocytes to the patient (reviewed in Ref. 1). Various lines of evidence indicate that such cells are capable of mediating regression of the cancer.² The effectors of adoptive immunotherapy that have been studied most extensively are lymphocytes isolated from the tumor or ascites (tumor-infiltrating lymphocytes, tumor-associated lymphocytes) and expanded in culture in the presence of cytokines, lymphocytes that have been sensitized *ex vivo* with a variety of tumor antigens (Ags) and Ag-presenting cells (e.g., dendritic cells), or nonspecific effectors such as lymphokine-activated killer cells. Both clinical and experimental experience indicate that it is difficult to consistently generate tumor-specific cells from cancer patients, especially with advanced disease.

The difficulty of generating tumor-specific lymphocytes by methods currently in existence could be surmounted by genetically modifying a naive population of lymphocytes to express tumor-specific receptors. Such receptors have been engineered by the genetic fusion of an Ag recognition domain with a molecule capable of transducing activation signals in lymphocytes.³ Prototypes of engineered tumor-specific receptors have been those resulting from fusions of oncoprotein-specific single-chain Fv (variable domain of antibodies) and either the CD3 ζ -chain or the Fc receptor-associated γ -chain. The single-chain Fv was derived from mouse monoclo-

nal antibodies (Abs) that bind either the Her2 receptor^{4,5} or the 38-kDa folate binding protein,⁶ molecules that are associated with breast or ovarian cancers, respectively. Cytotoxic T lymphocytes expressing these chimeric receptors were shown to be capable of killing tumor cells overexpressing the target Ags.⁴

We report here on a new class of tumor-specific chimeric receptors that use the natural ligands of molecules found to be overexpressed by cancer cells. Heregulin is one such ligand that was initially identified for its ability to interact with the Her2 receptor.⁷ It was later demonstrated that heregulin does not bind Her2 directly, but binds Her3 and Her4, two other members of the human epidermal growth factor (EGF) receptor family.^{8,9} Various isoforms of heregulin have been identified, including $\alpha 1$, $\beta 1$, $\beta 2$, and $\beta 3$.⁷ Her3 and Her4 overexpression has been documented in many breast cancer cell lines and recently in breast, ovarian, prostatic, and gastric cancers of patients.^{10,11} Chimeric heregulin- ζ (hrg- ζ) receptors were engineered to generate molecules capable of conferring Her3 and Her4 specificity in a non-major histocompatibility complex-restricted manner to T cells. Such T cells are potentially capable of mediating tumor immunity in adoptive immunotherapy of cancer.

MATERIALS AND METHODS

Cell lines

All cell lines were acquired from the American Type Culture Collection (Manassas, Va). The Jurkat, MDA-MB453, MDA-MB231, and COS cell lines were cultured in RPMI 1640 (BioWhittaker, Walkersville, Md) and 10% fetal bovine serum

Received November 17, 1998; accepted March 6, 1999.

Address correspondence and reprint requests to Dr. Sohel Talib, Cerus Corporation, 2525 Stanwell Drive, Suite 300, Concord, CA 94520. E-mail address: talibs@ceruscorp.com

(FBS) (HyClone Laboratories, Inc., Logan, Utah). BT-474 was cultured in Dulbecco's modified Eagle's medium (BioWhittaker), 10% FBS, and 10 μ g/mL bovine insulin (Life Technologies, Gaithersburg, Md). SKOV3 was cultured in McCoy's 5A medium (BioWhittaker) and 10% FBS. All cell lines were cultured at 37°C and 5% CO₂.

Plasmid constructs and recombinant vaccinia

Reverse transcription coupled with polymerase chain reaction (RT-PCR) was used to amplify DNA fragments encoding the immunoglobulin (Ig)-like and EGF-like domains (amino acids 35–239) of two isoforms of heregulin, α 1 and β 1 (hrg α and hrg β , respectively).¹² The same sense primer, 5'-AACGGCTAGCAT-TGCCTCCCCAATTGAAAGAGATGAAAAGCCAG-3', was used to amplify both hrg α and hrg β . The antisense primers used to amplify hrg α and hrg β were 5'-TCGGATCCTGGTACAGCT-GCTCCGCCCTTTCTTGGTTTGGGA-3' and 5'-TCGGATC-CCCCTCCATAAATTCAATCCCAAGATGCTTGTAGAA-3', respectively. The primers incorporated restriction sites (5', *Nhe*I; 3', *Bam*HI) for cloning of the heregulin DNA between sequences encoding the CD5 leader and a fusion of the CH2CH3 domains of IgG, the CD34 transmembrane region, and the CD3 ζ -chain (B.B. and B.S., manuscript in preparation). The entire fusion gene was subcloned into the pCDNA3 vector, a standard mammalian expression vector that contains the selectable marker neo^r (Invitrogen, San Diego, Calif). The constructs are named p3-hrg α ch ζ and p3-hrg β ch ζ . The hrg α ch ζ and hrg β ch ζ fusion genes were subcloned into the vaccinia virus expression plasmid pTKG, which contains the *gpt* gene as a selectable marker.³ Recombinant vaccinia virus was generated by homologous recombination¹³ with the WR strain (mouse neurotropic strain, American Type Culture Collection) and selection by mycophenolic acid (Sigma, St. Louis, Mo). All stocks were at high titers, and expression of hrg- ζ was confirmed by flow cytometric analysis of infected cells. The recombinant vaccinia virus expressing the CD4/CH2CH3/ ζ fusion molecule that was used as a control in these studies will be described elsewhere (B.B. and B.S., manuscript in preparation). RT-PCR was used to amplify the DNA encoding the Her2, Her3, and Her4 receptors. The primer pairs (sense/antisense) used to amplify Her2, Her3, and Her4 were as follows: 5'-ATAACGCGTGCCACCATGGAGCTGGCG-GCCTTG-3'/5'-ATATCTAGATCACAATGGCAGTC-CAG-3'; 5'-ATAACGCGTGCCACCATGAGGGCGAACG-ACGCT-3'/5'-ATATCTAGATTACGTTCTCTGGGCATT-3'; and 5'-ATAACG CGTGCCACCATGAAGCCGGCGACAG-GA3'/5'-ATATCTAGATTACACCACAGTATTCCG-3', respectively. The sense primers also included a consensus kozak sequence and a *Mlu*I restriction site. The antisense primers included a *Xba*I restriction site. The DNA amplified by RT-PCR was cloned into the pCI-Neo vector (Promega, Madison, Wis).

Transfection of hrg- ζ chimeras and T-cell activation assay

The hrg- ζ chimeras were permanently introduced into the Jurkat cell line (Jurkat heregulin α (J.HRG α) and Jurkat heregulin β (J.HRG β)) by liposome-mediated transfection and selection under 1 mg/mL G418 (Life Technologies). When cell death was evident, the remaining live cells were harvested by Ficoll density gradient separation and expanded in the presence of additional G418. Expression of the constructs was verified by flow cytometry. Functional studies were performed with both bulk-selected populations and clones isolated by limiting dilution cloning. J.HRG α and J.HRG β cells (2.5×10^4 cells) were incubated with various tumor cell lines (5×10^4

cells) in the presence of 5 ng/mL phorbol myristate acetate (Sigma) for 16 hours at 37°C and 5% CO₂. Interleukin-2 (IL-2) release by activated T cells was quantitated by an IL-2 enzyme-linked immunosorbent assay (ELISA) (Genzyme, Cambridge, Mass). Peptides consisting of the binding domains of heregulin or EGF (R&D Systems, Minneapolis, Minn) were added to the incubations to test for their ability to inhibit T-cell activation by the MDA-MB453 cell line. J.HRG α and J.HRG β were also incubated with COS cells transiently transfected with Her2, Her3, Her4, or vector alone. COS cells (1.5×10^4) were transfected with 1 μ L of lipofectamine (Life Technologies) and 200 ng of plasmid DNA in low sera OPTI-MEM media (Life Technologies). Transfected COS cells were incubated for 24 hours before T cells were added; IL-2 release was measured by ELISA after incubating for 16 hours.

Flow cytometry

Cells (5×10^5) were stained with fluorescein isothiocyanate-conjugated goat anti-human IgG γ -chain-specific Ab (Sigma) in phosphate-buffered saline and 1% FBS for 30 minutes at 4°C. Cells were washed once and fixed in 1% paraformaldehyde. Stained cells were analyzed on a FACScan instrument (Becton Dickinson, Mountain View, Calif).

Vaccinia-redirected cytotoxicity

CD8⁺ T lymphocytes were isolated from the peripheral blood of a healthy donor. Peripheral blood mononuclear cells were separated from red blood cells by standard Ficoll density gradient separation. CD8⁺ cells were captured on a CD8⁺ separation device (RPR Gencell, Santa Clara, Calif) and grown in AIM-V media (Life Technologies), 10% FBS, and 300 international units of IL-2 per milliliter (Chiron, Emeryville, Calif). At 2 days after CD8⁺ capture, the cells were depleted of contaminating CD4⁺ cells and captured on a CD3⁺ separation device (RPR Gencell), which provides a mitogenic signal to CD8⁺ T cells. The expanding population of CD8⁺ T cells was maintained at $\sim 1 \times 10^6$ to 2×10^6 cells/mL and was provided with fresh media and IL-2 every 2–3 days. Cells were grown for ≥ 7 days before use in cytotoxicity assays. Vaccinia infection of the T cells and cytotoxicity assays were performed essentially as described by Romeo and Seed.³ Briefly, T cells were infected with either nonrecombinant vaccinia WR virus or one of the recombinant viruses. T cells were incubated for 4 hours (including an initial 1-hour incubation with virus) to express the receptors. Target cells were labeled for 1 hour with ⁵¹Cr and washed three times. Infected T cells were incubated at various effector to target ratios (serial 2-fold dilution) with 7.5×10^3 labeled target cells for 4 hours at 37°C and 5% CO₂. Specific lysis was calculated as follows: (sample release – spontaneous release)/(maximum release – spontaneous release). Effector to target ratios were calculated after accounting for the exact proportion of T cells expressing the chimeric receptors (quantified by flow cytometry).

RESULTS AND DISCUSSION

To create the chimeric receptor, the genes for the Ig-like and EGF-like domains of hrg α and hrg β were fused with the genes for the CH2CH3 domains of IgG and the CD3 ζ -chain (Fig 1A). Heregulin provided the Ag recognition domain, CH2CH3 provided an epitope tag, and ζ provided the signal-transducing activity. It was recently reported that a spacer region such as CH2CH3 may be

Figure 1. Construction of hrg- ζ chimeric receptors and surface expression in transfected Jurkat and 293T cells. **A:** The Ig-like and EGF-like domains of hrg α or hrg β , sufficient for binding with the Her3 and Her4 receptors, were fused with the CH₂CH₃ domains of the IgG heavy chain and the CD3 ζ -chain. The leader and transmembrane regions were derived from CD5 and CD34, respectively (B.B. and B.S., manuscript in preparation).²⁸ **B:** Surface expression of the chimeric receptors in stable Jurkat transfectants was verified by flow cytometry. The indicated clones were stained with a fluorescein isothiocyanate-conjugated anti-human IgG γ -chain-specific Ab (solid lines). Background fluorescence was assessed by staining parental Jurkat cells with the same anti-IgG Ab (dotted lines). **C:** Greater surface expression of the chimeric receptors was observed in transient transfections of the 293T cell line. Flow cytometry analysis was performed by staining (anti-IgG Ab) 293T cells transfected either with constructs encoding the chimeric receptor (solid lines) or with vector alone (dotted lines).

required for efficient Ag binding and signaling by chimeric receptors;¹⁴ therefore, we included it in the chimeric construct. The fusion gene was cloned into a standard mammalian expression vector (pCDNA3),

which also contains a selectable marker (neo^r). Stable expression of the chimeric hrg α - ζ and hrg β - ζ receptors in Jurkat cells was achieved by liposome-mediated transfection and selection under G418. When the Jurkat

Table 1. Hrg- ζ -Transfected Jurkat Cell Reactivity Toward Various Tumor Targets*

Tumor line	IL-2 release (pg/mL)			Receptor overexpression†		
	J.HRG α	J.HRG β	Jurkat	Her2	Her3	Her4
MDA-MB453 (breast)	1743	2243	<10	+	+	+
BT-474 (breast)	1765	964	<10	+	+	+
MDA-MB231 (breast)	31	14	<10	—	—	—
SKOV3 (ovarian)	50	27	<10	+	—	—

*Jurkat cells were transfected either with the hrg α - ζ (J.HRG α) or hrg β - ζ (J.HRG β) receptors. A total of 25,000 transfected or parental Jurkat cells were cocultured with 50,000 tumor cells for 16 hours. Culture supernatants were assayed for IL-2 content by ELISA to measure the extent of T-cell activation.

†The status of Her2, Her3, and Her4 overexpression on the various tumor cell lines has been determined previously by other investigators¹⁷ and is summarized above.

stable transfectants named J.HRG α and J.HRG β were stained with IgG γ -chain-specific Ab and analyzed by flow cytometry (Fig 1B), relatively low surface expression of the chimeras was observed. In contrast, high levels of surface expression were observed in transiently transfected 293T cells (Fig 1C).

Jurkat cells expressing either the hrg α - ζ or hrg β - ζ chimera were capable of responding specifically to tumor cell lines that overexpress Her3 and Her4. When J.HRG α and J.HRG β cells were incubated with the MDA-MB453 or BT-474 breast cancer cell lines, there was significant IL-2 release from Jurkat cells, which is a marker of T-cell activation (Table 1). Importantly, only negligible IL-2 release was observed by J.HRG cells incubated with MDA-MB231 or SKOV3 (Table 1), two cancer cell lines that do not overexpress Her3 and Her4. Parental Jurkat cells did not secrete significant levels of IL-2 under these conditions (Table 1). The pattern of heregulin reactivity revealed by these T-cell activation assays is in agreement with other assays of heregulin binding. For instance, several groups have reported that heregulin-toxin fusions are toxic only to tumor cells overexpressing Her3 or Her4.¹⁵⁻¹⁷

Although a correlation between J.HRG reactivity and Her3/Her4 overexpression was established, additional experiments were performed to formally demonstrate the specificity of the hrg- ζ chimeras. A recombinant soluble form of heregulin- α was tested for its ability to compete with hrg α - ζ or hrg β - ζ recognition of Her3/Her4. When soluble heregulin- α peptide was added to the coculture of J.HRG cells and MDA-MB453 cells, there was a significant dose-dependent inhibition of the response by J.HRG α but not of the response by J.HRG β (Fig 2, A and B). A recombinant soluble EGF peptide did not have any effect on the reactivity of J.HRG α and J.HRG β reactivity to MDA-MB453 (Fig 2, A and B). The inability of the heregulin- α peptide to compete with hrg β - ζ function is likely a consequence of the binding of the β isoform to Her3 and Her4 receptors with a very high affinity. Thus, it has been reported that the β isoforms of heregulin bind Her3/Her4 with an $\sim$ 10-fold higher affinity than the α isoforms.¹⁸

The specificity of the hrg- ζ receptors was also confirmed by the independent expression of Her2, Her3, and Her4 in COS cells, which do not normally express

heregulin receptors. COS cells were transiently transfected with constructs encoding Her2, Her3, Her4, or the vector alone. J.HRG α and J.HRG β cells were cocultured with the transfected COS cells. Significant IL-2 release was only observed when the J.HRG cells were cultured with COS cells expressing Her3 or Her4 (Fig 2C). This emphasizes the fact that heregulin binds Her3 and Her4, but not Her2. However, this experiment does not exclude the possibility that there are other unidentified receptors for heregulin.

The hrg- ζ receptor chimera redirects T cells to recognize and respond to tumor cells that overexpress Her3 or Her4. In our experiments, recombinant vaccinia virus containing the chimeric genes was used to express the receptors in CD8⁺ T cells isolated from the peripheral blood of a healthy individual. Flow cytometry verified expression of the chimeras in T cells infected with recombinant vaccinia (Fig 3A). These T cells were then tested for their ability to kill MDA-MB453 cells in a standard 4-hour ⁵¹Cr release cytotoxicity assay and were found to mediate significant specific killing of MDA-MB453 (Fig 3B). T cells expressing the CD4- ζ chimera or infected with the nonrecombinant WR vaccinia strain showed no appreciable lytic capability (Fig 3B). As expected, hrg- ζ -expressing T cells did not kill the MDA-MB231 cell line (data not shown), which does not overexpress Her3 or Her4. Although in the present study, cytolysis of target cells by gene-modified T cells was used as an indicator of their effector function, cytokine release by the effector cells in conjunction with *in vitro* cytolytic activity may provide additional proof of their antitumor reactivity *in vivo*. Using this strategy, it may be possible to generate potent antitumor killer cells by engineering the cytotoxic T lymphocytes of patients with cancer to express the chimera. Our experiments suggest that an *ex vivo* gene therapy strategy that expresses these and similar chimeras in lymphocytes may generate potent effectors for adoptive immunotherapy.^{19,20}

Current research suggests that a potential impediment to the successful therapeutic delivery of gene-modified cells to patients is the immune response observed against proteins expressed by certain transgenes. The tumor-specific chimeric receptors described by others use single-chain Fv's that are derived from mouse Ab genes. It is expected that T cells expressing these genes

Figure 2. Hrg- ζ activity involves specific interaction of the heregulin domain with either the Her3 or Her4 receptors. **A:** A soluble recombinant hrg α peptide, but not a soluble EGF peptide, effectively competes with hrg α - ζ recognition of molecules (Her3 and Her4) on the MDA-MB453 breast cancer cell line. The peptides were added to the coincubation of J.HRG α with MDA-MB453 cells. **B:** There was no significant competition of hrg β - ζ activity (in J.HRG β) by either hrg α or EGF peptides. **C:** J.HRG α and J.HRG β cells were activated by COS cells transfected with genes for either the Her3 or Her4 receptors, but not the Her2 receptor or vector alone.

would likely become targets of an anti-mouse Ab (human anti-mouse Ab) immune response.²¹ Even humanization of these Abs does not exclude the possibility of an anti-idiotypic response. There is new evidence docu-

Figure 3. Redirected cytotoxicity of a breast cancer cell line by CD8⁺ T cells expressing hrg- ζ chimeric receptors. **A:** Recombinant vaccinia virus containing the genes for CD4-Ig- ζ , hrg α - ζ , or hrg β - ζ was used to infect CD8⁺ T cells isolated from a healthy donor. Expression of the receptors in infected cells was determined by staining with an anti-IgG Ab and flow cytometry. The proportion of cells staining positive is indicated. **B:** CD8⁺ T cells expressing either the hrg α - ζ or hrg β - ζ receptors are capable of lysing the MDA-MB453 breast cancer cell line in a standard 4-hour chromium release assay. T cells expressing the CD4-Ig- ζ chimera and T cells infected by nonrecombinant virus (WR) were not cytotoxic toward MDA-MB453 cells.

menting the rapid elimination of T cells expressing foreign proteins in patients.²² In contrast, the hrg- ζ chimera is less likely to be a target of an immune response, because it uses human genes exclusively. Additional experiments are necessary to determine whether

the junctions of the gene fusions are immunogenic in humans.

A potential problem with engineering auto-specific T cells is that they may be reactive not only with tumor cells but also with normal tissues that express low levels of the targeted Ag. It should be noted, however, that both humoral and cell-mediated immune responses to overexpressed tumor-associated Ags such as Her2 and Melan-A/Mart1, which are expressed in normal tissues as well, can be documented in cancer patients.²³⁻²⁶ Indeed, a variety of current immunotherapeutic strategies actively seek to target such Ags.²⁷ Hrg- ζ -expressing T cells will be tested in an animal model of a cancer that overexpresses Her3/Her4. Although the studies performed here used vaccinia virus vectors to transfer the hrg- ζ gene into T cells to establish the proof of concept of ligand-mediated cytolysis, incorporation of the chimeric gene into more appropriate transducing vectors, such as retroviral or lentiviral vectors, will be required to monitor the long-term activity of modified T cells in animal models. Although efficacy in controlling the cancer will be determined, indications of autoimmune reactions will also be monitored.

The hrg- ζ chimera is a novel tumor-specific receptor that is capable of redirecting lymphocytes to recognize and respond to tumor cells in a non-major histocompatibility complex-restricted manner. Adoptive immunotherapy mediated by lymphocytes armed with such receptors may enhance clearance of solid tumors and limit metastatic disease. The principle advantage to using a natural ligand-based chimera, as opposed to Ab-based chimeras, is a reduction in the likelihood of an immune response that would eliminate the T cells expressing the transgene. Based on this work, other ligand based receptors may be designed for the redirected cytolysis of malignant tissues.

ACKNOWLEDGMENTS

We thank Drs. James Allison, Nilabh Shastri, Theresa Whiteside, and Subramaniam Malarkannan for helpful comments on the manuscript.

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