A potential therapy for chordoma via antibody-dependent cell-mediated cytotoxicity employing NK or high-affinity NK cells in combination with cetuximab

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OBJECTIVE Chordoma is a rare bone tumor derived from the notochord and is resistant to conventional therapies such as chemotherapy, radiotherapy, and targeting therapeutics. Expression of epidermal growth factor receptor (EGFR) in a large proportion of chordoma specimens indicates a potential target for therapeutic intervention. In this study the authors investigated the potential role of the anti-EGFR antibody cetuximab in immunotherapy for chordoma.

METHODS Since cetuximab is a monoclonal antibody of the IgG1 isotype, it has the potential to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) employing natural killer (NK) cells as effectors. Polymorphisms in the CD16 allele expressed on NK cells have been shown to influence the degree of ADCC of tumor cells, with the high-affinity valine (V)/V allele being responsible for more lysis than the V/phenylalanine (F) or FF allele. Unfortunately, however, only approximately 10% of the population expresses the VV allele on NK cells. An NK cell line, NK-92, has now been engineered to endogenously express IL-2 and the high-affinity CD16 allele. These irradiated high-affinity (ha)NK cells were analyzed for lysis of chordoma cells with and without cetuximab, and the levels of lysis observed in ADCC were compared with those of NK cells from donors expressing the VV, VF, and FF alleles.

RESULTS Here the authors demonstrate for the first time 1) that cetuximab in combination with NK cells can mediate ADCC of chordoma cells; 2) the influence of the NK CD16 polymorphism in cetuximab-mediated ADCC for chordoma cell lysis; 3) that engineered haNK cells—that is, cells transduced to express the CD16 V158 FcγRIIIa receptor—bind cetuximab with similar affinity to normal NK cells expressing the high-affinity VV allele; and 4) that irradiated haNK cells induce ADCC with cetuximab in chordoma cells.

CONCLUSIONS These studies provide rationale for the use of cetuximab in combination with irradiated haNK cells for therapy for chordoma.

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KEY WORDS chordoma; antibody-dependent cell-mediated cytotoxicity; ADCC; epidermal growth factor receptor; EGFR; cetuximab; immunotherapy; oncology

Chordoma, a rare bone tumor, is derived from residual notochord. It accounts for 20% of primary spinal tumors and only 1%–4% of all malignant bone tumors. Approximately 300 new chordoma cases per year are diagnosed in the US, with approximately 2400 chordoma patients alive in the US annually. Median overall survival from the time of diagnosis is an estimated 6–7 years. Surgery followed by radiation therapy is the usual “standard of care,” but the anatomical location and size of the tumor often prevent curative excision with clear margins. Thus, relapse is common and metastases have been reported in up to 40% of cases. The US Food and Drug Administration has approved no therapeutic agent for chordoma, and the disease entity is largely resistant to standard cytotoxic chemotherapy, creating an urgent need for novel therapeutic modalities.

Cytotoxic chemotherapy’s ineffectiveness in chordoma has spurred efforts to identify new therapeutic modalities. mTOR, b-type platelet-derived growth factor receptor, vascular endothelial growth factor, and epidermal growth factor receptor (EGFR) have been identified as potentially relevant therapeutic targets for chordoma. Previous stud-
ies have shown that a large proportion of chordomas express EGFR.\textsuperscript{14,37,41} Cetuximab, an IgG1 anti-EGFR monoclonal antibody (mAb), is the only anti-EGFR agent that both blocks the EGFR-dependent proliferation pathway and has the potential to induce antibody-dependent cell-mediated cytotoxicity (ADCC).\textsuperscript{12} In vitro studies, cetuximab mediated ADCC in several types of cancer cells that express EGFR, including esophageal cancer, non–small cell lung cancer, and squamous cell carcinoma of the head and neck.\textsuperscript{26} Several therapeutic agents targeting EGFR, including erlotinib, gefitinib, lapatinib, and sapanitinib, have been shown to inhibit the proliferation of chordoma cells.\textsuperscript{33,35} To date, however, the use of radiation and/or these and other agents has resulted in an extremely low response rate in patients, that is, less than 5%. The potential of cetuximab-mediated ADCC in chordoma has not been investigated.

Antibody-dependent cell-mediated cytotoxicity is mediated by the binding of a human IgG1 antibody with its ligand on tumor cells and with the CD16 Fc receptor on natural killer (NK) cells. Interaction between IgG1 antibody-bound tumor cells and Fcγ receptor triggers the activation and degranulation of the NK cells (Fig. 1). Natural killer cells from healthy donors can express 3 types of polymorphism in the CD16 allele: 1) endogenous alleles for the CD16 high-affinity Fc receptor FcγRIIIa (V158) only (VV genotype), 2) endogenous alleles for the lower-affinity phenylalanine (F) allele only (FF genotype), or 3) both alleles (VF genotype). In general, the NK cells of the VL allele are the most efficient effectors in ADCC. Unfortunately, only approximately 14% of humans express the VV allele on NK cells.\textsuperscript{8,25,29,30,40,44} An NK cell line derived from a lymphoma patient has been shown, as an irradiated, adaptively transferred agent, to be safe and has provided preliminary evidence of clinical benefit.\textsuperscript{2,15,39} The NK-92 cell line, however, does not express CD16 and also requires IL-2 for propagation. This cell line has now been engineered to express the high-affinity (ha) CD16 V158 FcγRIIIa receptor, as well as further engineered to express IL-2, and is designated haNK.\textsuperscript{14}

Here we demonstrate for the first time 1) that cetuximab in combination with NK cells can mediate ADCC of chordoma cells; 2) the influence of the NK CD16 polymorphism in cetuximab-mediated ADCC for chordoma cell lysis; 3) that engineered haNK cells—that is, cells transduced to express the CD16 V158 FcγRIIIa receptor—bind cetuximab with similar affinity to normal NK cells expressing the high-affinity VV allele; and 4) that irradiated haNK cells induce ADCC with cetuximab in chordoma cells. Our findings suggest that while chordoma responds poorly to conventional therapies, the combination of adaptively transferred, irradiated haNK cells plus cetuximab may have clinical benefit for chordoma patients (Fig. 1).

Methods

Cell Culture and Reagents

The chordoma cell lines JHC7 and UM-Chor1 were obtained from the Chordoma Foundation. The chordoma cell lines U-CH2 (ATCC CRL-3218) and MUG-Chor1 (ATCC CRL-3219) were obtained from American Type Culture Collection. All cell lines were passaged for fewer than 6 months and were maintained as previously described.\textsuperscript{11} The haNK cells were provided through a Cooperative Research and Development Agreement (CRADA) between the National Cancer Institute and NantBioScience. The haNK cells were cultured in phenol red–free and gentamicin-free X-Vivo 10 medium (Lonza) supplemented with 5% heat-inactivated human AB serum (Omega Scientific Inc.) at a concentration of 5 × 10^5/ml. Twenty-four hours before all experiments, the haNK cells were irradiated at 10 Gy. Peripheral blood mononuclear cells (PBMCs) from healthy volunteer donors were obtained from the National Institutes of Health Clinical Center Blood Bank (NCT00001846).

Flow Cytometry

The following anti-human mAbs were used: phycoerythrin (PE)-EGFR (BD Biosciences), fluorescein isothiocyanate (FITC)-CD16 clone 3G8 (BD Biosciences), allopurinol (APC)-CD56 (BioLegend), PE-CD226 (DNAM-1; BD Biosciences), PerCP-Cy5.5-NKG2D (BD Biosciences), and PE-Cy7-perforin (eBioscience). Samples were acquired on a FACS Calibur or FACSVerse flow cytometer (both Becton Dickinson) and analyzed using FlowJo software (TreeStar Inc.). Isotype control staining was < 5% for all samples analyzed.

Antibody-Dependent Cellular Cytotoxicity Assay

The ADCC assay was performed as previously described,\textsuperscript{8} with indicated modifications. Natural killer effector cells were isolated from healthy donor PBMCs using the human NK Cell Isolation Kit (negative selection, Miltenyi Biotec) according to the manufacturer’s protocol, resulting in > 80% purity, and were allowed to rest overnight in RPMI-1640 medium containing 10% fetal bovine serum. Tumor cells were harvested and labeled with 111In. Cells were plated as targets at 2000 cells/well in 96-well round-bottom culture plates and incubated with 10 μg/ml of cetuximab (Erbitux, Eli Lilly) or unresponsive rituximab (Rituxan, Biogen) as a control isotype antibody at room temperature for 30 minutes. Either NK cells or haNK cells were added as effector cells. Various effector cell/target cell (E/T) ratios were used in the study. After 4 or 20 hours, supernatants were harvested and analyzed for the presence of 111In using a WIZARD2 Automatic Gamma Counter (PerkinElmer Inc.). Spontaneous release was determined using the following equation, where cpm stands for counts per minute: percent lysis = \left[\frac{[\text{experimental cpm} - \text{spontaneous cpm}]}{[\text{complete cpm} - \text{spontaneous cpm}] \times 100}\right].

To verify that CD16 (FcγRIII) on NK cells engages ADCC lysis mediated by cetuximab, CD16 mAb was used to block CD16. The NK cells were incubated with 2 μg/ml of CD16 mAb (clone B73.1, eBioscience) and haNK cells were incubated with 50 μg/ml of CD16 mAb, for 2 hours before being added to target cells.
CD16 (FcyRlla) Genotyping

DNA was extracted from PBMCs of healthy donors using a QiAamp DNA Blood Mini Kit (Qiagen) and stored at –80°C until use. The polymorphism of CD16 that is a valine (V) versus phenylalanine (F) substitution at amino acid position 158 was determined using allele-specific droplet digital polymerase chain reaction (PCR) employing the TaqMan array for CD16 (Life Technologies). A master reaction mix was prepared, and 1 μl of genotyping DNA was added. The PCR reaction was performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories) for 40 cycles at 95°C for 10 minutes, 94°C for 30 seconds, and 60°C for 1 minute. The plate was read on a Bio-Rad QX200 droplet reader. Data were analyzed with Bio-Rad QuantaSoft software version 1.5.

Cetuximab-Binding Properties of FcγRIIIa

Cetuximab-binding properties of FcγRIIIa were examined as previously reported, with indicated modifications. Natural killer cells or haNK cells were incubated with varying concentrations of cetuximab at 4°C for 30 minutes. Cells were then incubated with FITC-conjugated CD16 3G8 at 4°C for 30 minutes, washed twice with 1% bovine serum albumin/phosphate-buffered saline, and analyzed by flow cytometry. Results were expressed as the percentage inhibition of CD16 3G8 binding: (MFI in the absence of cetuximab – MFI in the presence of cetuximab) × 100/(MFI in the absence of cetuximab), where MFI represents mean fluorescence intensity.

Statistical Analysis

Significant differences in the distribution of data acquired by ADCC assays were determined by paired Student t-test with a 2-tailed distribution and reported as p values, using Prism 6.0f software (GraphPad Software Inc.). A p value < 0.05 was significant.

Results

Cetuximab Increases NK Cell Lysis of Chordoma Cell Lines via ADCC

It has been shown that chordoma cell lines express EGFR. We qualitatively confirmed and extended this finding employing 4 human chordoma cell lines: JHC7, UM-Chor1, U-CH2, and MUG-Chor1 (Fig. 2). The 4 chordoma cell lines expressed between 13% and 80% EGFR as determined by flow cytometry, although the absolute expression levels of EGFR can modulate with tissue culture density and time in culture.

We next performed an in vitro assay to determine cetuximab-mediated ADCC in chordoma cell lines employing NK cells from healthy donors as effectors. As shown in Fig. 3A, cetuximab significantly increased NK cell lysis relative to the isotype control antibody in JHC7 cells (13.7-fold, p < 0.01), UM-Chor1 cells (10.5-fold, p < 0.01), U-CH2 cells (83.5-fold, p < 0.01), and MUG-Chor1 cells (59-fold, p < 0.01). Cetuximab alone (no NK cells) did not mediate lysis of chordoma cells (data not shown). Natural killer cell lysis via ADCC occurs when CD16 (FcγRIII) on NK effector cells interacts with the Fc portion of antibodies recognizing target cells (Fig. 1). As shown in Fig. 3B, the addition of CD16 neutralizing antibody inhibited cetuximab-enhanced NK cell lysis in both the JHC7 and UM-Chor1 cell lines analyzed, indicating that cetuximab-induced NK cell lysis was mediated by ADCC.

CD16 Polymorphism of NK Cells is Associated With Cetuximab-Mediated ADCC of Chordoma Cells

The CD16 (FcγRIIIa) polymorphism expressed on NK cells is associated with cetuximab-mediated ADCC of chordoma cells.
cells is associated with affinity of CD16 for IgG1 mAbs (Fig. 1). The FCGR3A gene, which encodes FcγRIIIa, displays a functional allelic dimorphism that generates allotypes with either an F or a V residue at amino acid position 158. Previous studies have shown that the magnitude of response to cetuximab-mediated ADCC is related to FcγRIIIa polymorphism of NK cells in head and neck squamous cell carcinoma cells. We performed in vitro assays for ADCC activity mediated by cetuximab using FCGR3A-genotyped healthy-donor NK cells that expressed the FcγRIIIa-158 FF, VF, or VV allele. With the control isotype antibody, UM-Chor1 cells were killed at very low levels by NK cells regardless of NK phenotype (Fig. 4A). However, cetuximab increased NK cell lysis in all the NK cell phenotypes. Cetuximab-induced lysis by NK cells from 3 donors expressing the FcγRIIIa-158 FF allele was 24%, 17%, and 15%, respectively. In contrast, cetuximab-induced ADCC lysis by NK cells using 3 VF donors was 34%, 49%, and 32%, respectively, and 51%, 66%, and 59% lysis, respectively, using NK cells from 3 VV donors. There was a significant positive correlation ($R^2 = 0.85$) for the mean of cetuximab-mediated ADCC lysis induced by NK cells from 3 FF (19%), 3 VF (38%), and 3 VV (59%) donors (Fig. 4B). Taken together, these results suggest that NK cells that express the FcγRIIIa-158 V allotype exhibit enhanced cetuximab-mediated ADCC in chordoma cells.

**Phenotype of CD16 Polymorphism-Genotyped NK Cells and haNK Cells**

Natural killer cells from some individuals can be potent cytotoxic effectors for cancer therapy. However, there can be technical challenges in obtaining sufficient numbers of functionally active NK cells from patients. As an alternative, several cytotoxic NK cell lines have been generated, including NK-92. These NK-92 cells have recently been engineered to endogenously express IL-2 and haNK cells. We compared the phenotype (CD56, DNAM-1, NKG2D, perforin, and CD16) of CD16 polymorphism-genotyped healthy-donor NK cells with that of haNK cells. While there were only minor differences in the percentage of cells expressing a given marker, substantial differences in the levels of expression were observed, as determined by MFI. Compared with NK VV donors, haNK cells had a 20-fold higher MFI of CD56 (Fig. 5A), 2.9-fold higher expression of DNAM-1 (Fig. 5B), and 1.8-fold higher expression of NKG2D (Fig. 5C). There was no difference in perforin expression between NK cells and haNK cells (Fig. 5D). The mean MFI of CD16 was 1.5-fold higher in VV donors compared with FF donors and haNK cells (Fig. 5E).

**haNK Cell–Mediated ADCC of Chordoma Cells With Cetuximab**

To examine the potential utility of haNK cells for cetuximab therapy for chordoma, we performed an in vitro assay for cetuximab-mediated ADCC using haNK cells as effectors (Fig. 6A). Lysis by haNK cells with the isotype
FIG. 4. Cetuximab-mediated ADCC by CD16 polymorphism-genotyped NK cells. A: ADCC assays were performed with UM-Chor1, using NK cells from 3 FF, 3 VF, and 3 VV healthy donors at an E/T ratio of 10:1. Indicated groups were incubated with cetuximab. B: Correlation of cetuximab-mediated ADCC with NK cells from 3 FF, 3 VF, and 3 VV donors. *p < 0.05, Student t-test. Error bars indicate mean ± standard deviation for triplicate measurements.

FIG. 5. The phenotype of CD16 polymorphism-genotyped NK cells and haNK cells. Expression levels of CD56 (A), DNAM-1 (B), NKG2D (C), perforin (D), and CD16 (E) of NK cells from 3 FF, 3 VF, 3 VV healthy donors and haNK cells. Numbers indicate percentage of positive cells (upper) and MFI (lower). Bars indicate the mean.
control was 11.8% of JHC7 cells and 2.6% of UM-Chor1 cells. Cetuximab significantly enhanced haNK cell lysis compared with isotype control in both JHC7 (1.7-fold, \( p < 0.01 \)) and UM-Chor1 cells (2.6-fold, \( p < 0.01 \)). The addition of CD16 neutralizing antibody inhibited cetuximab-enhanced haNK cell lysis in both JHC7 and UM-Chor1 cell lines (data not shown). As NK cells have previously been shown to be “serial killers”—that is, 1 NK cell can lyse up to 5 target cells—an 2-hour release assay were also performed (Fig. 6B). In those tests, lysis of the 2 chordoma cell lines was markedly greater. These results indicate that haNK cells induce ADCC via cetuximab in chordoma cells. To determine relative affinities, we next compared the ability of cetuximab to inhibit the binding of FITC-conjugated CD16 mAb to CD16 polymorphism-genotyped healthy-donor NK cells and haNK cells (Fig. 7A). A 50% inhibition of CD16 antibody binding to NK cells from 4 FF donors was achieved with 220 \( \mu g/ml \) of cetuximab. Compared with FF donors, a 4.5-fold lower (49.2 \( \mu g/ml \)) concentration of cetuximab showed a 50% inhibition of CD16 antibody binding to healthy NK cells from a VV donor and haNK cells, respectively (Fig. 7B). These results show that both NK cells expressing FcγRIIIa-158 VV and haNK cells bind cetuximab with higher affinity than NK cells expressing FcγRIIIa-158 FF.

Discussion

Based on preclinical evidence of the role of EGFR in chordoma pathogenesis and immunohistochemical evidence that over 70% of chordoma specimens express EGFR, several clinical trials targeting EGFR have been undertaken in chordoma patients. Because these trials were not randomized or well controlled, however, no consensus has been reached concerning the therapeutic benefit of EGFR inhibition in chordoma. In 2 separate case reports, the combination of the EGFR mAb cetuximab and gefitinib, a tyrosine kinase inhibitor of EGFR, demonstrated partial radiographically defined responses. However, the potential of cetuximab-induced ADCC in chordoma was not directly examined. Here, we showed that cetuximab markedly and significantly increases NK cell lysis via ADCC in 4 of 4 chordoma cell lines (Fig. 3). Moreover, NK cells obtained from healthy donors carrying FcγRIIIa-158 VV induced higher cetuximab-mediated ADCC lysis in chordoma (Fig. 4). Previous in vitro studies have indicated that the FcγRIIIa-158 VV phenotype of NK cells enhances the affinity of CD16 to IgG1,14 inducing ADCC mediated via IgG1 mAb in several types of cancer cell lines.8,14,28 Some clinical studies have also shown that FcγRIIIa polymorphisms of NK cells correlated with response to IgG1 mAb therapy. Musolino et al. reported that patients with metastatic breast cancer who had FcγR2A-131 histidine (H)/H and/or FcγR3A-158 VV genotypes had a significantly better objective response rate and progression-free survival with trastuzumab therapy than patients with neither genotype.28 In a study of 49 patients with follicular lymphoma, FcγR3A-158 VV patients had an improved response to rituximab.6 Three retrospective studies in metastatic colorectal cancer patients treated with cetuximab reported that VV is the most beneficial FcγR3A-158 genotype.4,10,32 Taken together, our observations indicate that cetuximab has potential clinical benefit for chordoma patients, especially in those 14% of patients with the FcγR3A-158 VV genotype or in combination with haNK cell infusion.

Although ADCC induction can be observed in vitro, clinical translation raises some obstacles. First, recruiting sufficient numbers of functionally active NK cells to target tumor tissues is technically challenging since they represent only 10% of lymphocytes and are often dysfunctional in a cancer-induced immunosuppressive environment.9 Moreover, chemotherapy and radiation therapy, first-line treatment for metastatic and/or advanced chordoma, could also reduce the number and activity of lymphocytes. Adoptive NK cell therapies have been developed to supply sufficient numbers of functional NK cells for patients. The cytotoxic NK-92 cell line was generated for adoptive transfer therapy from a 50-year-old man with progressive non-Hodgkin’s lymphoma. Four Phase I trials in different malignancies have been conducted using irradiated NK-92 cells. The infusions were well tolerated, and clinical responses were observed in patients with hematological malignancies, melanoma, lung cancer, and kidney cancers. Since NK-92 cells do not express the FcγRIIIa receptor, they cannot mediate ADCC. The NK cell line designated haNK was established by inducing high-affinity CD16 V158 FcγRIIIa receptor to NK-92 cells.13 Since only approximately 14% of the population is homozygous for the high-affinity FcγRIIIa receptor (FcγR3A-158 VV),18,20 there is a clear rationale for infusing haNK cells into patients who carry the genotype of the low- or intermediate-affinity FcγRIIIa receptor to maximize mAb efficacy. Our results show that haNK cells have a 2.8-fold higher affinity to cetuximab than NK cells from healthy donors carrying
the FCGR3A-158 FF genotype (Fig. 7B). Consistent with their high cetuximab-binding ability, haNK cells significantly induced ADCC via cetuximab in chordoma cells (Fig. 6). Moreover, since $10^9$–$10^{10}$ irradiated NK-92 cells were safely administered to cancer patients, the potential for similar levels of adoptive transfer of irradiated haNK cells exists, even in patients whose endogenous NK cells express the VV phenotype.

NK-92 cells have been shown to express large numbers of activating receptors such as NKp30, NKp46, and NK-G2D.22 NKG2D and DNAM-1 are the best-characterized activating NK cell receptors implicated in the immune response against cancers. Both receptors recognize their ligands expressed on tumor cells and induce target cell lysis.27 Our data show that haNK cells, as compared with normal NK cells, have higher expressions of NKG2D and DNAM-1 according to MFI (Fig. 5A and B), indicating a greater ability to recognize and lyse tumor cells. Without cetuximab, NK cells from healthy donors lysed chordoma cells at extremely low levels (Figs. 3 and 4). In contrast, haNK cells induced greater lysis of chordoma cells without cetuximab (Fig. 6B).

**Conclusions**

Here we show for the first time that cetuximab can induce ADCC in chordoma cells, whereas chordoma cells cannot be killed in significant numbers by NK cells alone. Moreover, NK cells that express FcγRIIa-158 VV induced higher cetuximab-mediated ADCC of chordoma cells. An engineered NK-92 cell line transduced with haNK cells bound cetuximab with high affinity, resulting in haNK cell–induced ADCC via cetuximab in chordoma cells. Our results also indicate that cetuximab therapy could lead to a better clinical outcome for chordoma patients who have NK cells expressing the CD16 V158 FcγRIIIa receptor allele. Adaptively transferred, irradiated haNK cells could provide sufficient numbers of functional NK cells for all chordoma patients and could functionally convert FCGR3A-158 FF carriers to VV carriers. Our findings provide the rationale for cetuximab plus irradiated haNK cell–mediated immunotherapy for clinical benefit in patients with chordoma.

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Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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