Anti-EGFR Antibody Cetuximab Enhances the Cytolytic Activity of Natural Killer Cells toward Osteosarcoma

Jens H.W. Pahl1, S. Eriaty N. Ruslan1, Emilie P. Buddingh1, Susy J. Santos1, Karoly Szuhai2, Massimo Serra5, Hans Gelderblom3, Pancras C.W. Hogendoorn4, R. Maarten Egeler1, Marco W. Schilham1, and Arjan C. Lankester1

Abstract

Purpose: Osteosarcoma and Ewing’s sarcoma are the most common bone tumors in children and adolescents. Despite intensive chemotherapy, patients with advanced disease have a poor prognosis, illustrating the need for alternative therapies. Sarcoma cells are susceptible to the cytolytic activity of resting natural killer (NK) cells which can be improved by interleukin (IL)-15 stimulation. In this study, we explored whether the cytolytic function of resting NK cells can be augmented and specifically directed toward sarcoma cells by antibody-dependent cellular cytotoxicity (ADCC).

Experimental Design: Epidermal growth factor receptor (EGFR) expression was examined on osteosarcoma and Ewing’s sarcoma cell lines by flow cytometry and in osteosarcoma biopsy and resection specimens by immunohistochemistry. Cetuximab-mediated ADCC by NK cells from osteosarcoma patients and healthy controls was measured with 4-hour 51Cr release assays.

Results: EGFR surface expression was shown on chemotherapy-sensitive and chemotherapy-resistant osteosarcoma cells (12/12), most primary osteosarcoma cultures (4/5), and few Ewing’s sarcoma cell lines (2/7). In the presence of cetuximab, the cytolytic activity of resting NK cells against all EGFR-expressing sarcoma cells was substantially increased and comparable with that of IL-15–activated NK cells. Surface EGFR expression on primary osteosarcoma cultures correlated with EGFR expression in the original tumor. The cytolytic activity of osteosarcoma patient-derived NK cells against autologous tumor cells was as efficient as that of NK cells from healthy donors.

Conclusion: Our data show that the cytolytic potential of resting NK cells can be potentiated and directed toward osteosarcoma cells with cetuximab. Therefore, cetuximab-mediated immunotherapy may be considered a novel treatment modality in the management of advanced osteosarcoma.

Introduction

Osteosarcoma and Ewing’s sarcoma most frequently arise in adolescents and young adults and represents the majority of all malignant primary bone tumors in this patient group (1–3). The current treatment consists of a combination of systemic multidrug chemotherapy and complete surgical resection (3–5). In cases with localized disease, up to 70% of the patients achieve persistent remission. In contrast, patients with advanced, metastasized, and recurrent disease experience a very poor prognosis, which has not improved during the last decades despite intensification of chemotherapy regimens. Therefore, novel treatment strategies with a favorable toxicity profile are warranted. In this perspective, we and others have recently reported on the potential exploitation of cellular immunotherapy against sarcomas by natural killer (NK) cells (6–8).

NK cells can respond to and kill cells undergoing cellular stress due to virus infection or malignant transformation. The cytotoxic activity of NK cells is regulated by the equilibrium of inhibiting and activating signals conveyed by target cells. On tumor cells, MHC class I expression (NK cell inhibitory signal) may be downregulated to evade cytotoxic T-cell recognition. Conversely, the expression of stress ligands (NK cell–activating signal) may be upregulated on tumor cells. Both of these processes may lead to increased sensitivity of tumor cells to NK cells (9, 10). In addition, the interplay with other immune cells and the pro- or anti-inflammatory microenvironment may modulate the function and activity of NK cells (10, 11).
Recently, we and others have shown that sarcoma cell lines are moderately susceptible to the cytolytic potential of resting NK cells (6–8). The cytolytic activity of NK cells can be directly potentiated by activating cytokines, such as interleukin (IL)-15, leading to increased lysis of sarcoma cells (6–8). In this study, we set out to explore whether the cytolytic activity of resting NK cells can be improved and directed specifically toward sarcoma cells. Therefore, we intended using a monoclonal antibody (mAb) of the human IgG1 subtype which recognizes an antigen expressed on sarcoma cells and is able to induce antibody-dependent cellular cytotoxicity (ADCC) by NK cells. Because the administration of cetuximab has already resulted in objective responses in patients with other tumors, in association with only mild adverse effects, these findings provide a rationale for the implementation of cetuximab-mediated immunotherapy in the treatment of advanced osteosarcoma.

**Materials and Methods**

**Patient samples**

Formalin-fixed, paraffin-embedded tumor samples were obtained from one biopsy (obtained at the time of diagnosis, prechemotherapy) and 4 resections of local recurrent or metastatic tumors (postchemotherapy) from 4 high-grade osteosarcoma patients (diagnosed between 2008 and 2010) by the Department of Pathology, Leiden University Medical Center. Five short term grown primary osteosarcoma cell cultures (between passage 2 and 13) were generated from the tumor material as previously described (6, 14). Clinicopathologic details of these patients and samples are summarized in Table 1. Peripheral blood samples from these patients were collected prior to the initiation of chemotherapy after written informed consent approved by the Review Board on Medical Ethics of the Leiden University Medical Center and used for cytotoxicity experiments (Table 1). Tumor specimens were obtained and analyzed according to the ethical guidelines of the national organization of scientific societies (FEDERA, http://www.federa.org/gedragscodes-codes-conduct-en).

**Cell lines**

The following extensively characterized sarcoma cell lines were included in this study: osteosarcoma cell lines OHS, OSA (SISA-1), SAOS-2, U2-OS, ZK-58 (15) and the Ewing’s sarcoma cell lines A673, CADO-ES, SK-ES-1, SK-N-MC, STA-ET2.1, TC71 (15) and L1062 (14). All sarcoma cell lines were obtained from the EuroBoNeT cell line repository (by 2007) and were confirmed for their identity by short tandem repeat DNA fingerprinting in 2011. The cell line TC71 was maintained in IMDM medium (Invitrogen). All other cell lines were grown in RPMI-1640 medium (Invitrogen). Both media were supplemented with 10% chimeric IgG1 type mAb cetuximab (13). So far, the application of anti-EGFR mAb–targeted therapy in bone sarcomas has not been reported. We focused on exploring whether the cytotoxic potential of allogeneic and autologous NK cells can be specifically directed toward sarcoma cells with cetuximab.

### Table 1. Clinicopathologic details of patient material

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<th>Osteosarcoma patient</th>
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fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all Invitrogen). All Ewing’s sarcoma cell lines were grown in 0.1% gelatin-coated tissue culture flasks. The chemotherapy-resistant variant cell lines of SAOS-2 and U2-OS (16–18) were cultured in IMDM medium with 10% FCS and penicillin/streptomycin and maintained in chemotherapeutic drugs as follows: SAOS-2-DX580 and U2-OS-DX580 with 580 μg/mL of doxorubicin (DX); SAOS-2-MTX1000 and U2-OS-MTX300 with 1,000 and 300 ng/mL of methotrexate (MTX), respectively; SAOS-2-CDDP6 (SAOS-2-CDDP6μg) and U2-OS-CDDP4 (U2-OS-CDDP4μg) with 6 and 4 μg/mL of cisplatin (cis-diaminedichloroplatinum, CDDP), respectively. Drug sensitivities of each cell line were calculated from the drug dose–response curves and expressed as IC50 (drug concentration resulting in 50% inhibition of cell growth) after 96 hours of incubation (in vitro treatment). Fold increases in drug resistance, quantified as the ratio between IC50 of each drug-resistant variant to that of its corresponding parental cell line, were as follows: 315 for U2-OS-DX580, 135 for U2-OS-MTX300, 281 for SAOS-2-MTX1μg, 63 for U2-OS-CDDP4μg, and 112 for SAOS-2-CDDP6μg.

All cell lines were negative for Mycoplasma infection as regularly checked by PCR. The primary osteosarcoma cultures were maintained in RPMI-1640 medium supplemented with 20% FCS and penicillin/streptomycin in gelatin-coated culture tissue culture flasks.

Cell isolations and stimulations

Peripheral blood mononuclear cells (PBMC) were isolated from osteosarcoma patients’ blood samples (autologous) oruffy coats of healthy adult donors (allogeneic; Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands) by Ficoll-Hypaque density gradient centrifugation. NK cells were purified by negative selection, depleting non-NK cells through a combination of biotin-conjugated monoclonal anti-human antibodies and MicroBeads using the “Human NK cell Isolation Kit” (Miltenyi Biotech); NK cell purity was more than 95% as determined by flow cytometry, analyzing NK cells as CD56+, CD3-, CD14-, and CD19- cells. NK cells were depleted from PBMCs (NKD PBMCs) by positive selection using anti-CD56 MicroBeads (Miltenyi Biotech); NKD PBMCs contained less than 0.1% of NK cells as analyzed by flow cytometry. IL-15–activated NK cells were obtained by incubating purified NK cells in AIM-V medium with 2 mmol/L of glutamine (Invitrogen) supplemented with 10% of pooled human AB serum (Sanquin), penicillin/streptomycin and 10 ng/mL of IL-15 (Peprotech) for 2 to 3 weeks in 24-well format tissue culture plates without feeder cells.

To measure NK cell activation after cetuximab cross-linking, upregulation of CD69 expression on NK cells (300,000) was measured after coculture with STA-ET2.1 (150,000), L1062 (80,000), and OSA (75,000) cells for 48 hours in 24-well plates in the absence or presence of cetuximab (10 μg/mL).

Flow cytometry

Determination of NK cell percentages in PBMCs, validation of NK cell purity, and expression of the NK cell activation marker (CD69) was analyzed phenotypically by staining with fluorescently labeled antibodies followed by fluorescence-activated cell sorting (FACS). The following antibodies were applied according to the manufacturer’s instructions: anti-CD3FITC (SK7), anti-CD3PerCP-Cy5.5 (SK7), anti-CD14PerCP-Cy5.5 (M5E2), anti-CD19PE (4G7), CD69FITC (L78; Becton Dickinson); anti-CD56PE (N901 NKH1), anti-NKG2DPE (ON72; IOTEST Immunotech). Expression of EGFR on the surface of sarcoma cell lines and primary osteosarcoma cultures was measured using the anti-EGFR mAb cetuximab (Erbitux; Merck KGaA) followed by the Alexa Fluor 647 goat anti-human IgG secondary antibody (A21445; Invitrogen). The anti-CD20 mAb rituximab (MabThera; Roche) was used as an IgG1 isotype-matched negative control for cetuximab. FACS measurements were carried out with the FACS Calibur (BD Biosciences) and analyzed with the “BD Cell Quest Pro” software (version 5.2.1).

51Cr release assay

The cytolytic activity of PBMCs, NKD PBMCs, and purified NK cells against sarcoma cell lines and primary osteosarcoma cultures was measured in 4-hour 51Cr release assays. Target cells were labeled with 100 μl Na-chromate (51Cr, 3.7 MBq) for 1 hour. After washing, 2.5 × 104 target cells were added to the effector cells in duplicate or triplicate at the indicated effector–target (E:T) ratios and incubated in the presence or absence of cetuximab (10–7 to 10 μg/mL as indicated) or the control mAb rituximab (10 μg/mL) for 4 hours at 37°C. Supernatants were collected, and the release of 51Cr was measured with a beta-counter (Wallac/PerkinElmer). Spontaneous and total release were obtained by incubation with medium and Triton X-100 (2.5%; Merck Chemicals), respectively. The specific lysis was calculated by the following formula: percentage of specific lysis = 100 × (experimental release–spontaneous release)/total release–spontaneous release.

Immunohistochemistry

Sections of 4 μm of representative tumor sections were deparaffinized and pepsin antigen retrieval was done. Expression of EGFR was assessed using a mouse monoclonal anti-EGFR antibody (31G7, 1:10; Zymed; Invitrogen) followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP linker antibody conjugate (DPVO-110HRP; Immunologic) and DAB+Substrate Chromagen System (Dako) detection. The sections were examined with a Leica DM5000 fluorescence microscope and LAS-AF acquisition program (Leica).

Statistical analysis

Statistical analyses were carried out with GraphPad Prism version 5.04 or SPSS version 16.0 (IBM) using paired student t tests, comparing means between groups of samples and linear regression analysis. Error bars represent the
SEM. A P value less than 0.05 was considered statistically significant. ns, not statistically significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

EGFR is expressed on the surface of osteosarcoma cell lines
ADCC by NK cells requires antibody binding to an antigen expressed on the tumor cell surface. Therefore, surface expression of EGFR, as detected by cetuximab, was measured on a panel of osteosarcoma (n = 12) cell lines by flow cytometry. All osteosarcoma cell lines expressed EGFR on the cell surface, with the highest expression on the cell lines HOS, OHS, and OSA (Fig. 1). The chemoresistant variants of SAOS-2 and U2OS also expressed EGFR. Previously, EGFR has been reported undetectable in Ewing’s sarcoma cell lines (n = 3; ref. 19). To extend these findings, surface EGFR expression was assessed on a panel of Ewing’s sarcoma cell lines (n = 7). EGFR expression was not detectable on 5 of 7 Ewing’s sarcoma cell lines. Correspondingly, EGFR expression was not detectable in Ewing’s sarcoma biopsy and resection specimens, as determined by immunohistochemistry (data not shown).

Cetuximab enhanced cytolysis of EGFR-expressing osteosarcoma cell lines by NK cells
To investigate whether cetuximab can enhance cytolysis of EGFR-expressing osteosarcoma and Ewing’s sarcoma cell lines by NK cells, resting NK cells were incubated with one EGFR− and several EGFR+ cell lines in the presence of cetuximab or the isotype-matched, nonbinding anti-CD20 control mAb in a 4-hour 51Cr release assay. As compared with cytolysis in the absence of mAb, the negative control mAb did not alter killing of sarcoma cells by NK cells (data not shown). In contrast, the addition of cetuximab increased the lysis of EGFR-expressing sarcoma cells but not of EGFR-negative cell lines (Fig. 2, panel A and B, i; data not shown). The lysis of the chemoresistant-resistant variant cell lines of SAOS-2 and U2OS was equally enhanced by cetuximab. Cetuximab-enhanced lysis by resting NK cells was comparable with the lysis by IL-15–activated NK cells (Fig. 2, panel A and B, ii). The addition of cetuximab to IL-15–activated NK cells hardly led to a further increase of the cytolytic activity. As an alternative parameter for NK cell activation, it was observed that the percentage of CD69-expressing NK cells increased after coculture of NK cells with EGFR-expressing sarcoma cells in the presence of cetuximab (Fig. 3, panel A).

Thus, the cytotoxic function of resting NK cells toward EGFR-expressing sarcoma cell lines as well as their activation status was substantially augmented in the presence of cetuximab.

Cetuximab-mediated lysis is independent of EGFR expression intensity
Despite different sensitivities to NK cell killing, the magnitude of cetuximab-enhanced lysis by resting NK cells by NK cells, resting NK cells were incubated with one EGFR− and several EGFR+ cell lines in the presence of cetuximab or the isotype-matched, nonbinding anti-CD20 control mAb in a 4-hour 51Cr release assay. As compared with cytolysis in the absence of mAb, the negative control mAb did not alter killing of sarcoma cells by NK cells (data not shown). In contrast, the addition of cetuximab increased the lysis of EGFR-expressing sarcoma cells but not of EGFR-negative cell lines (Fig. 2, panel A and B, i; data not shown). The lysis of the chemoresistant-resistant variant cell lines of SAOS-2 and U2OS was equally enhanced by cetuximab. Cetuximab-enhanced lysis by resting NK cells was comparable with the lysis by IL-15–activated NK cells (Fig. 2, panel A and B, ii). The addition of cetuximab to IL-15–activated NK cells hardly led to a further increase of the cytolytic activity. As an alternative parameter for NK cell activation, it was observed that the percentage of CD69-expressing NK cells increased after coculture of NK cells with EGFR-expressing sarcoma cells in the presence of cetuximab (Fig. 3, panel A).
Cetuximab-mediated lysis is dependent on NK cells

In the absence of effector cells, cetuximab did not elicit cytolytic effects on EGFR-expressing cell lines during the 4-hour cytotoxicity assay (Fig. 4). FcRIIIα/CD16 expression is required to elicit ADCC by NK cells. Because FcRIIIα/CD16 can also be expressed by monocytes, it was studied whether cetuximab-mediated ADCC by PBMCs is dependent on NK cells. In the presence of cetuximab, lysis of EGFR-expressing sarcoma cells by PBMCs was comparable with the cetuximab-enhanced lysis by purified NK cells (Fig. 4, panel A). In contrast, NK cell depletion abolished killing by PBMCs both in the absence and presence of cetuximab, indicating that in this in vitro system cetuximab-mediated killing was strictly dependent on NK cells present in the PBMCs.

Next, the dependence of cetuximab-mediated lysis on the concentration of cetuximab was investigated in a serial dilution experiment. The lysis induced by cetuximab was comparable between 10 and 1 μg/mL of cetuximab, but it was reduced by at least 50% at a concentration of 1 μg/mL (Fig. 4, panel B). Lower cetuximab concentrations failed to enhance lysis. Hence, 0.01 μg/mL of cetuximab expression.
EGFR+ primary cultures derived from EGFR+ osteosarcoma tumors are highly susceptible to cetuximab-mediated lysis by autologous PBMCs

Primary tumor cell cultures (n = 5) were generated from osteosarcoma biopsy (n = 1) and resection (n = 4) specimens derived from 4 different osteosarcoma patients (Table 1). EGFR expression in these osteosarcoma samples was membranous, as determined by immunohistochemistry, and correlated to the EGFR densities on the corresponding primary cultures, as determined by flow cytometry between passages (p) 3 and 13.

Cetuximab was the minimal concentration to substantially enhance cytolysis by NK cells.

Discussion

The identification of antigens specifically expressed on tumor cells has fueled the development of tumor-specific, mAb-based targeted therapies. The introduction of anti-CD20 mAb (Rituximab, MabThera) and anti-Her2 mAb (Trastuzumab, Herceptin) against B-cell malignancies and breast cancer, respectively, has improved patient prognosis (20, 21).

Surface expression of EGFR has been shown in several tumors, such as colorectal cancer, head and neck squamous cell carcinoma, and is specifically recognized by the chimeric IgG1 mAb cetuximab (13, 20). In numerous clinical phase II and III studies, the addition of cetuximab to conventional multidrug chemotherapy or radiotherapy has led to a significant improvement of clinical response rates, progression-free survival, and overall survival. Therefore, cetuximab therapy was approved for the treatment of recurrent, refractory, and metastatic colorectal cancer (22–25) as well as head and neck squamous cell carcinoma (26, 27) by the U.S. Food and Drugs Administration (FDA).

Previously, we and others have shown the cytolytic potential of NK cells against sarcoma cells (6–8). In this study, we sought to explore whether this cytolytic activity can be more specifically targeted toward sarcoma cells using a sarcoma-reactive mAb, with a human Fc portion that can bind to FcRIIa/CD16 on human NK cells. As several studies have described surface EGFR expression in osteosarcoma tumors and on osteosarcoma cell lines (12, 19, 28–31), we explored the potential of the anti-EGFR mAb cetuximab to specifically direct NK cell–mediated killing to sarcoma cell lines.

In agreement with previous studies on other tumor types, cetuximab induced NK cell–dependent ADCC against EGFR-expressing sarcoma cells. Similar to previous studies (32–35), we show that 0.01 μg/mL of cetuximab was the minimal concentration to induce cetuximab-mediated lysis by NK cells. These concentrations have been reported in sera of patients treated with cetuximab and in the tumor environment in a xenograft model (36, 37), indicating that cetuximab-mediated ADCC could be a functional anticancer mechanism in vivo. Although in other studies the magnitude of cetuximab-induced ADCC correlated with the level of EGFR expression (34, 35, 38), this correlation was...
not evident in osteosarcoma, despite the use of highly comparable methods to assess ADCC (32, 39). In fact, minimal EGFR densities were sufficient for the cetuximab-induced lysis of sarcoma cells (32, 39). Cetuximab-induced ADCC was comparable with the maximal killing achieved by IL-15–activated NK cells. In contrast to some other models (32, 34, 40, 41), we did not observe an additive effect of cetuximab on the lysis by cytokine (IL-15)-activated NK cells. Multiple mechanisms may account for the antitumor effect of cetuximab in patients. Masking of the EGFR extracellular binding site from its natural ligand EGF inhibits the activation of the receptor tyrosine kinase and downstream signaling pathways (13, 42). EGFR blockage has been shown to arrest cell-cycle progression and lead to apoptosis (13). Cetuximab can inhibit tumor angiogenesis, neovascularization and invasion, and sensitizes tumor cells to radiation and chemotherapy-induced growth inhibition and apoptosis in vivo (13). Finally, cetuximab may induce complement-dependent cytotoxicity or cytolytic effects by immune cells via ADCC (13, 32, 38, 39, 43). An advantage of cetuximab-mediated ADCC is that it would be independent of the EGFR mutation status (34, 38) and persistently activated EGFR signaling pathways (13, 40).

The primary mode of action of cetuximab in vivo is difficult to determine. In a murine model, the anticancer effect of cetuximab was presumed to be mediated by NK cells (41). Depletion of NK cells in murine osteosarcoma xenograft models or in mice with syngeneic mesenchymal stem cell–induced osteosarcoma could address whether an antitumor effect of cetuximab or murine anti-EGFR mAb relies on the presence of NK cells (44–46). In humans, the relevance of ADCC has been suggested by the finding that FcγRIIIa/CD16 polymorphism of NK cells correlated with the survival of colorectal cancer patients (47, 48), as well as with the efficacy of cetuximab-mediated ADCC by NK cells in vitro (34). Interestingly, the intratumoral NK cells have recently been associated with improved survival when colorectal cancer patients had been treated with cetuximab (49). In this study, we used a unique combination of tumor
specimens, primary tumor cultures, and PBMCs from osteosarcoma patients. This allowed us to establish that cetuximab treatment can improve the lysis of EGFR-expressing, autologous primary osteosarcoma cells by patient-derived NK cells via cetuximab-mediated ADCC. Cetuximab treatment is associated with relatively mild adverse effects and has been approved for clinical usage by the FDA (37, 50). Therefore, in the treatment of osteosarcoma patients, cetuximab-mediated immunotherapy could be scheduled in the presence of endogenous or adoptively transferred NK cells. As such, cetuximab may provide an interesting treatment modality for patients with chemotherapy-resistant or metastatic EGFR+ sarcomas.

Disclosure of Potential Conflicts of Interest

P.C.W. Hogendoorn is a consultant and is on the advisory board of Amgen, Inc. The other authors disclosed no potential conflicts of interest.

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