Lenalidomide Enhances Natural Killer Cell and Monocyte-Mediated Antibody-Dependent Cellular Cytotoxicity of Rituximab-Treated CD20+ Tumor Cells

Lei Wu, Mary Adams, Troy Carter, Roger Chen, George Muller, David Stirling, Peter Schafer, and J. Blake Bartlett

Abstract

Purpose: Lenalidomide has significant activity in myelodysplastic syndromes, multiple myeloma, and non-Hodgkin’s lymphoma (NHL). In previous studies, natural killer (NK) cell expansion by lenalidomide was shown to enhance the cytotoxic effect of rituximab. This study assessed the ability of lenalidomide to enhance antibody-dependent cellular cytotoxicity (ADCC) in rituximab-treated NHL cell lines and primary tumor cells from patients with B-cell chronic lymphocytic leukemia (B-CLL) in vitro.

Experimental Design: An in vitro ADCC system was used to assess the ability of lenalidomide to enhance human NK cell and monocyte function in response to rituximab.

Results: Lenalidomide directly enhanced IFN-γ production via Fc-γ receptor-mediated signaling in response to IgG. It was also a potent enhancer of NK cell-mediated and monocyte-mediated tumor cell ADCC for a variety of rituximab-treated NHL cell lines in vitro, an effect that was dependent on the presence of antibody and either interleukin-2 or interleukin-12. Lenalidomide also enhanced the ability of NK cells to kill primary tumor cells derived from three patients with B-CLL who have been treated previously with fludarabine plus cyclophosphamide. Enhanced NK cell ADCC was associated with enhanced granzyme B and Fas ligand expression and could be inhibited by a granzyme B inhibitor and partially inhibited by antibody to FasL. Enhanced NK cell Fc-γ receptor signaling is associated with enhanced phosphorylated extracellular signal-related kinase levels leading to enhanced effector function.

Conclusions: These findings suggest that lenalidomide has the potential to enhance the rituximab-induced killing of NHL cell lines and primary B-cell chronic lymphocytic leukemia cells via a NK cell-mediated and monocyte-mediated ADCC mechanism in vitro, providing a strong rationale for the combination of lenalidomide with IgG1 antibodies to target tumor-specific antigens in patients with cancer.

Monoclonal antibodies (mAb) are important weapons in the chemotherapeutic arsenal, targeting specific receptors on cancer cells (1). Rituximab is a chimeric anti-CD20 mAb that has significant clinical activity when used alone or in combination with chemotherapy in patients with relapsed or refractory non-Hodgkin’s lymphoma (NHL; ref. 2). In patients with NHL and B-cell chronic lymphocytic leukemia (B-CLL), rituximab enhances antibody-dependent cellular cytotoxicity (ADCC) as a major mechanism of action (2). However, the relevant cell populations and effector mechanisms leading to tumor cell death during ADCC are the subject of ongoing research.

Experiments using a novel flow cytometric assay have shown that an increased number of natural killer (NK) cells degranulate in response to CD20+ lymphoma cells in rituximab-treated patients with NHL (3). In ADCC, immunoglobulins complexed on a cell surface activate Fc-γ receptors on both host NK cells and monocytes. This triggers a cytolytic response mediated by perforins and granzymes as well as the induction of apoptosis through Fas ligand (FasL) and oxidative mechanisms (4–7).

Lenalidomide (Revlimid; Celgene) has been shown to have significant activity in the treatment of myelodysplastic syndromes in patients with the 5q deletion (8). Administration of 10 mg lenalidomide, given either daily (continuous treatment) or for 21 days every 4 weeks, reduced the requirement for transfusion and also decreased cytologic and cytogenetic abnormalities in these patients. Lenalidomide (25 mg given for 21 days every 4 weeks) in combination with dexamethasone was also highly effective in patients with multiple myeloma (9, 10). Other studies suggest that lenalidomide monotherapy may have clinical efficacy in relapsed or refractory, indolent or aggressive NHL (11).

Potential mechanisms of action of lenalidomide include immunomodulatory and nonimmunomodulatory activities.
Lenalidomide has been shown to enhance T cells and NK cell activation markers in patients with advanced cancers (12). NK cell expansion by lenalidomide has also been associated with an increase in the cytotoxic effect of rituximab in a severe combined immunodeficient mouse lymphoma model in vivo (13). In other studies, lenalidomide was shown to enhance the cytotoxicity of an anti-CD40 mAb (SGN-40) in CD40-expressing human multiple myeloma cell lines (14). The action of lenalidomide in these studies was attributed to direct anti-proliferative and apoptotic effects as well as to increased sensitivity of multiple myeloma cells to ADCC by functional cytotoxic NK effector cells.

The present study was undertaken to assess the ability of lenalidomide to enhance ADCC of rituximab-treated NHL cell lines and primary B-CLL cells in vitro in an attempt to better elucidate the mechanism of action of lenalidomide.

Materials and Methods

NK cell and monocyte preparation. NK cells and monocytes were isolated from fresh, buffy-coated, whole blood by 30-min incubation with RosetteSep cocktail (Stem Cell Technologies) followed by Ficoll-Hypaque density gradient centrifugation. CD56+ NK cells were isolated with RossetteSep cocktail (Stem Cell Technologies) followed by Ficoll-isolated from fresh, buffy-coated, whole blood by 30-min incubation and Ficoll.

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NK IgG-induced cytokine/chemokine assays. Flat-bottomed plates were coated with 100 µg/mL human IgG (Sigma-Aldrich) overnight at 4°C; unbound IgG was washed away. NK cells were plated at 2 × 10^5 per well into 96-well plates, and 10 ng/mL either interleukin (IL)-2 or IL-12 (R&D Systems) was added. Lenalidomide was added to the plate wells in the concentration range of 0.00032 to 5 µmol/L. After 48-h incubation, the supernatants were harvested and analyzed for levels of IFN-γ (R&D Systems). In some of the experiments, the supernatants were also analyzed for the levels of granzyme B or perforin (Cell Sciences) and analyzed for the level of various cytokines [granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-6] and chemokines [IL-8, inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES] using ELISA (R&D Systems) or Lumines. Statistical analysis was done to determine the significance of the addition of lenalidomide to IL-2 plus IgG or to IL-2 alone using the unpaired t test algorithm from GraphPad Prism. All donors tested were used in the analysis.

ADCC assay. Purified NK cells (1 × 10^5) were seeded in 96-well U-bottomed plates in 100 µL RPMI 1640 supplemented with 2% human AB serum. The cells were treated with lenalidomide or thalidomide (0.001-10 µmol/L) plus 10 ng/mL IL-2 or IL-12. The cells were then incubated at 37°C overnight.

Fig. 1. In vitro production of NK cell IFN-γ in response to lenalidomide or thalidomide incubated for 48 h with IgG and IL-2 or IL-12. Mean ± SE.

Results

Enhancement of IFN-γ production by NK cells. The addition of lenalidomide (0.000382-5 µmol/L) to human IgG incubated with either IL-12 or IL-2 enhanced IFN-γ production by 3- to 8-fold, respectively, in comparison with lenalidomide plus IgG, IL-12, or IL-2 (Fig. 1). The enhancing effect of lenalidomide was...
dose dependent for both IL-2 and IL-12. Similar results were observed with thalidomide, which enhanced IFN-γ production by 3-fold in the presence of IL-12 and 2-fold in the presence of IL-2 (Fig. 1). There was no IFN-γ production in response to lenalidomide alone, immobilized IgG alone, or lenalidomide plus IgG. Either IL-2 or IL-12 plus immobilized IgG was required for IFN-γ production (Fig. 1).

Enhancement of NK cell cytotoxicity in rituximab-coated NHL cell lines. The killing of Namalwa cells by lenalidomide (1 µmol/L), rituximab, and lenalidomide (1 µmol/L) plus rituximab was 18.8%, 7.4%, and 35.1%, respectively, in the presence of IL-2 (Fig. 2A). In addition, in the presence of IL-2, lenalidomide dose-dependently increased the NK cell (E:T = 10:1) specific lysis of rituximab-coated Farage cells (Fig. 2B) and Raji cells (Fig. 2C). For Farage cells, cell killing by lenalidomide (1 µmol/L), rituximab, and both was 15.4%, 1.8%, and 32.6%, respectively, in the presence of IL-2. For Raji cells, cell killing by lenalidomide (1 µmol/L), rituximab, and both was 26.6%, 34.5%, and 70.1%, respectively, in the presence of IL-2. In the presence of IL-12, the killing of Namalwa cells by lenalidomide (1 µmol/L), rituximab, and lenalidomide (1 µmol/L) plus rituximab was 11.5%, 7.1%, and 28.0%, respectively (Fig. 2D). Lenalidomide also dose-dependently increased the NK cell (E:T = 10:1) specific lysis of rituximab-coated Farage cells (Fig. 2E) and Raji cells (Fig. 2F), in the presence of IL-12. For Farage cells, cell killing by lenalidomide (1 µmol/L), rituximab, and both was 20.5%, 2.0%, and 32.5%, respectively. For Raji cells, cell killing by lenalidomide (1 µmol/L), rituximab, and both was 16.5%, 29.9%, and 65%, respectively, in the presence of IL-12. Enhancement of NK cell-mediated killing against rituximab-coated Namalwa cells was not observed with thalidomide.
Additionally, lenalidomide did not enhance NK cell-mediated killing of trastuzumab (HER-2/neu, IgG1) or mouse IgG1 coated Farage cells (Fig. 2H). There was also no killing of Jurkat (T cells, CD20+) cells in the presence of rituximab (Fig. 2I).

Additionally, lenalidomide (1 μmol/L) with or without rituximab also enhanced the ability of NK cells to kill primary tumor cells derived from three patients with B-CLL who have been treated with fludarabine plus cyclophosphamide, two of whom had relapsed after treatment (Fig. 3A). In further experiments, lenalidomide (1 μmol/L) in the presence of IL-2 increased the NK cell cytotoxicity of rituximab-coated CD20+ Jeko-1 (mantle cell lymphoma) cells from 70% to 95% at an E:T ratio of 50:1, from 60% to 85% at an E:T ratio of 25:1, and from 40% to 55% at an E:T ratio of 10:1 (Fig. 3B). Lenalidomide alone had a negligible (<5%) effect on tumor cell killing relative to the control (background killing = 40%). Rituximab-only treatment increased tumor cell killing by ~ 30% above the background. Therefore, the combined killing effects of lenalidomide and rituximab acted synergistically, enhancing the NK cell-mediated killing of tumor cells by 55% above background.

**Enhancement of monocyte-mediated lysis of NHL cells.** Mono
cyte-mediated lysis of rituximab-coated Farage cells showed very good killing activity, from 5% up to 20% at a 50:1 ratio, in the presence of lenalidomide (10 μmol/L; Fig. 3C). Unlike the NK cell assay, the addition of IL-2 or IL-12 was not required to show ADCC. However, the specific lysis of rituximab-coated Farage cells by lenalidomide-pretreated monocytes was completely blocked by neutralizing anti-IL-12 antibody.

Lenalidomide also enhanced monocyte-mediated lysis of rituximab-coated Raji cells from ~30% killing with rituximab alone to >60% at an E:T ratio of 50:1 (data not shown).

**Enhancement of NK cell cytokine/chemokine production by lenalidomide.** Enhancement of ADCC by lenalidomide was associated with increased NK cell expression of IL-8, MCP-1,

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**Fig. 3.** A. Lenalidomide-enhanced the ability of NK cells to kill primary tumor cells from patients with B-CLL who have been treated with fludarabine plus cyclophosphamide. Mean ± SD of three donors. In vitro enhancement by lenalidomide of (B) NK cell cytotoxicity of rituximab-coated CD20+ Jeko-1 (mantle cell lymphoma) cells and (C) monocyte-mediated lysis of Farage (human B-cell lymphoma) cells by lenalidomide. Mean ± SD of two donors.
RANTES, IP-10, and GM-CSF but decreased expression of IL-6 (Fig. 4). For IL-8, IP-10, and RANTES, enhancement was only statistically significant with immobilized IgG at the highest lenalidomide dose (10 μmol/L). In the case of GM-CSF, IP-10, MIP-1α, and IL-6, marked effects were also observed with lenalidomide plus IL-2 but in the absence of the antibody. No significant change was observed with MIP-1α production.

Mechanism of enhancement of ADCC by lenalidomide. Lenalidomide (10 μmol/L) together with immobilized IgG increased the percentage of CD56+ NK cells expressing FasL from ~5% (at a lenalidomide concentration of 0.1 μmol/L) to ~10% (Fig. 5A). IL-2 (10 ng/mL) treatment with immobilized IgG also increased the NK cell FasL expression comparable with lenalidomide treatment. Moreover, the combination of lenalidomide (0.1-10 μmol/L), IL-2 (10 ng/mL), and immobilized IgG enhanced the NK cell FasL expression to a level greater than that achieved by either agent alone, showing partially additive responses at 0.1 and 10 μmol/L lenalidomide (Fig. 5A). Notably, lenalidomide (1 μmol/L) in conjunction with IL-2 and immobilized IgG showed a clear synergistic enhancement of NK cell FasL expression [IgG alone, ~5%; IL-2 + IgG, ~8%; lenalidomide (1 μmol/L) + IgG, ~5%; and lenalidomide (1 μmol/L) + IgG + IL-2, ~25%].

The addition of lenalidomide was shown to enhance granzyme B production over that observed with IL-2 alone in a dose-dependent manner, but it had no effect on perforin production (Fig. 5B and C), although IL-2 alone had a strong enhancing effect on both. Addition of the granzyme B inhibitor II (40 μmol/mL; Calbiochem) to the NK cells 1 h before lenalidomide and IL-2 treatment abrogated the effect of IL-2 on granzyme B production. As expected, the granzyme B inhibitor II was inactive against the IL-2-stimulated perforin production. ADCC was totally prevented in the presence of a granzyme inhibitor (Fig. 5D) and partially prevented by the addition of anti-FasL (Fig. 5E).

Analysis by Western blot showed that lenalidomide increased levels of pERK with a clear dose-dependent response (Fig. 6A and B).

Discussion

Treatment options for patients with malignant lymphoma have been substantially enriched by the development of mAbs. In particular, the chimeric anti-CD20 antibody rituximab has direct antilymphoma activity and is highly active in indolent and aggressive lymphoma in combination with chemotherapy (15). ADCC is suggested as one of the in vivo mechanisms of action of rituximab, although the exact pathways involved are not fully understood (16).

In this study, we found that both lenalidomide and thalidomide enhanced in vitro NK cell production of IFN-γ induced by interaction of IgG with the Fc-γ receptor but only in the presence of IL-2 or IL-12. We then investigated the ability of lenalidomide and thalidomide to enhance the ADCC of rituximab. When multiple NHL cell lines were precoated with rituximab and exposed to NK cells pretreated with...
lenalidomide, there was strongly enhanced NK cell-mediated cytotoxicity. The specificity of this effect was confirmed by the lack of tumor cell killing by NK cells pretreated with lenalidomide in the absence of either concurrent cytokine signaling (via IL-2 or IL-12 receptors) or an antibody directed against the relevant tumor surface antigen. Thus, enhancement of NK cell-mediated ADCC of NHL cells in vitro appears to require both antibody binding to Fc-γ receptors plus IL-2 or IL-12, as lenalidomide alone does not cause an effect.

These findings are consistent with previous NK cell depletion studies, which showed that the antitumor activity of rituximab is enhanced in the presence of lenalidomide (13) and can be attributed to an enhancing effect of lenalidomide on purified human effector cells. Interestingly, thalidomide was inactive in

![Fig. 5. In vitro effect of lenalidomide on NK cell expression of (A) FasL, (B) granzyme B, and (C) perforin production from NK cells in the presence of immobilized IgG and IL-2. Effect of (D) granzyme B inhibitor and (E) anti-FasL on ADCC. Mean ± SD of two donors. INH II, proteinase inhibitor II.](image-url)
the ADCC assay despite being a potent enhancer of IFN-γ production by NK cells. This was partly explained by the inability of an IFN-γ mAb to block ADCC, indicating that IFN-γ is not required for ADCC in this system (data not shown). Thalidomide has been shown previously to enhance NK cell expansion and effector function in patients with multiple myeloma who respond to treatment (17) and this may be linked to its ability to enhance NK cell IFN-γ. We also found that lenalidomide enhanced the ability of monocytes to initiate ADCC of rituximab-coated Farage tumor cells, an effect that did not require exogenous cytokine but was completely blocked by a neutralizing anti-IL-2 mAb.

To elucidate the mechanism of action of lenalidomide, we analyzed the effect of lenalidomide on the expression of pERK, which has been implicated as having a role in NK cell-mediated ADCC (18). ERK2 is activated in primary NK cells in humans by stimulation of the low-affinity receptor for the Fc fragment of IgG (Fc-γRIIA), and this in turn regulates NK cell cytotoxicity (18).

Based on the results presented here, it may be postulated that the NK cell stimulatory effect of lenalidomide is due to enhanced Fc-γ receptor signaling, which is likely to elevate pERK, leading to enhanced expression of granzyme B and FasL. Notably, lenalidomide in combination with IL-2 and immobilized IgG resulted in the enhancement of NK cell FasL expression. The enhanced expression of granzyme B is thought to be associated with activation of caspase-dependent pathways of apoptosis as well as with direct effects on mitochondria and DNA degradation (4). Blocking granzyme B function by a specific soluble inhibitor appeared to totally block the ability of lenalidomide to enhance ADCC, whereas anti-Fasl was able to only partially block ADCC. Perforin was enhanced by IL-2 alone and was not further enhanced by lenalidomide.

Our findings support previous reports that NK cells produce T-cell-recruiting chemokines in response to antibody-coated tumor cells (19). Further enhancement of chemokine production by IgG-stimulated NK cells during ADCC in the presence of lenalidomide is suggestive of concomitant promotion of chemotraction and potential infiltration of tumor-specific T cells. Specifically, in the presence of lenalidomide and antibody (IgG), we observed large increases in NK cell production of MCP-1 and GM-CSF, smaller increases in IL-8, IP-10, and RANTES, and decreased production of IL-6. In particular, it could be postulated that the enhanced production of MCP-1 by NK cells within the tumor site may also attract effector T cells (20), whereas increased GM-CSF may enhance dendritic cell function and antitumor immunity. Also, increased levels of serum GM-CSF have been observed in solid tumor patients treated with lenalidomide (21).

Furthermore, lenalidomide also enhanced the ability of NK cells to kill primary tumor cells derived from three patients with B-CLL who have been treated previously with fludarabine plus cyclophosphamide. This suggests that lenalidomide could be used as salvage therapy in patients who relapse after other treatment regimens, and clinical trials using lenalidomide in this setting are currently under way.

In conclusion, the observation that lenalidomide enhances the rituximab-induced killing of NHL cell lines and primary B-CLL cells via a NK cell-mediated and monocyte-mediated ADCC mechanism in vitro provides a strong rationale for the combination of these drugs in patients with NHL and CLL.

Disclosure of Potential Conflicts of Interest

Authors are employees of Celgene and hold stock options in Celgene.

References

1. Leone G, Sica S, Voso MT, Rutella S, Pagano L. Treatment of acute leukemias with monoclonal antibodies: current status and future prospects. Cardiovasc Hematol Agents Med Chem 2006;4:33–52.
2. Jazirehi AR, Bonavida B. Cellular and molecular signal transduction pathways modulated by rituximab (Rituxan, anti-CD20 mAb) in non-Hodgkin’s lymphoma: implications in chemosensitization and therapeutic intervention. Oncogene 2005;24: 2121–43.
3. Fischer L, Penack O, Gentilini C, et al. The anti-lymphoma effect of antibody-mediated immunotherapy is based on an increased degradation of peripheral blood natural killer (NK) cells. Exp Hematol 2006;34:793–9.
4. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 2003;3:361–70.
5. Dallepè F, Patrone F, Frumento G, Sacchetti C. Antibody-dependent killing of tumor cells by polymorphonuclear leukocytes. Involvement of oxidative and nonoxidative mechanisms. J Natl Cancer Inst 1984; 73:331–9.
6. Barker E, Reisfeld RA. A mechanism for neutrophil-mediated lysis of human neuroblastoma cells. Cancer Res 1993;53:362–7.
7. Mocchegiani E, Malavolta M, NK and NKT cell functions in immunosenescence. Aging Cell 2004;3: 177–84.
8. List A, Dewald G, Bennett J, et al. Lenalidomide in cancer therapy: preclinical rationale and rationale for the combination of these drugs in patients with NHL and CLL.
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the myelodysplastic syndrome with chromosome 5q deletion. N Engl J Med 2006;355:1456–65.
9. Dimopoulos MA, Spencer A, Attal M, et al. Study of lenalidomide plus dexamethasone versus dexamethasone alone in relapsed or refractory multiple myeloma (MM): results of a phase 3 study (MM-010) [abstract]. Blood 2006;106:6.
10. Weber D, Chen C, Niesvizky R, et al. Lenalidomide plus high-dose dexamethasone provides improved overall survival compared to high-dose dexamethasone alone for relapsed or refractory multiple myeloma (MM): results of a North American phase III study (MM-009) [abstract]. J Clin Oncol 2006;24:7521.
11. Wiernik PH, Lossos IS, Justice G, et al. Preliminary results from two phase II studies of lenalidomide monotherapy in relapsed/refractory non-Hodgkin’s lymphoma [abstract]. J Clin Oncol 2006;24:17569.
12. Bartlett JB, Dredge K, Dalgleish AG. The evolution of thalidomide and its IMiD derivatives as anticancer agents. Nat Rev Cancer 2004;4:314–22.
13. Hernandez-Ilizaliturri FJ, Reddy N, Holkova B, Ottman E, Czuczman MS. Immunomodulatory drug CC-5013 or CC-4047 and rituximab enhance antitumor activity in a severe combined immunodeficient mouse lymphoma model. Clin Cancer Res 2005;11:5984–92.
14. Tai YT, Li XF, Catley L, et al. Immunomodulatory drug lenalidomide (CC-5013, IMiD) augments anti-GD40 SGN-40-induced cytotoxicity in human multiple myeloma: clinical implications. Cancer Res 2005;65:11712–20.
15. Buske C, Dreyling M, Unterhalt M, Hiddemann W. Monoclonal antibody therapy for malignant lymphoma [in German; English abstract]. Med Klin (Munich) 2006;100:14–24. Erratum in: Med Klin (Munich) 2005;100:346.
16. Marcus R, Hagenbeek A. The therapeutic use of rituximab in non-Hodgkin’s lymphoma. Eur J Haematol 2007;78:5–14.
17. Davies FE, Raje N, Hideshima T, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood 2001;98:210–6.
18. Trotta R, Puoro KA, Paroli M, et al. Dependence of both spontaneous and antibody-dependent, granule exocytosis-mediated NK cell cytotoxicity on extracellular signal-regulated kinases. J Immunol 1998;161:6648–56.
19. Roda JM, Parihar R, Magro C, Nuovo GJ, Trindandapani S, Carson WE III. Natural killer cells produce T cell-recruiting chemokines in response to antibody-coated tumor cells. Cancer Res 2006;66:517–26.
20. Brown CE, Vishwanath RP, Aguilar B, et al. Tumor-derived chemokine MCP-1/CCL2 is sufficient for mediating tumor tropism of adoptively transferred T cells. J Immunol 2007;179:3332–41.
21. Bartlett JB, Michael A, Clarke IA, et al. Phase I study to determine the safety, tolerability and immunostimulatory activity of thalidomide analogue CC-5013 in patients with metastatic malignant melanoma and other advanced cancers. Br J Cancer 2004;90:955–61.