Brief report

Milatuzumab immunoliposomes induce cell death in CLL by promoting accumulation of CD74 on the surface of B cells

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Chronic lymphocytic leukemia (CLL) is an incurable progressive disease for which new therapies are required. Therapy with monoclonal antibodies (mAbs) has improved the outcome of patients with CLL, making further investigation of novel antibodies directed against alternative and specific targets on B cells an important area of translational research. We now describe functional properties of an antagonistic humanized mAb to CD74, milatuzumab, showing that milatuzumab combined with a crosslinking antibody induces cytotoxicity in vitro in CLL cells in a caspase- and stromal-independent manner associated with aggregation of CD74 on the cell surface. Furthermore, incorporation of milatuzumab into an immunoliposome induces even more of a cytotoxic response than in vitro crosslinking, representing a novel therapeutic formulation for this mAb. Based on these data, future development of the milatuzumab-immunoliposome formulation as a therapeutic agent for CLL is warranted.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia, and is a progressive and incurable disease. CLL treatments include alkylating drugs, purine analogs, and more recently, monoclonal antibodies (mAbs). mAbs such as rituximab that target the CD20 antigen selectively expressed on CLL cells augment the cytotoxicity of traditional chemotherapy agents, and are associated with improved response and progression-free survival.1-4 However, nearly all patients eventually relapse after such treatments, indicating a need for novel and specific therapeutic agents.

CD74 is a type II transmembrane protein expressed on B cells that has recently been pursued as a target for antibody-mediated therapy.5 It associates with the α and β chains of HLA-DR, and normally functions as a major histocompatibility complex class II chaperone. Signaling through CD74 is also implicated in B-cell proliferation, nuclear factor κB activation, and cell survival.6,7 CD74 expression is increased on the surface of leukemic B cells, making it an attractive target for CLL and other B-cell malignancies. CD74 signaling is initiated after engagement with macrophage migration-inhibitory factor (MIF) and subsequent activation of survival pathways to inhibit apoptosis and stimulate proliferation.8,9 In addition, a recent study demonstrates that CD74 signaling induces TAp63 and VLA-4 to enhance CLL proliferation.8,9 In addition, a recent study demonstrates that CD74 signaling represents a potential therapeutic option in CLL and other CD74-expressing malignancies.5

Here we describe an antagonistic humanized mAb to CD74, milatuzumab. Milatuzumab has demonstrated antiprofiterative activity in non-Hodgkin lymphoma (NHL) and multiple myeloma (MM) cell lines and extends the survival of severe combined immune-deficient (SCID) mice injected with NHL and MM cells.5,7,11 However, little is known about the efficacy of milatuzumab in CLL. Our data demonstrate that milatuzumab mediates direct cytotoxicity in CLL cells by a mechanism involving aggregation of CD74 on the cell surface. Furthermore, incorporation of milatuzumab into a liposome potentiates the cytotoxic effect of this antibody, suggesting a novel therapeutic formulation.

Methods

Patients, cell separation, culture conditions, and reagents

For in vitro studies, written, informed consent was obtained in accordance with the Declaration of Helsinki to procure cells from patients with previously diagnosed CLL, as defined by the modified National Cancer Institute criteria, under an Institutional Review Board–approved protocol at The Ohio State University.12 Patient characteristics are available in supplemental Table 1 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Isolated mononuclear cells were negatively B-cell selected and placed in culture, as previously described by our group.13 HS-5 stromal cells were obtained from ATCC. CD40L was purchased from PeproTech. Milatuzumab was provided by Immunomedics Inc. Goat anti-human IgG antibody (Fc gamma fragment-specific, anti-Fc) was purchased from Jackson ImmunoResearch Laboratories. Q-VD-OPH pan-caspase inhibitor was purchased from MP Biomedicals.
Flow cytometric assays
Viability was determined by flow cytometry using propidium iodide (PI). For surface staining, CLL cells were washed in phosphate-buffered saline and stained with antibodies to CD20 or CD74 (BD Biosciences).

Immunoblot analysis
Immunoblots were performed as described.14 Antibodies used included PARP (Calbiochem); caspase 3 and 9 (R&D Systems), caspase 2, 6 and 8 (Cell Signaling), and tubulin (Santa Cruz Biotechnology).

Preparation of ILs
Immunoliposomes (ILs) were prepared as previously described.15 A postinsertion method was used to incorporate milatuzumab into preformed liposomes, and targeted milatuzumab-IL was prepared with an antibody-to-lipid ratio of 1:1000. Further details are available in supplemental Methods.

Statistical analysis
All reported statistical evaluations were performed by the Center for Biostatistics at The Ohio State University. Because the observations from the same patient are correlated, linear mixed models were used for analysis to take account of this within patient correlation. Treatment differences were estimated and tested from these models. The Holm step-down procedure was used to adjust for multiple comparisons or multiple endpoints when necessary. P values less than .05 for single comparisons or after adjustment for multiple comparisons were considered significant.

Results and discussion
We first determined the in vitro survival of primary CLL cells after milatuzumab treatment. As shown in Figure 1A and B, milatuzumab (mila) + anti-Fc crosslinker rapidly induces significant cell death compared with anti-Fc alone (difference of 18% averaged across time points; N = 26; P < .0001). This result was verified by MTT assay (supplemental Figure 1). This effect is dependent on crosslinking, since milatuzumab alone had no cytotoxic effect on the cells, and the effect of mila + anti-Fc was greater than that of rituximab + anti-Fc. Milatuzumab-mediated killing appears to be caspase-independent, because treatment does not increase cleavage of caspases 3, 6, 8, or 9 or the caspase substrate PARP relative to the Fc crosslinker control, although processing of caspase 2 is observed (Figure 1C). While the pan-caspase inhibitor, Q-VD-OPH, is able to block cleavage of both of PARP and caspase 2, it has no significant effect on milatuzumab-induced cell death (Figure 1D; N = 5; P = .03).

The role of microenvironmental factors in the survival and drug resistance of CLL cells is becoming increasingly studied.16 Importantly, the effect of milatuzumab is not significantly diminished by coculture with a stromal cell line (Figure 1Ei; 37.3% more cell death with mila + anti-Fc vs anti-Fc alone in the absence of HS-5; 48.5% more cell death with mila + anti-Fc vs anti-Fc alone in the presence of HS-5; N = 11; P = .10). In contrast, fludarabine-induced cytotoxicity is noticeably diminished by coculture with stroma despite the variation in fludarabine responsiveness among individual samples (Figure 1Ei; 19.3% more cell death with fludarabine vs vehicle control in the absence of HS-5; 2.2% more cell death with fludarabine vs vehicle control in the presence of HS-5). Furthermore, treatment with CD40L, commonly found to protect CLL cells from cell death, is unable to prevent milatuzumab-induced cytotoxicity (Figure 1Eii; 23% more cell death with mila + anti-Fc vs anti-Fc alone in the absence of CD40L; 29% more cell death with mila + anti-Fc vs anti-Fc alone in the presence of CD40L; N = 17; P = .09; N = 17; P = .11). Together, these data indicate that milatuzumab may be effective in vivo despite the presence of an intact microenvironment.

We next investigated whether milatuzumab was able to mediate antibody-dependent cellular cytotoxicity (ADCC). Similar to reports in lymphoma cell lines,17 no ADCC was detected with either mononuclear cells or granulocytes at any effector to CLL target cell ratio (data not shown). These findings indicate that direct cell death via CD74 ligation is likely the principal contributor to milatuzumab efficacy.

We next sought to determine how CD74 ligation by milatuzumab promoted cell death. We observed that upon milatuzumab treatment in the presence of crosslinking, CLL cells aggregated in culture (supplemental Figure 2) and the mean fluorescent intensity (MFI) of CD74 on the cell surface increased significantly (Figure 2A-B left; 1.50 vs 5.03 MFI [anti-Fc vs mila + anti-Fc]; N = 14; P = .0003). No significant change in surface expression of CD74 was observed after treatment with anti-Fc alone, while milatuzumab alone lead to a slight decrease in surface CD74, potentially due to increased receptor internalization in the absence of crosslinker. In addition, significant surface retention of CD20 was not observed after in vitro crosslinking with milatuzumab (Figure 2B right; 6.36 vs 5.56 MFI [anti-Fc vs mila + anti-Fc] N = 14; P = .14), indicating that the increased surface CD74 is antigen-specific and not due to nonspecific antibody trapping between clustered cells. These data suggest that milatuzumab promotes the maintenance and/or accumulation of CD74 on the cell surface, which likely initiates downstream signaling pathway(s) leading to cell death. The lack of this effect after treatment with milatuzumab alone indicates that crosslinking is necessary for milatuzumab-induced cell death. However, association with Fc receptors on other cells in the microenvironment may not be sufficient to mediate this effect, as indicated by the lack of ADCC with milatuzumab.17 We therefore investigated whether other methods to promote receptor accumulation with milatuzumab induce a similar in vitro cytotoxic effect as crosslinking antibody. Similar to previous studies with the anti-CD22 mAb, epratuzumab,18 we found that milatuzumab immobilized on a plastic cell culture plate increased cell death compared with the nonimmobilized antibody, but this was not as active in cell killing compared with using soluble anti-Fc (supplemental Figure 3; 18.2% vs 43.2% PI positive, immobilized mila vs mila + anti-Fc averaged across time points; N = 6; P < .0001). These results suggest that association of multiple receptors may be required to initiate a death signal, which is limited when antibodies are fixed on cell culture plates. Enhanced cell death through Fas signaling has been described for CD44,19 a binding partner for CD74, and is a suggested mechanism for milatuzumab.20 Therefore activation of this signaling pathway in CLL after milatuzumab treatment is currently being evaluated.

Incorporation of internalizing antibodies into liposomes has been described as a method for targeted drug delivery in B-cell malignancies.15,21 We found that incorporation of milatuzumab into a liposome (mila-IL) was able to mediate the same receptor aggregation on the cell surface as milatuzumab with anti-Fc (Figure 2C), an effect that was not evident with liposome alone (mila-IL vs liposome; 4.23 vs 1.39 MFI; N = 11; P = .0001). Furthermore, mila-IL induced significantly more cell death in CLL cells compared with IgG incorporated liposomes (Figure 2D; mila-IL vs IgG-IL; 36% vs 4.8% PI positive; N = 11; P = .0001). Importantly, cell death induced by mila-IL was significantly higher than that caused by milatuzumab plus crosslinking in vitro (Figure 2D; mila-IL vs mila + anti-Fc; 36% vs 25.5% PI positive; N = 11;
This cytotoxicity was evident even without packaging the immunoliposome with a chemotherapeutic agent, such as doxorubicin, which has been described previously with this antibody.21 Here, we provide evidence that the incorporation of milatuzumab into a liposome may induce cell death without a dependence on other cell types in the microenvironment. Together, these data...
support the clinical application of mila-IL-based therapy in CLL and possibly other CD74-positive malignancies.

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Authorship

Contribution: E.H. designed the research, performed experiments, analyzed data, wrote the manuscript, and approved the final version of the manuscript; G.T. designed the research, performed experiments, analyzed data, reviewed drafts, and approved the final version of the manuscript; E.J.S. and J.D.H. performed experiments and approved the final version of the manuscript; X.Z. and D.J. performed statistical analysis and approved the final version of the manuscript; D.M.L. and N.M. designed components of the research, reviewed drafts, and approved the final version of the manuscript; D.M.G. provided reagents, reviewed drafts, and approved the final version of the manuscript; R.J.L. reviewed drafts and approved the final version of the manuscript; and J.C.B. designed and supervised the research, obtained funding for the research work, reviewed drafts, and approved the final version of the manuscript.

Conflict-of-interest disclosure: D.M.G. is an officer and member of the Board of Directors of Immunomedics Inc, which owns milatuzumab. The remaining authors declare no competing financial interests.

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