The Ch14.18-GM-CSF Fusion Protein Is Effective at Mediating Antibody-dependent Cellular Cytotoxicity and Complement-dependent Cytotoxicity in Vitro

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ABSTRACT

Granulocyte/macrophage-colony stimulating factor (GM-CSF) is very effective at enhancing antibody-dependent cellular cytotoxicity (ADCC) mediated by granulocytes and monocytes. Recently, a fusion protein consisting of GM-CSF and chimeric human/mouse anti-ganglioside G D2 antibody Ch14.18 (Ch14.18-GM-CSF) has been generated to improve the effectiveness of immunotherapy by directing GM-CSF to the tumor microenvironment and prolonging its relatively short half-life. In this study, we examined the ability of this fusion protein to enhance the in vitro killing of G D2 -expressing human neuroblastoma cells by granulocytes and mononuclear cells, as well as by complement. The Ch14.18-GM-CSF fusion protein was equally effective as the combination of equivalent amounts of free Ch14.18 and GM-CSF in mediating the killing of NMB7 neuroblastoma cells by granulocytes from seven of eight neuroblastoma patients. The fusion protein was also equally effective as the combination of Ch14.18 and GM-CSF in mediating ADCC by neuroblastoma patients’ mononuclear cells. In addition, the fusion protein was as effective as Ch14.18 alone in directing complement-dependent cytotoxicity against NMB7 cells. Our results demonstrate that the biological activities expressed by ADCC and complement-dependent cytotoxicity of both monoclonal antibody Ch14.18 and GM-CSF are retained by the Ch14.18-GM-CSF fusion protein and lend further support for future clinical trials of this fusion protein in patients with neuroblastoma.

INTRODUCTION

Advances in cancer therapy in the last three decades have transformed the majority of childhood cancers from uniformly fatal diseases to largely curable illnesses. Unfortunately, the outcome of advanced stage neuroblastoma, which comprises more than one-half of all neuroblastoma, remains dismal despite surgery, radiation, intensive chemotherapy, and bone marrow transplantation. Clearly, new therapeutic strategies for the treatment of neuroblastoma are urgently needed. A promising approach is targeted therapy with mAbs directed against human tumor-associated antigens. In this regard, immunotherapy with mAbs directed against the neuroblastoma-associated antigen, disialoganglioside G D2 , has been actively pursued (1–3). The G D2 antigen is ideal for mAb-mediated therapy of neuroblastoma because it is expressed at high density in the vast majority of human neuroblastoma cells but is absent in normal tissues excluding neurons, skin melanocytes, and peripheral pain fibers, where it is poorly expressed (4, 5). To minimize immunogenicity, a human-mouse chimeric antibody directed against ganglioside G D2 (Ch14.18) was developed by fusing the cDNA sequences encoding the constant portion of human γ1 heavy chain and κ light chain with those encoding the variable portions of immunoglobulin from the murine hybridoma 14.18 (6). Ch14.18 was demonstrated to be very effective in mediating ADCC against neuroblastoma cells in the presence of human granulocytes and mononuclear cells (7), as well as in directing complement against neuroblastoma and melanoma (8). Phase I clinical trials of Ch14.18, conducted by us and others in children with neuroblastoma, revealed that Ch14.18 had therapeutic efficacy and was fairly well tolerated with proper supportive care (9, 10).

Many cytokines enhance effector cell functions, particularly in mediating ADCC (11–14). Our recent studies demonstrated that GM-CSF enhances anti-G D2 -mediated ADCC by granulocytes of normal individuals as well as those of neuroblastoma patients (15). These and other encouraging results have prompted the use of GM-CSF, in conjunction with mAbs in clinical trials, for the treatment of colorectal carcinoma (16, 17) and neuroblastoma (18). GM-CSF augmented ADCC activity of mononuclear cells and granulocytes against colorectal

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The abbreviations used are: mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL-2, interleukin 2; TNF, tumor necrosis factor; LU, lytic unit(s); HAMA, human antimouse antibody.
Biological Activities of mAb Ch14.18-GM-CSF Fusion Protein

Granulocytes and mononuclear cells isolated from peripheral blood or bone marrow of neuroblastoma patients were used as effector cells against NMB7 human neuroblastoma cells in the presence of Ch14.18, Ch14.18 plus GM-CSF, or Ch14.18-GM-CSF fusion protein. Whether added individually or as a fusion protein, the Ch14.18 concentration was maintained at 1 μg/ml, and the GM-CSF concentration was 260 ng/ml. In the absence of Ch14.18 and GM-CSF, lytic activity of granulocytes of all eight neuroblastoma patients was equal to background (0.2 ± 0.5 LU), and that of mononuclear cells of patient U13 averaged 6.9 ± 1.8 LU, in four independent experiments. Specific target cell lysis by Ch14.18 or Ch14.18-GM-CSF fusion protein at 1 μg/ml, in the absence of effector cells, was 1.0 ± 1.4%.

Table 1 Antibody-dependent lytic activity of GM-CSF-stimulated granulocytes and mononuclear cells

<table>
<thead>
<tr>
<th>No.</th>
<th>Site of tumor</th>
<th>BMT</th>
<th>Ch14.18</th>
<th>Ch14.18 + GM-CSF</th>
<th>Ch14.18-GM-CSF fusion protein</th>
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<tr>
<td>P1006</td>
<td>BM, bone</td>
<td>No</td>
<td>55.8 ± 5.6</td>
<td>81.8 ± 10.8</td>
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<td>P1009</td>
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<td>9.1 ± 1.7</td>
<td>14.4 ± 3.3</td>
<td>14.7 ± 3.4</td>
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<td>10.0 ± 1.0</td>
<td>8.6 ± 0.7</td>
<td>7.9 ± 1.4</td>
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<tr>
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<td>No</td>
<td>10.7 ± 3.7</td>
<td>31.3 ± 6.2</td>
<td>21.7 ± 4.8</td>
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<td>P1013</td>
<td>BM, bone</td>
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<td>7.0 ± 2.0</td>
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<tr>
<td>P1014</td>
<td>Chest</td>
<td>No</td>
<td>44.4 ± 2.5</td>
<td>107 ± 12.0</td>
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<td>42.6 ± 4.5</td>
<td>59.0 ± 4.4</td>
<td>64.8 ± 6.4</td>
</tr>
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</table>

* BM, bone marrow; BMT, bone marrow transplant.

Table 1: Antibody-dependent lytic activity of GM-CSF-stimulated granulocytes and mononuclear cells

MATERIALS AND METHODS

Cell Lines and Antibodies. The neuroblastoma cell line NMB7 was graciously provided by Dr. Shuen-Kuei Liao, Chang Gung Medical College, Tao-Yuan, Taiwan. The human-mouse chimeric anti-GD2 antibody, Ch14.18 (6), and the Ch14.18-GM-CSF fusion protein (19) were developed and characterized as described previously.

Patient Population. The patient population consisted of eight high-risk neuroblastoma patients who had recurrent or refractory disease after at least one regimen of multiagent chemotherapy and autologous bone marrow transplant in four or eight patients (Table 1).

Isolation of Granulocytes and Mononuclear Cells. Granulocytes were isolated from eight neuroblastoma patients characterized in Table 1. Briefly, patients’ heparinized whole blood or bone marrow were centrifuged on a Ficoll-Paque density gradient at 400 × g for 30 min. The resulting pellet, consisting of RBCs and granulocytes, was washed with RPMI 1640, resuspended in hemolytic buffer (0.15 mM NH₄Cl and 0.01 mM NaHCO₃), and incubated for 2 min at 37°C to lyse RBCs. The remaining granulocytes were washed twice with RPMI 1640 and resuspended in medium supplemented with 10% FBS and 2 mM glutamine (complete media). Mononuclear cells were isolated from the interphase after Ficoll-Paque centrifugation and then washed twice with RPMI 1640 before being suspended in complete media. Granulocyte and mononuclear cell populations were determined to be >99% pure by differential cell counting after staining cells with Wright’s stain. Cell viability was >99%, as determined by propidium blue exclusion.

ADCC. The lytic activity of neutrophils and mononuclear cells was determined by a 51Cr-release assay using a neuroblastoma cell line, NMB7, as target cells (1 × 10⁵) that were labeled with 0.2 mCi of Na₂⁵¹CrO₄ for 4 h at 37°C. After
To determine whether the immune-modulatory effect of the Ch14.18-GM-CSF fusion protein and Ch14.18 to establish that there was no significant difference in the binding of Ch14.18 in a previous study (19). The results of an ELISA assay subtracting the percentage of tumor cell lysis attributable to complement alone.

**RESULTS**

**ADCC.** The binding of the Ch14.18-GM-CSF fusion protein to the target antigen $G_{14.18}$ was compared with that of Ch14.18 in a previous study (19). The results of an ELISA assay established that there was no significant difference in the binding of the Ch14.18-GM-CSF fusion protein and Ch14.18 to $G_{14.18}$. To determine whether the immune-modulatory effect of GM-CSF and the biological activities of Ch14.18 are preserved in the Ch14.18-GM-CSF fusion protein, we performed ADCC assays using as effector cells granulocytes isolated from eight neuroblastoma patients and target cells from the neuroblastoma cell line NMB7. In two representative experiments (Fig. 1), the capacity of the Ch14.18-GM-CSF fusion protein to mediate tumor cell lysis by granulocytes was equivalent to that of the mixture of Ch14.18 and GM-CSF at all concentrations tested. Antibody-dependent tumor cell lysis peaked at 0.5–1 μg/ml of Ch14.18 with a GM-CSF concentration of 130–260 ng/ml.

![Graph](https://example.com/graph.png)

**CDC.** To determine whether the Ch14.18-GM-CSF fusion protein is able to direct CDC and whether this ability compares with that of mAb Ch14.18 alone, we performed CDC assays using sera from two normal donors as the complement source and cultured NMB7 human neuroblastoma cells as target.
The results of a representative experiment among three experiments shown in Fig. 2 indicated that complement-dependent lysis was similar when mediated by either the Ch14.18-GM-CSF fusion protein or mAb Ch14.18. Interestingly, however, in two of three experiments, at concentrations of Ch14.18 >0.2 μg/ml, the fusion protein appeared to be somewhat less effective than Ch14.18 alone in directing complement-dependent lysis.

**DISCUSSION**

The Ch14.18-GM-CSF fusion protein was reported previously to bind GD2 to a similar extent as mAb Ch14.18 (19). We now demonstrate for the first time that this same fusion protein is capable of mediating ADCC against human neuroblastoma cells by granulocytes and mononuclear cells of neuroblastoma patients. Furthermore, the Ch14.18 moiety of the Ch14.18-GM-CSF fusion protein was equally effective as equivalent amounts of mAb Ch14.18 in mediating ADCC, and the GM-CSF moiety of this fusion protein was as effective as GM-CSF in enhancing ADCC mediated by granulocytes and mononuclear cells, when the mAb Ch14.18 and GM-CSF concentrations were matched on a molar basis. Previous in vitro studies with Ch14.18-IL-2 fusion protein (21) or a mAb-TNF-α conjugate (23) demonstrated that these were more effective in mediating ADCC compared with cytokine alone (21, 23) or mAb alone (21). A more meaningful comparison of ADCC activities is to compare those achieved with either the fusion protein or the combination of mAb and cytokine at equivalent concentrations. As observed previously by us (7, 15, 18), GM-CSF generally enhanced Ch14.18-mediated ADCC by granulocytes; however, now we demonstrated that this activity of GM-CSF was not diminished when fused to Ch14.18. The Ch14.18-GM-CSF fusion protein performed as well as Ch14.18 alone in directing CDC. However, at concentrations of Ch14.18 generally >0.2 μg/ml, the fusion protein appeared to be somewhat less effective in directing CDC than free Ch14.18. It is possible that at high concentrations, the structural features of the fusion protein may impose some steric hindrance to complement fixation in vitro.

Functions of immune effector cells are usually suppressed in most cancer patients, and many chemotherapeutic drugs induce immunosuppression and neutropenia (24, 25). Consistent with this observation, we found that granulocytes from six of eight neuroblastoma patients mediated very low ADCC with mAb Ch14.18 alone, yet in all but one case, GM-CSF enhanced granulocyte and mononuclear cell ADCC. The ability of GM-CSF to increase the production of granulocytes and mononuclear cells as well as to enhance their cytotoxic activities against tumor cells is well documented (11–16). In addition, GM-CSF can also affect the migration of granulocytes (7, 26), resulting in their increased accumulation at tumor sites (27). In view of the effects of GM-CSF on these effector cells, particularly granulocytes, the use of a Ch14.18-GM-CSF fusion protein in the treatment of neuroblastoma would be of considerable interest, especially because our present study indicates that the Ch14.18-GM-CSF fusion protein is equally effective in mediating ADCC in vitro as are mixtures of Ch14.18 and GM-CSF at equivalent concentrations. More importantly, on the basis of our in vivo studies with Ch14.18-IL-2 (28, 29), the Ch14.18-GM-CSF fusion protein would be expected to target GM-CSF and thereby direct granulocytes and mononuclear cells to the tumor microenvironment far more effectively than a combination of Ch14.18 and GM-CSF. In this regard, in addition to studies with Ch14.18-IL-2, earlier studies demonstrated that mAb-cytokine conjugates administered to mice could target cytokines to tumor sites more effectively than the administration of free cytokine (30). Furthermore, the fusion of GM-CSF to Ch14.18 would not only increase the half-life of GM-CSF but would also avoid or minimize toxicities of GM-CSF, as was observed with a bispecific antibody targeting TNF-α to tumor sites (31). A HAMA or an anti-GM-CSF response may occur against the Ch14.18-GM-CSF fusion protein. However, on the basis of results of in vivo studies (27, 28) obtained with a similar fusion protein, Ch14.18-IL-2, it does not appear that a HAMA or anti-GM-CSF response would occur or occur to any significant level to affect the antitumor effect of the Ch14.18-GM-CSF fusion protein. The results of the studies on the Ch14.18-IL-2 fusion protein demonstrated that the fusion protein was much more effective than the mixture of Ch14.18 and IL-2 in suppressing the growth of disseminated metastases of neuroblastoma to bone marrow and liver. Furthermore, the occurrence of a HAMA response may actually be beneficial because of the generation of an anti-id response. Recent studies have shown that generating an anti-id response was effective in obtaining an antitumor response (32, 33). Taken together, our results lend strong support for conducting clinical trials with a Ch14.18-GM-CSF fusion protein in neuroblastoma patients.

**REFERENCES**


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