Cytokine enhancement of in vitro antibody-dependent cellular cytotoxicity mediated by chimeric anti-GD3 monoclonal antibody KM871

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Abstract

The chimeric KM871 monoclonal antibody targets the GD3 disialoganglioside antigen and is under investigation for its immunotherapeutic potential in melanoma. Preclinical and phase I studies have demonstrated the biodistribution and specific tumour targeting of KM871 to metastatic melanoma in vivo, with a long half-life and lack of immunogenicity making it an attractive candidate for further clinical trials. In vitro studies have demonstrated KM871 induces high levels of cytotoxicity in both antibody-dependent cellular-cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays. In order to investigate the potential for cytokine upregulation of KM871-mediated ADCC, freshly isolated healthy donor PBMC effector cells were cultured in the presence or absence of the cytokines interleukin-2, interleukin-12 and granulocyte/macrophage-colony stimulating factor and the ADCC determined over a 10-day period. In the absence of these cytokines, ADCC activity of 1 µg/ml KM871 on ⁵¹Cr-labeled SK-MEL-28 target cells could not be detected after 72 hrs of culture of PBMC effector cells in media. In contrast, ADCC mediated by KM871 was significantly enhanced and maintained for the 10-day study period upon culturing PBMCs with media containing IL-2 and/or IL-12, but not with GM-CSF. FACS analysis of the effector cell population indicated CD3-/CD16+56+ NK cells were primarily responsible for the KM871-mediated ADCC activity and a direct correlation was observed between the percentage of NK cells and the level of cytotoxicity mediated by the PBMCs. Furthermore, ADCC was significantly reduced using NK-depleted PBMCs. These results suggest combining IL-2 or IL-12 with KM871 may enhance KM871 immune-mediated cell killing in patients with metastatic melanoma.
Introduction

Gangliosides are cell surface glycosphingolipids with putative roles in cell growth, adhesion and proliferation. Ganglioside expression changes during development and differentiation in many normal tissues and organs, but upon malignant transformation elevated expression of gangliosides has been documented in tumours of neuroectodermal origin (melanoma, neuroblastoma and astrocytoma)\(^1\), \(^2\), \(^3\), \(^4\), \(^5\). Gangliosides modulate the transmembrane signalling essential for tumour cell growth, invasion and metastasis\(^6\) and their high cell surface expression renders them attractive targets for antibody-based cancer therapies.

Monoclonal antibodies (mAbs) directed against the disialoganglioside GD3 have been evaluated as therapeutic agents in melanoma since the generation of the mouse mAb R24\(^4\). An initial phase I clinical trial with mAb R24 in patients with advanced melanoma showed marked inflammatory reactions at tumour sites in some patients with cutaneous disease, and responses in 19% of patients\(^7\). Although additional trials of mAb R24 therapy alone\(^8\), \(^9\) or in combination with cytokines\(^10\), \(^11\), \(^12\) or chemotherapeutic agents\(^13\) have been conducted with occasional responses observed, assessment of the potential therapeutic value of R24 has been limited because of the strong immunogenicity of R24 in humans, accompanied by the development of high titres of anti-mouse antibodies in treated patients and related toxicities.

The chimeric IgG1 anti-GD3 monoclonal antibody KM871 has been developed with the aim of targeting the GD3 antigen in metastatic melanoma patients with reduced potential immunogenicity\(^14\). KM871 has potent immune effector functions, including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), and displays minimal difference in affinity but superior effector function compared to the parent murine IgG3 antibody KM641\(^14\), \(^15\). KM871 has been shown to specifically target GD3-positive melanomas\(^16\) and demonstrated a substantial anti-tumour effect in animals\(^14\), \(^17\). A phase I dose escalation trial in patients with metastatic melanoma was recently completed and demonstrated specific tumour targeting and lack of immunogenicity of KM871, as well as evidence of biologic effect in vivo\(^18\). Three patients (20%) had clinically evident inflammatory responses at the tumour site and at dose levels above 5mg/m\(^2\). The observed trough serum KM871 levels at one week post-infusion were >1 µg/ml, which would be sufficient for CDC and ADCC activity to be achieved over this period\(^14\), \(^17\).

The in vitro cytotoxicity and in vivo anti-tumour effects of mAbs are primarily mediated by triggering ADCC via activation of Fc receptors of macrophages and natural killer (NK) cells\(^19\), \(^20\). In vitro studies have shown that a variety of cytokines, including IL-2, IL-12 and GM-CSF, upregulate the expression of Fc receptor and thereby enhance ADCC, and several cytokines have been investigated as potential adjuvants in melanoma.

Interleukin-2 (IL-2) can potentiate the NK activity of PBLs in vitro\(^21\) and augment ADCC against melanoma\(^22\), \(^23\). IL-2 therapy leads to the induction of tumour infiltrating lymphocytes (TILs) including lymphokine activated cells (LAKs) and cells with specificity for melanoma antigens\(^24\). IL-2-based immunotherapy has been approved by the FDA as therapy for metastatic melanoma on the basis of a 16% objective response rate with complete clinical responses and durable remissions in 6% of melanoma patients\(^25\). IL-12 has been demonstrated to induce secretion of IFN-gamma from resting and activated T and NK cells, to enhance NK/LAK cell cytolytic activity and to increase cytolytic T-cell responses\(^26\). Animal models have demonstrated the potent anti-tumour activity of IL-12\(^27\) most likely effected through IFN-gamma and the direct killing by activated lymphocytes, the antiangiogenic effects of IL-12-induced IFN-gamma, and injury to both the tumour and its microcirculation by activated neutrophils. Phase I clinical trials with IL-12 have observed transient complete responses in melanoma in a small number of patients\(^28\). The hematopoietic growth factor GM-CSF controls the proliferation and survival of neutrophils, eosinophils and monocytes\(^29\). GM-CSF stimulates various mature haematopoietic cell functions including augmentation of monocyte antitumour activity for human melanoma cells\(^30\), \(^31\) and has been reported to have potential benefit as an adjuvant in melanoma\(^32\).
The immunological specificity of cytokine-activated PBMCs in patients receiving cytokine therapy may be enhanced through the co-administration of monoclonal antibodies with tumour specificity. By targeting the immunologically activated effector cells to the tumour cells via the Fc portion of the mAb, ADCC of the tumour cells occurs. Accordingly, the immune effector functions mediated through the Fc region of KM871 may have an important therapeutic role in vivo, and the enhancement of this activity by the co-administration of cytokines in the clinic could enhance the immunotherapeutic effectiveness of KM871 in melanoma.

This study investigated the in vitro effects of IL-2, IL-12 and GM-CSF, individually and in combination, on KM871 mAb-mediated ADCC of melanoma SK-MEL-28 target cells, and evaluated the major PBMC effector cell population involved in cell killing.

Results

IL-2 and IL-12 enhance and maintain the ADCC activities of KM871

To analyse the effects of cytokines on ADCC and LAK activities over a period of 10 days, freshly isolated healthy donor PBMCs were cultured with medium alone or IL-2, IL-12, and GM-CSF individually, or in combination, at clinically relevant concentrations. Background LAK and ADCC were determined on days 1, 2, 3, 5, 7 and 10 for each treatment group. In the absence of cytokines, PBMCs at an effector to target ratio of 25:1 effected KM871-mediated ADCC activity for only the early stage of culture (days 1-2) with no ADCC activity observed after day 3 (Figure 1A). PBMCs cultured with IL-2 effected strongly enhanced ADCC activity on SK-MEL-28 target cells in the presence of the GD3-specific antibody KM871 compared with the control KM966 antibody. This marked enhancement of ADCC was maintained throughout the 10-day investigation period (Figure 1B). After culturing PBMCs for 3 days with IL-2 (10 ng/ml), low levels (10-20%) of LAK activity were detected. Increasing the IL-2 concentration to 100 ng/ml further enhanced both ADCC and LAK activities (data not shown). IL-12 did not show significant effect on ADCC activity initially (days 1-2), but enhanced and maintained ADCC activity at later time points compared with the control (days 3-10) (Figure 1C). GM-CSF did not show any effects on the ADCC activity of PBMCs (Figure 1D) compared with that of PBMCs cultured with medium alone (Figure 1A). These studies were repeated using purified granulocytes. No cytotoxic activity of granulocytes on SK-MEL-28 target cells was observed in the presence or absence of any of the cytokines investigated during the observation period (days 1-7, data not shown).

Effects of cytokine combinations on KM871-mediated ADCC

Purified PBMCs were cultured with IL-2 and IL-12, IL-2 and GM-CSF, IL-12 and GM-CSF, or IL-2 plus IL-12 and GM-CSF. Cytokine concentrations were 10 ng/ml (24 IU/ml) IL-2, 100 ng/ml IL-12 and 500 ng/ml GM-CSF. The observed KM871-mediated in vitro ADCC activities determined on days 1, 2, 3, 5, 7 and 10 for each treatment group are shown in Figure 2. IL-2 combined with other cytokines (IL-2 and IL-12 in Figure 2A, IL-2 and GM-CSF in Figure 2C, IL-2 plus IL-12 and GM-CSF in Figure 2D) did not show any additive or synergistic enhancement of KM871-mediated ADCC activity compared with that of PBMCs cultured with IL-2 alone (Figure 1B) over the 10-day study period. In addition, no further enhancement of ADCC activity was demonstrated by IL-12 combined with GM-CSF (Figure 2B) compared with that of PBMCs under the stimulation of IL-12 alone (Figure 1C). However, when PBMCs were cultured with IL-2 and IL-12, marked augmentation of LAK activity was detected over the entire assay period (Figure 2A). The addition of GM-CSF to the culture media did not result in further enhancement (Figure 2D).
Figure 1. KM871-mediated ADCC activity of PBMCs cultured in the presence of individual cytokines. PBMCs (2.5 x 10^5/well) were cultured for 1 to 10 days at 37°C with (A) medium alone, (B) 10 ng/ml IL-2, (C) 100 ng/ml IL-12, or (D) 500 ng/ml GM-CSF. ADCC activities were assayed at each time point at an E:T ratio of 25:1 and with 1 µg/ml KM871 mAb (open bars). Control mAb KM966 (///) and medium only (\) were also included. The data, expressed as the mean % cytotoxicity of triplicate samples with error bars denoting standard deviation, is representative of over 3 experiments conducted.

Figure 2. KM871-mediated ADCC activity of PBMCs cultured in the presence of cytokine combinations. PBMCs (2.5 x 10^5/well) were cultured for 1 to 10 days at 37°C with (A) IL-2 (10 ng/ml) and IL-12 (100 ng/ml), (B) IL-12 (100 ng/ml) and GM-CSF (500 ng/ml), (C) IL-2 (10 ng/ml) and GM-CSF (500 ng/ml), or (D) IL-2 (10 ng/ml) plus IL-12 (100 ng/ml) and GM-CSF (500 ng/ml). ADCC activities were determined on days 1, 2, 3, 5, 7 and 10 at an E:T ratio of 25:1 and with 1 µg/ml KM871 mAb (open bars). Control mAb KM966 (///) and medium only (\) were included. The data, expressed as the mean % cytotoxicity of triplicate samples with error bars denoting standard deviation, is representative of over 3 experiments conducted.
To assist in the interpretation of the ADCC achieved following culture of the effector PBMCs with the different cytokines, the specific KM871-mediated ADCC on SK-MEL-28 target cells was calculated by subtracting the background control IgG1 activity from the cytotoxicity determined for KM871. The specific ADCC for each treatment group of the individual or combination of cytokines is shown in Figure 3 for days 1, 3, 5 and 10. Analysis of variance determined significant differences between the specific KM871 ADCC achieved each day by the different groups. From days 3 to 10, incubation of PBMCs with 10 ng/ml IL-2 resulted in significantly enhanced specific ADCC compared to other cytokine treatment groups ($P<0.001$), except for IL-2 with GM-CSF where the activities were very similar to those with IL-2 alone. The specific ADCC following PBMC culture with IL-12 was significantly enhanced by the addition of IL-2 from day 3 onwards ($P<0.002$), but was not significantly greater than the activity with IL-2 alone.

**Figure 3. Specific ADCC mediated by 1 µg/ml KM871.** Specific ADCC was determined by subtracting the background LAK cytotoxicity from that observed with 1 µg/ml KM871. The KM871-specific release of $^{51}$Cr effected by PBMCs precultured with the cytokines IL-2 (10 ng/ml), IL-12 (100 ng/ml) or GM-CSF (500 ng/ml) individually or in combination is presented for days 1, 3, 5 and 10 as the mean percent cytotoxicity, with standard errors, for triplicate samples.

**CD3-/CD16+56+ NK cell levels relate to the ADCC activity of KM871**

In order to determine which cell types were responsible for the ADCC activity observed, PBMCs were then cultured in the presence of each cytokine or combinations of cytokines and the percentage of each subclass of lymphocytes in the PBMCs was analysed by FACS at days 1 and 7. The percentage of CD14+ macrophages was determined as a percentage of the total PBMCs analysed. CD3+/CD4+ T cells, CD3+/CD8+ T cells, CD3-/CD16+56+ NK cells and CD3+/CD16+56+ NKT cells were determined as a percentage of total monocytes determined by FACS analysis of forward and side scatter. The results are presented in Table 1. Data in Figure 4 detail the analysis of ADCC activities observed for the different cytokine treatments on days 1 and 7 of culture and the percentage of CD3-/CD16+56+ NK cells present. On day 1, no marked differences were observed between treatment groups for the proportions of CD3-/CD16+56+ NK cells (Figure 4A), CD3+/CD4+ T cells, CD3+/CD8+ T cells, CD15+ granulocytes or CD14+ macrophages (Table 1). NK cells were approximately 8% of PBMCs in all treatment groups. Higher levels of ADCC activities were observed in the groups treated with IL-2 alone or in combination with IL-12 (Figure 4B). Similar levels of KM871-mediated ADCC on SK-MEL-28 target cells were observed for PBMCs incubated for 24 hours with IL-12, GM-CSF, IL-12 and GM-CSF or medium alone (Figure 4B).
Table 1. Percentage of lymphocyte subclasses in PBMCs on days 1 and 7 of culture with cytokines.

<table>
<thead>
<tr>
<th>PBMC Culturec</th>
<th>CD3+/CD4+a</th>
<th>CD3+/CD8+a</th>
<th>CD14+b</th>
<th>CD3-/CD16+56+a</th>
<th>CD3+/CD16+56+a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Day 7</td>
<td>Day 1</td>
<td>Day 7</td>
<td>Day 1</td>
<td>Day 7</td>
</tr>
<tr>
<td>IL-2 (10 ng/ml)d</td>
<td>48.2</td>
<td>62.1</td>
<td>17.5</td>
<td>21.2</td>
<td>11.8</td>
</tr>
<tr>
<td>GM-CSF (500 ng/ml)</td>
<td>56.6</td>
<td>71.7</td>
<td>18.3</td>
<td>22.1</td>
<td>12.1</td>
</tr>
<tr>
<td>IL-12 (100 ng/ml)</td>
<td>55.0</td>
<td>66.3</td>
<td>16.5</td>
<td>23.5</td>
<td>11.7</td>
</tr>
<tr>
<td>IL-2 + GM-CSF</td>
<td>52.5</td>
<td>65.0</td>
<td>18.4</td>
<td>22.1</td>
<td>12.4</td>
</tr>
<tr>
<td>IL-2 + IL-12</td>
<td>56.8</td>
<td>68.6</td>
<td>18.3</td>
<td>22.7</td>
<td>11.1</td>
</tr>
<tr>
<td>GM-CSF + IL-12</td>
<td>52.3</td>
<td>70.5</td>
<td>16.0</td>
<td>22.7</td>
<td>10.6</td>
</tr>
<tr>
<td>IL-2 + GM-CSF + IL-12</td>
<td>57.1</td>
<td>72.6</td>
<td>18.1</td>
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<td>12.4</td>
</tr>
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<td>55.3</td>
<td>74.7</td>
<td>16.5</td>
<td>22.1</td>
<td>13.1</td>
</tr>
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</table>

aResults expressed as percent of total monocytes determined by FACS analysis of forward and side scatter.
bResults expressed as a percentage of total PBMC analysed by FACS.
cPBMCs were freshly isolated on day 0 and cultured in medium alone or medium supplemented with cytokines.
dThe final concentration of IL-2 in culture was 10 or 100 ng/ml when used alone and 10 ng/ml when used in combination. IL-12 and GM-CSF were used at 100 and 500 ng/ml, respectively for both single and combination treatments.
Figure 4. PBMCs cultured with cytokines: Relationship of the percentage of NK cells to the ADCC activity on days 1 and 7. Shown are the percentage of NK cells in PBMCs for each cytokine treatment group on day 1 (Panel A) and day 7 (Panel C), as well as the corresponding ADCC activities on target SK-MEL-28 cells for each treatment group on day 1 (Panel B) and day 7 (Panel D). The data are expressed as the mean % cytotoxicity mediated in the presence of 1 µg/ml KM871 of triplicate samples, with error bars denoting one standard deviation.

At the later stage of the effector cell culture (day 7), the percentage of CD3-/CD16+56+ (NK) cells had markedly altered for each group (Figure 4C). The largest percentage (7-10%) of NK cells was observed in the IL-2 alone or IL-2 combination treatment groups. The percentage of NK cells in the IL-12 or IL-12 plus GM-CSF treatment groups had diminished to <4% of PBMCs. Less than 1% of NK cells remained in the PBMCs cultured with GM-CSF or medium alone after 7 days (Figure 4C). The percentage of NK cells clearly paralleled the ADCC activity (Figure 4) with higher ADCC activity of PBMCs observed when higher levels of NK cells were present, as in the IL-2 treatment groups. When the percentage of NK cells was low, such as following GM-CSF culture, negligible ADCC activity was detected. A similar phenomenon was not observed for other PBMC lymphocyte subclasses (Table 1). A marked increase in the percentage of CD3+/CD16+56+ and CD14+ cells in the GM-CSF treated PBMCs was observed (Table 1) and increased levels of CD3+/CD16+56+ cells were also observed with medium alone on day 7. However, no correlation was found between the level of CD3+/CD16+56+ cells or CD14+ macrophages and KM871-mediated ADCC or LAK activity.

The role of NK cells in effecting the ADCC observed in the presence of KM871 was further examined with NK cell-depleted PBMCs. Healthy donor PBMCs were purified and a portion of the preparation was then depleted of NK cells. FACS analysis determined that the CD3-/CD56+/CD16+ NK cell population was 23.9% of the freshly purified PBMCs and was reduced to 6.49% in the NK-depleted PBMCs. Both PBMC populations were cultured with 10 ng/ml IL-2, 100 ng/ml IL-12 or medium alone and the ADCC mediated by 1 µg/ml KM871 or isotype control antibody on target SK-MEL-28 cells determined on days 0, 1 and 7. Results for days 1 and 7 are presented in Figure 5. The KM871-mediated ADCC achieved by PBMCs cultured in the presence of IL-2 or IL-12 (Figure 5A) was similar to that observed in Figure 1. However, significantly reduced ADCC was observed with the NK-depleted PBMCs after 1 (Figure 5C) and 7 (Figure 5D) days of culture with IL-2 or IL-12 (P<0.001 on day 1 and day 7 for each cytokine), correlating with the markedly reduced NK cell effector levels.
**Figure 5. Effect of NK cell depletion on KM871-mediated ADCC.** Freshly purified PBMCs (Panels A and B; 23.9% CD3-/CD56+/CD16+) and NK cell-depleted PBMCs (Panels C and D; 6.49% CD3-/CD56+/CD16+) were cultured for 7 days with the cytokines IL-2 (10 ng/ml), IL-12 (100 ng/ml), or media alone (control). ADCC activities were determined on days 1 (Panels A and C) and 7 (Panels B and D) at an E:T ratio of 25:1 and with 1 µg/ml KM871 mAb (open bars). Control isotype-matched mAb (/>) and medium only (\/) were included. The data, expressed as the mean % cytotoxicity of triplicate samples with error bars denoting standard deviation, is representative of 2 experiments.

**Discussion**

The administration of cytokines as adjuvants or as therapy in metastatic melanoma has been the focus of many immunotherapy trials; however cytokine-activated effector cells may achieve a superior directed anti-tumour response through the Fc receptor binding of co-administered tumour targeting mAbs. The GD3 antigen expressed in over 90% of melanomas is an attractive antigen for targeted immunotherapy, and the chimeric mAb KM871 has potent immune effector function *in vitro* and *in vivo*. We have previously shown that KM871 specifically targets GD3-expressing tumours in animal models (16), and causes marked growth inhibition of xenografts in nude mice (17). A phase I clinical trial of KM871 demonstrated specific targeting of KM871 to tumour sites, minimal normal tissue uptake and a long serum half-life (18). Serum levels sufficient for CDC and ADCC activity were observed, and 3/15 evaluable patients showed clinically evident inflammatory responses at tumour sites. These characteristics, as well as the lack of HACA response in any patient, indicate that immune-mediated therapy of metastatic melanoma with KM871 has great potential, and mechanisms to increase efficacy are highly desirable.

The results of this study indicate that the NK cells activated *in vitro* by IL-2-containing media (24 IU/ml, 10 ng/ml) effected significantly enhanced KM871-mediated ADCC for up to 10 days. In contrast, control effector cells demonstrated declining ADCC activity over a 3-day period only. A variety of regimens using differing dose schedules of IL-2 have produced measurable anti-tumour responses in patients with melanoma (33). Phase I clinical trials in malignant melanoma have investigated the combination of IL-2 with the anti-melanoma murine R24 (10, 11) or chimeric 14.18 (34) antibodies and reported responses in several patients. Enhanced ADCC and increased NK or CD16+ and CD56+ cell numbers were associated with the treatments.
Enhancement of ADCC of human PBMCs by GM-CSF has been described (30, 31), but negative results have also been reported (35). Immunotherapy trials in melanoma combining GM-CSF with the chimeric 14.18 anti-GD2 mAb (36) or the R24 anti-GD3 mAb (37) were able to demonstrate increased numbers of monocytes and significant enhancement of patient effector cell function ex vivo by day 8, but no anti-tumour activity was observed. Using higher R24 doses in combination with GM-CSF in a phase Ib trial, two partial responses were observed at the MTD of 50 mg/m² (12); however further studies with R24 were restricted by the development of HAMA in patients treated with the murine mAb.

In the current 10-day study, in vitro culture with GM-CSF failed to show any enhancement of PBMC or granulocyte ADCC activities on target SK-MEL-28 cells, despite doubling the PBMC monocyte population by day 7. Increasing the effector to target ratio from 25:1 to 100:1, or the GM-CSF concentration to 1000 ng/ml, did not demonstrate any effect on ADCC. In addition, GM-CSF did not show any synergistic effect on the ADCC activity of PBMCs when used in combination with IL-2 and/or IL-12. We conclude that granulocytes do not mediate KM871 antibody-dependent cytolysis under the conditions of this study.

The anti-tumour effect of IL-12 appears to be dependent on the induction of interferon-gamma and the activation of either CD8+ T cells or NK cells in vivo. Patients receiving IL-12 exhibit increased circulating interferon-gamma, but levels attenuate with subsequent IL-12 treatment (28). The anti-tumour activity of IL-12 therapy has been associated with the ability to sustain interferon-gamma levels (38). Our results indicate that IL-12 activation of PBMCs significantly enhances and maintains KM871-mediated ADCC in vitro with negligible LAK activity, although these results were not superior to those obtained with IL-2 alone or the combination of IL-2 and IL-12.

A detailed analysis of effector cell subtypes associated with the KM871-mediated ADCC activity indicated CD3-/CD16+56+ NK cells play a significant role. A direct correlation between the percentage of CD3-/CD16+56+ NK cells and the level of ADCC activity was observed. NK cells participate in both the innate and adaptive immune systems. NK cells are remarkably efficient effector cells, equipped for killing and capable of rapid response to exogenous or endogenous signals by producing a variety of cytokines and factors involved in interactions between immune and non-immune cells (39).

The presence of "brisk" tumour infiltrating lymphocytes (TILs) within the vertical growth phase of primary melanoma cell nodules (40) correlates significantly with delayed time to metastasis and a longer survival period (41, 42). Similarly the presence of brisk TILs has a strong predictive value for melanoma metastases in lymph nodes (43), subcutaneous tissue (44), and the presence of lymphocytosis, primarily as higher CD4+/CD8+ ratios, immediately following IL-2 therapy in patients with metastatic melanoma is a factor associated with anti-tumour response (45). However, low levels of NK cells in tumour stroma and an almost complete absence of NK cells in melanoma lesions has been reported, despite the high peripheral blood levels of NK cells observed following interferon (44) or IL-2 treatment (46). The administration of an anti-melanoma antibody such as KM871 may improve the tumour localisation of the peripheral NK cells and focus anti-tumour activity.

In conclusion, the chimeric KM871 mAb specifically recognises the GD3 antigen and mediates strong in vitro ADCC against the SK-MEL-28 GD3+ target cells. IL-2 and IL-12 strongly enhanced and maintained this ADCC activity for 10 days when used alone or in combination with PBMCs in culture. In contrast, GM-CSF did not display any enhancement or induction of ADCC activity by PBMCs or granulocytes. The direct correlation between NK cell levels and the degree of KM871-mediated ADCC on target GD3+ melanoma cells indicate the importance of NK cells in this immune effector process. The potent and sustained immune effector activity observed with cytokines in this in vitro study and the anti-inflammatory responses observed in patients receiving KM871 suggest combination immunotherapy of KM871 with cytokines, particularly IL-2 or IL-12, has promise in the treatment of melanoma.
Abbreviations

CDC, complement dependent cytotoxicity; GD3, ganglioside GD3

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References


Materials and methods

Antibodies

Chimeric anti-GD3 monoclonal antibody KM871 (IgG1) and control isotype-matched chimeric anti-GM2 monoclonal antibody KM966 were prepared by the Division of Immunology, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd. Tokyo, Japan. For the detection of cluster differentiation receptors on the surface of PBMC subsets, anti-CD3-FITC/CD4-PE, anti-CD3-FITC/CD8-PE, anti-CD19-FITC, anti-CD20-PE, anti-CD14-PE, anti-CD15-FITC, and the mouse negative control antibodies IgG1-FITC/IgG1-PE, IgM-FITC and IgG2a-PE were purchased from DAKO (Glostrup, Denmark). Anti-CD3-FITC/CD16+56-PE was purchased from Immunotech (Maine, USA).
Cell lines

The SK-MEL-28 melanoma cell line (ATCC HTB-72) was purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with 5% heat-inactivated bovine serum (TRACE Biosciences Pty. Ltd. Australia), 100 units/ml penicillin and 100 µg/ml streptomycin.

Preparation of human peripheral blood mononuclear cells

PBMCs and granular cells were isolated from healthy volunteer donor blood. Heparinised whole blood was fractionated by density centrifugation on Ficoll-Hypaque (ICN Biomedical Inc., Ohio, USA). PBMC and granular cell fractions were collected separately and washed three times with RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 5% heat-inactivated FCS.

NK cell depletion of PBMCs

Half a preparation of freshly isolated PBMCs was incubated with mouse anti-huCD56 antibody IM-1844 (20 µg/ml, Beckman Coulter, Fullerton, CA) for 30 min on ice. Following washing with PBS, the cells were incubated for 30 min on ice with Dynabeads coated with human anti-mouse IgG (Pan mouse IgG, Dynal, Oslo, Norway) and the NK cell-depleted PBMC population recovered according to the manufacturers instructions. The NK cell ratios in the PBMC and the NK cell-depleted PBMC populations were determined by FACS.

Flow cytometry analysis

To examine the phenotype of PBMCs during culture, cells were analysed by direct immunofluorescence for reactivity with a series of mAbs using standard techniques on a Coulter Epics Elite ESP flow cytometer (Coulter Corporation, Miami, Florida, USA). Briefly, 3 x 10^5 PBMCs were incubated for 30 minutes at 4°C with mAbs conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), washed three times, and resuspended in fixative solution for analysis. PBMC phenotypes were classified as helper T cell (CD3+/CD4+); cytotoxic T cell (CD3+/CD8+); macrophage (CD14+), Natural Killer (NK) cells (CD3-/CD16+56+) or NKT cells (CD3+/CD16+56+). The percentage of CD14+ macrophages was determined as a percentage of total PBMCs analysed. CD3+/CD4+ T cells, CD3+/CD8+ T cells, CD3-/CD16+56+ NK cells and CD3+/CD16+56+ NKT cells were determined as a percentage of total monocytes determined by FACS analysis of forward and side scatter.

Cytokines

Following purification on day 0, PBMCs were cultured in media (RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 5% heat-inactivated FCS) alone or enriched with cytokines. Human recombinant IL-2 (hu r IL-2), IL-12 (hu rIL-12) and GM-CSF (hu rGM-CSF) were purchased from R & D Systems (Minneapolis, MN). The final concentration of IL-2 in the PBMC culture was 10 or 100 ng/ml (24 or 240 IU/ng) when used alone and 10 ng/ml (24 IU/ml) when used in combination with other cytokines. The concentrations of IL-12 and GM-CSF in the culture were 100 ng/ml and 500 ng/ml for both single and combination treatments investigated. NK cell-depleted PBMCs were cultured in the presence of IL-2 (10 ng/ml) or IL-12 (100 ng/ml). These concentrations encompass clinically achievable concentrations of the individual cytokines determined through serum level measurements, in the case of IL-2 (47), or extrapolation from doses administered to patients, in the case of IL-12 (28) and GM-CSF (12).

Antibody-dependent cell cytotoxicity (ADCC) and lymphocyte activated killer (LAK) assays

ADCC and LAK activities of human PBMCs, NK cell-depleted PBMCs and granular cells were measured in 4-hr ^{51}Cr release assays. GD3-positive SK-MEL-28 melanoma target cells (4 x 10^6) were radiolabelled with 200 µCi...
$^{51}$Cr for 2 hr at 37°C and then washed three times with culture medium. Labelled target cells were plated with the effector cells in 96-well U-bottomed microtitre plates (NUNC, Roskilde, Denmark) at effector/target (E:T) cell ratios of 25:1. For ADCC activity measurements, 1 µg/ml (final concentration) antibody was added in triplicate to each well. LAK activity refers to any enhanced cytotoxicity effected by lymphocytes following exposure to cytokines measured in the absence of antibody. Plates were incubated at 37°C for 4 hours, 50 µl supernatant was then harvested from each well and the $^{51}$Cr released determined by gamma counting (Cobra II Autogamma counter, Packard Instruments, Melbourne, Australia). Controls included in the assay corrected for spontaneous release (medium alone) and total release (10% Tween20 in PBS). Appropriate controls with the same antibody subclass were run in parallel. The percentage cell lysis (cytotoxicity) was calculated according to the formula:

$$\% \text{ Cytotoxicity} = \left( \frac{\text{Sample Counts} - \text{Spontaneous Release}}{\text{Total Release} - \text{Spontaneous Release}} \right) \times 100$$

The specific cytotoxicity mediated by KM871 was calculated by subtracting the background percentage cytotoxicity mediated by the subclass control mAb from that observed in the presence of 1 µg/ml KM871.

**Statistical analysis**

ADCC assays were all performed in triplicate, and the mean values and standard deviations reported. One way Analyses of variance (ANOVA) were performed on the mean specific cytotoxic activities on days 1, 3, 5 and 10 of the different cytokine treatment groups to assess any differences observed for statistical significance. The subsequent running of a Tukey multiple comparison test between the experimental groups defined any differences observed with $P$ values <0.05, indicating statistical significance.

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