Recombinant humanized anti-CD40 monoclonal antibody triggers autologous antibody-dependent cell-mediated cytotoxicity against multiple myeloma cells

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Summary. Multiple myeloma (MM) is currently incurable, and novel therapies are needed. In this study, we examined a novel recombinant humanized monoclonal antibody against CD40 (rhuCD40 mAb) and demonstrate for the first time that rhuCD40 mAb induces antibody-dependent cell-mediated cytotoxicity (ADCC) against CD40-positive MM cells. Importantly, we show that rhuCD40 mAb induces autologous ADCC against primary patient MM cells, without triggering ADCC against normal B cells. This study, therefore, both demonstrates that rhuCD40 mAb triggers autologous ADCC against patient MM cells and provides the framework for the clinical evaluation of rhuCD40 mAb immunotherapy to improve patient outcome in MM.

Keywords: ADCC, CD40, multiple myeloma.

MATERIALS AND METHODS

Antibodies. Recombinant humanized anti-CD40 mAb [Immunoglobulin G1 (IgG1)] and control human monoclonal IgG1 were provided by Genentech (South San Francisco, CA, USA). The murine anti-human CD40 mAb SGN-14 (Seattle Genetics, Seattle, WA, USA) was humanized to construct rhuCD40 mAb. Heavy and light chain complementarity-determining regions (CDRs) from SGN-14 murine mAb were swapped into plasmids encoding a human light and human heavy immunoglobulin chain. Additional individual substitutions were made in the light chain.
Fig 1. Effects of humanized anti-CD40 mAb on MM cell lines. (A and B) The expression of CD40 on human MM cell lines was analysed by flow cytometry. MM.1S (A) and RPMI8226 (B) cells were incubated with phycoerythrin-conjugated anti-CD40 Ab for 20 min on ice. After washing with cold PBS once, cells were fixed with 1% paraformaldehyde and analysed using a flow cytometer. Percentage positivity (grey) is relative to an isotype-matched control Ab (white). (C and D) The ADCC activity of humanized anti-CD40 Ab against MM cell lines MM.1S (C) and RPMI8226 (D) was assessed using the 51Cr-release assay. MM cells labelled with 100 μCi 51CrO4– were co-cultured with MNC from normal donors at various effector to target ratios (E/T ratio) in AIM-V medium alone (white), with control mAb (grey), or with rhuCD40 mAb (black) for 4 h at 37°C. Radioactivity in the supernatants was then counted using a Wizard 3 gamma counter. The spontaneous release of target cells was less than 20%. Results shown are representative of three experiments, and values indicate the mean ± SD of triplicate wells. A statistically significant increase in the cytotoxicity of MM.1S cells (*P = 0.02) was observed in the presence of rhuCD40 mAb compared with control mAb. (E and F) The proliferation of MM cell lines induced by anti-CD40 Ab was assessed by 3H proliferation assay. MM.1S (2 × 104 cells/well; E) and RPMI8226 (1 × 105 cells/well; F) cells were cultured in RPMI1640 with 5% FCS medium alone, with control mAb (grey), with rhuCD40 mAb (black), or with CD40L (dotted) at the indicated concentrations. 3H thymidine (0.5 μCi) was added to each well for the last 8 h of 48-h cultures. Cells were then harvested and radioactivity was counted. Results shown are the mean count per min (cpm) ± SD relative to control culture without Abs in triplicate wells, and they are representative of three experiments. A statistically significant increase in the proliferation of MM.1S cells (***P = 0.03) was observed in the presence of CD40L compared with control mAb.
and heavy chain variable regions to restore binding. The variants were expressed and assayed for binding to human CD40. The selected variants were cloned into mammalian expression vectors and co-transfected for the expression of full-length immunoglobulin in Chinese hamster ovary (CHO) cells.

Primary cells. Primary MM cells (>90% CD138 positive) and B cells (>85% CD19 positive) were purified from bone marrow (BM) aspiration specimens of MM patients and from normal donor peripheral blood (PB), respectively, using a RosetteSep negative selection system (StemCell Technologies, Vancouver, BC, Canada).

ADCC assay. ADCC activity against MM cell lines was assessed using the 51Cr release assay. 51Cr-labelled target cells (5 × 10^5 cells/well) were incubated with 10 µg/ml rhuCD40 mAb and then co-cultured with mononuclear cells (MNC) from normal donors at various effector to target ratios in the presence of 1 µg/ml rhuCD40 mAb in serum-free AIM-V medium (Invitrogen, Carlsbad, CA, USA) for 4 h at 37°C. Co-cultures with the same concentration of control mAb or without mAb were performed at the same time. Supernatants were then harvested and the radioactivity was counted. Per cent specific lysis was calculated as:

\[
\frac{(\text{experimental release} - \text{spontaneous release})}{\text{(maximum release} - \text{spontaneous release}) \times 100
\]

RhuCD40 mAb-mediated ADCC against primary MM cells and B cells using autologous MNC as effector cells was assessed by the colorimetric lactate dehydrogenase (LDH) assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega, Madison, WI, USA), because of the low 51Cr uptake by primary cells, as described for the 51Cr release assay. Per cent cytotoxicity was calculated as:

\[
\frac{(\text{experimental} - \text{effector spontaneous})}{\text{(target maximum} - \text{target spontaneous release}) \times 100
\]

Cell proliferation assay. MM cell proliferation was measured by 3H thymidine (NEN Life Science Products, Boston, MA, USA) incorporation for the last 8 h of 48-h cultures in the presence or absence of rhuCD40 mAb, control Ab or CD40L, as previously described (Hayashi et al, 2002).

Statistical analysis. Statistical significance of the differences observed in the proliferation of MM cells and ADCC by anti-CD40 Ab was determined using the Mann–Whitney U-test. The minimal level of significance was \( P < 0.05 \).

RESULTS AND DISCUSSION

The effects of rhuCD40 mAb on MM cell lines
We first investigated the expression of CD40 on MM cell lines using flow cytometry. CD40 was expressed highly on MM.1S cells (Fig 1A), but only weakly expressed on RPMI8226 cells (Fig 1B). These results confirm and extend previous reports demonstrating the expression of CD40 on other MM cell lines (Urashima et al, 1995; Tai et al, 2002).

We next examined the ADCC activity of rhuCD40 mAb on these MM cell lines (Fig 1C and D). MM cells were co-cultured with PBMCs from normal donors in the absence or presence of rhuCD40 mAb or control mAb. The rhuCD40 mAb triggered ADCC against MM.1S cells (Fig 1C), but little, if any. ADCC against RPMI8226 cells (Fig 1D). This result supports the selective rhuCD40 mAb-induced ADCC on CD40-positive cells; alternatively, an as yet undefined resistance mechanism may manifest in RPMI8226 cells. Cytotoxicity in the absence of effector cells, i.e. the direct cytotoxic effect of rhuCD40 mAb against MM cells for 4 h, was < 5% (data not shown).

Since we and others have reported that another anti-CD40 mAb, clone G28-5, induced the proliferation of MM cell lines (Tong et al, 1994; Tai et al, 2002), we next examined the effect of rhuCD40 mAb on MM cell growth. As shown in Fig 1E and F, rhuCD40 mAb did not trigger the proliferation of either MM.1S or RPMI8226 cells; in contrast, CD40L triggered a modest [stimulation index (SI) = 1.14 ± 0.02 mean and standard deviation (SD)], but statistically significant \( P = 0.03 \), proliferation of CD40-positive MM.1S cells. These results support the potential clinical application of rhuCD40 mAb as a novel therapeutic for MM.

The effects of rhuCD40 mAb on primary MM cells
Based upon the results in MM cell lines, we next examined the effects of rhuCD40 mAb on primary patient MM cells. MM cells (>90% CD138 positive), purified from patient BM using an Ab combination for negative selection, highly expressed CD40 in two out of three patients (Fig 2A, patients 1 and 2). Previous reports by us and others (Tong et al, 1994; Tai et al, 2002) have suggested that CD40 is expressed on the majority of primary MM cells, suggesting it as a potential target for Ab-mediated immunotherapy.

We next examined the autologous ADCC activity of rhuCD40 mAb on primary patient MM cells. Target cells were assessed using the 51Cr release assay. The minimal level of significance was \( P < 0.05 \).
(10 × 10³ cells/well) were co-cultured with autologous MNC at various effector to target ratios in serum-free medium alone, with rhuCD40 mAb (1 µg/ml) or with control IgG (1 µg/ml) for 4 h at 37°C. As shown in Fig 2B, rhuCD40 mAb triggered significant (P = 0.02) autologous ADCC against primary MM cells expressing...
CD40 (Fig 2B, patients 1 and 2), but not against CD40-negative patient MM cells (Fig 2B, patient 3). The cytotoxicity of rhuCD40 mAb alone to MM cells was < 5% in an assay of 4 h (data not shown). These data, using primary tumour and effector cells from the same MM patients, demonstrate that rhuCD40 mAb induces ADCC selectively against CD40-positive MM cells. Importantly, ADCC against normal peripheral B cells, which also express > 90% CD40 (Fig 2A), was < 5% (Fig 2B), further supporting the potential clinical application of this mAb.

In summary, we have shown that rhuCD40 mAb triggers ADCC on CD40-positive MM cells, providing the framework for clinical trials evaluating rhuCD40 mAb-mediated immunotherapy to improve patient outcome in MM.

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