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Direct effect of bispecific anti-CD33 × anti-CD64 antibody on proliferation and signaling in myeloid cells

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Abstract

Bispecific anti-CD33 × anti-CD64 antibody (BsAb) directly inhibited proliferation and colony formation of human acute myeloid leukemia cell lines, without affecting the function of normal monocytes. Addition of BsAb to normal monocytes induced tyrosine phosphorylation of Cbl and Vav, association of these molecules with CD33, and downstream signaling. In leukemia cells that were insensitive to BsAb treatment, Vav and Cbl were constitutively phosphorylated and, therefore, constitutively associated with CD33. Direct growth inhibition is an additional mechanism by which BsAb may be useful in the therapy of AML. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Leukemia cells from patients with acute myeloid leukemia (AML) commonly express certain myeloid-specific antigens such as CD33 and CD64 [1–3]. Considerable attention has been focused on targeting these antigens with monoclonal antibodies (mAb) for therapeutic effects [4]. Bispecific antibodies (BsAb), targeting leukemia-associated antigens simultaneously with targeting activating antigens on cytotoxic effector cells, offer a novel and powerful approach to anti-leukemia therapy [5]. We have previously reported the production of BsAb CD33 × anti-CD64 [6,7] and have demonstrated its in-vivo and in-vitro activities in mediating lysis of AML cells by cytotoxic myeloid effector cells [6,8,9]. However, the direct effect of the BsAb on AML cells was not previously examined. Since both CD33 and CD64 are present on AML cells, the effects induced by this BsAb after binding to either CD33 alone, CD64 alone, or both antigens, could be determined by both the molecular signaling cascades of either one or both of the antigens.

CD64 is a member of the Fcγ receptor family of cell-surface proteins that play an important role in both host defense and autoimmune disorders. Fcγ receptor signaling can lead to downstream events such as phagocytosis, ADCC, enhancement of antigen presentation and the release of intracellular cytokines and reactive oxygen intermediates [10]. Ligation of monocyte-associated CD64 results in rapid tyrosine phosphorylation on several signal transduction molecules. These molecules include the immunoreceptor tyrosine-based activation motif (ITAM) of the γ chain [11,12], and several non-receptor PTKs: the src-family members Hck, Lyn [13] and Fgr [14]; Syk [14–16]; the protooncogenes c-Cbl [17,18] and Vav [14]; and phospholipase C (PLC) [19]. However, the leukocyte immunoglobulin-like receptors (LIR) -1 and -2, which are also expressed on monocytes, are bound to the tyrosine phosphatase SHP-1. Co-ligation of either LIR with CD64 inhibits tyrosine phosphorylation of the associated Fc receptor γ chain and Syk molecules, as well as intracellular calcium mobilization [20]

CD33 is a cell-surface antigen specifically expressed on myeloid cells including myeloid leukemia cells. It

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is the smallest member of the siglec (sialic acid-binding Ig-related lectins) family, and is a 67 kDa transmembrane glycoprotein expressed by the earliest myeloid progenitors. It continues to be present during myelomonocytic differentiation on monocytes and neutrophils. However, the expression of CD33 on neutrophils is much lower than on monocytes. It has been shown recently that the cytoplasmic tail of CD33 contains two tyrosine-based motifs, both potential ITIMs. MAb directed against CD33 are used in the diagnosis of leukemia and for therapeutic targeting and purging in AML. However, despite its clinical importance, little is known of its role in myeloid cells, except that it may be involved in sialic acid-dependent cell interactions. While the expression of CD33 on myeloid cells was first described in the early 1980s [1], only very recently have data regarding the possible function(s) of CD33 been reported [21–23]. Taylor demonstrated that CD33 becomes tyrosine phosphorylated in myeloid cells after both pervanadate treatment and CD33 receptor cross-linking, resulting in recruitment of tyrosine phosphatases SHP-1 and SHP-2 [22]. Phosphorylation of CD33 was specifically inhibited by an Src family tyrosine kinase inhibitor. Moreover, the first cytoplasmic tyrosine residue (LXY340XXL) of CD33 is dominant in SHP-1/SHP-2 binding, and mutation of this same tyrosine enhances CD33-mediated adhesion. Co-ligation of CD33 and CD64 on monocytes resulted in decreased phosphorylation and duration of phosphorylation of various proteins, suggesting that recruitment of SHP-1 to CD33 resulted in a general decrease in kinase activity [21]. This raises the possibility that, similar to CD22 in B cells and p75/AIMR1 in NK cells, CD33 might act as an inhibitory receptor in myeloid cell signaling leading to inhibition of cell growth and apoptosis.

Here, we report our findings on the direct effects of anti-CD33 × anti-CD64 BsAb on cells from AML cell lines and normal monocytes from healthy donors.

2. Materials and methods

2.1. Antibodies

The mAb anti-CD33, anti-CD64 and anti-CD33 × anti-CD64 BsAb were obtained from Medarex, Inc. (Annandale, NJ). The anti-Syk, anti-Vav, anti-Cbl, anti-CD33 rabbit or goat polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A horse-radish-peroxidase-conjugated anti-phosphotyrosine mAb, 4G10, was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). The fluorescein-labeled anti-CD14, anti-CD33 and anti-CD64 mAb was obtained from B&D (San Jose, CA).

2.2. Materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.3. Cells and cells lines

Human AML cell lines HL-60 and U-937 were obtained from ATCC. Cell-line DER was created earlier in our laboratory. The NB4 cell line was a gift from Dr. Lanotte (Hospital St. Louis of Paris, France). All leukemia cells were cultured under standard conditions in RPMI-1640 containing 10% FCS.

Monocytes were isolated via adherence from peripheral blood mononuclear cells collected from normal donors as described elsewhere [24].

2.4. Proliferation assay

AML cells were cultured in triplicate wells in 96-well round-bottomed plates, with or without various amounts of BsAb in a final volume of 200 μl of RPMI-10 at 37 °C in humidified 5% CO_2 in air, at 10^5 cells per well. The cultures were pulsed with 1 μCi [^{3}H] thymidine per well during the last 3 h of the 3 h culture. The amount of ^{3}H-thymidine incorporated into acid-precipitable DNA was assessed via liquid scintillation counting.

2.5. Colony formation assay

AML cells were cultured in 0.3% agarose as described elsewhere [25]. Briefly, 10^4 cells were placed in 35 mm tissue culture dishes containing tissue culture media RPMI-1640 supplemented with 10% FBS and agarose at final concentration of 0.3%. After a 10–14 day incubation in the presence of various concentration of antibodies at 37 °C, the number of colonies was estimated under the light microscope. Clusters containing less than 40 cells were excluded from analysis.

2.6. FACS analysis for CD64 and CD33

Normal or leukemic cells were washed and then suspended in staining media (SM), containing RPMI-1640, 3% FCS, 0.01% NaN_3 and 1 μg/ml of propidium iodide (Calbiochem, La Jolla, CA), plus saturating amounts of FITC-conjugated anti-CD33, anti-CD64 mAbs, or an isotype-matched control mAb of irrelevant specificity. After 30 min at 4 °C, the cells were washed in SM twice and then analyzed on a FACScan (Becton Dickinson). Dead cells and debris were excluded from analysis by characteristic forward and side scatter profiles and propidium iodide staining.

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2.7. Phagocytosis assay

Monocytes were cultured 5 days in the presence of 50 ng/ml of GM-CSF (Immunex Inc., Seattle, WA). After two washes with PBS, cells were stained with FITC-labeled anti-CD14 (green fluorescence) mAb for 30 min at room temperature then washed twice with PBS and transferred to a polypropylene 96-well plate at a concentration of 10^5/well. *E. coli* particles (Molecular Probes Inc., Eugene, OR) pre-stained with tetramethylrhodamine (red fluorescence) were added to monocytes at a 10:1 ratio. Various concentrations of BsAb or control antibody were added in a 10 μl volume. After incubation at 37 °C for 1 or 3 h, each well was flushed with medium and the cell suspension transferred to a fresh tube. After fixation with 2% paraformaldehyde, the cells were deposited on slides by cytocentrifugation and then analyzed by fluorescent microscopy. The percentage of double-stained cells was calculated.

2.8. Cell activation and immunoprecipitation

Monocytes from normal donors or AML cell lines ($5–6 \times 10^6$) were activated by 10 μg/ml BsAb anti-CD33 × anti-CD64 or unconjugated anti-CD33 and anti-CD64 mAb for 20 min at room temperature, followed by the addition of polyclonal anti-mouse IgG at 20 μg/ml for different time points (from 1 min to 1 h). The reaction was stopped by adding ice-cold PBS. After three washes in ice-cold PBS, cells were lysed in lysis buffer, containing 1% (v/v) Triton X-100, 0.15 M NaCl, 50 mM Tris–HCl [pH = 7.2], 0,1% SDS, 1 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% [v/v] EDTA, 1% [v/v] Aprotinin, and 0.03 mM Leupeptin. After 30 min on ice, the nuclear debris was removed by centrifugation for 15 min at $13{,}000 \times g$. Lysates were equalized with respect to the amount of protein, as assessed by O.D. at 280 nM. Specific immunoprecipitation was performed for 2 h to overnight in the presence of 30% (vol./vol.) 'Protein A/G', conjugated with agarose (Santa Cruz Biotech., CA). Immunoprecipitates were washed three times in lysis buffer and then suspended in an equal volume of Laemli sample buffer for SDS-PAGE.

2.9. SDS-PAGE and Western blotting

Total cell lysates or immune-precipitates were added to separate wells (8 μg/well) of SDS-PAGE (7.5–10% acrylamide) gel, electrophoretically size-separated under reducing conditions, and then transferred onto nitrocellulose for immunoblotting. The filters were first incubated for 1 h in 5% non-fat dry milk in PBS-T (PBS plus 0.01% Tween 20), and then incubated with the primary antibody for 2 h. After washing in PBS-T, the filters were incubated for 1 h in horse-radish peroxidase-conjugated matching secondary antibodies. The filters then were washed in PBS-T, incubated with the enhanced chemoluminescence detection reagents (Pierce, Rockford, IL), and exposed to X-ray film (Fuji Film, Fischer, Tustin, CA).

3. Results

3.1. Inhibition of AML cell proliferation and colony formation upon BsAb ligation

We examined AML cells for their response to receptor ligation measuring ^{3}H-thymidine incorporation. HL-60, U-937, NB4 and DER cells were incubated with the anti-CD33 × anti-CD64 BsAb, or unconjugated single mAb anti-CD33 or anti-CD64 or both mAB. We found that BsAb significantly ($P < 0.001$) inhibited proliferation of HL-60 and U-937 cells in a dose-dependant manner, but failed to decrease proliferation of DER cells (Fig. 1). In NB4 cells we also observed significant (about 60%) inhibition of proliferation initiated by BsAb (data not shown). Interestingly, the level of inhibition was maximal at BsAb concentrations of 0.01–0.1 μg/ml. These data suggest that the effect was not caused by antibody toxicity. Meanwhile, incubating HL-60 cells with the unconjugated anti-CD33 or anti-CD64 mAb did not decrease proliferation of leukemia cells. In contrast, U-937 cells were responsive to the combination of both unconjugated mAb. However, DER cells were not responsive to either BsAb or unconjugated mAb.

The ability of BSAB to inhibit the leukemia cells growth was confirmed by a similar dose-dependent reduction of colony formation in both HL-60 ($P < 0.001$) and U-937 ($P < 0.001$) cells (Fig. 2), but not in DER cells (data not shown). However, we found that incubation of leukemia cells with both unconjugated mAb did not affect colony formation. Importantly, in both proliferation and colony formation assays, the maximum BSAB activity was observed at a concentration of 0.01–0.1 μg/ml and therefore was probably not caused by antibody toxicity.

3.2. Effect of IFN-γ on sensitivity of AML cells to antibody treatment

We investigated the levels of CD33 and CD64 expression on three leukemia cell lines: HL-60, U-937 and DER. CD33 was expressed at 95, 97 and 96%, respectively (data not shown). CD64 was expressed at 79, 95 and 30%, respectively (data not shown). Since our experiments did not detect differences between AML cells in the level of CD33 expression, but indicated that the level of CD64 expression on DER cells was significantly lower, we addressed the question of whether the

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increased level of CD64 on DER cells would alter the cells' response to antibody ligation. After treatment of U-937 (responsive) and DER (unresponsive) cells with 200 μg/ml of INF-γ at 37 °C for 24 h, the level of CD64 expression was 98 and 64%, respectively. We performed proliferation assays in the presence or absence of 0.1 μg/ml of various antibodies. We found that IFN-γ was able to increase significantly ($P < 0.001$) the sensitivity of DER cells (which were initially unresponsive) to antibody ligation (Fig. 3). U-937 cells responded to BsAb and the combination of anti-CD33 and anti-CD64 mAb even without INF-γ treatment. However, the effects of BsAb and single mAb were increased after treatment of the cells with IFN-γ.

3.3. Stimulation of normal monocyte phagocytosis by BsAb

Since CD33 and CD64 antigens are expressed by both AML cells and normal monocytes, we examined whether or not BsAb ligation could affect the phagocytic function of monocytes. We incubated monocytes from five normal donors in the presence of 50 ng/ml of GM-CSF for 5 days and then analyzed their ability to

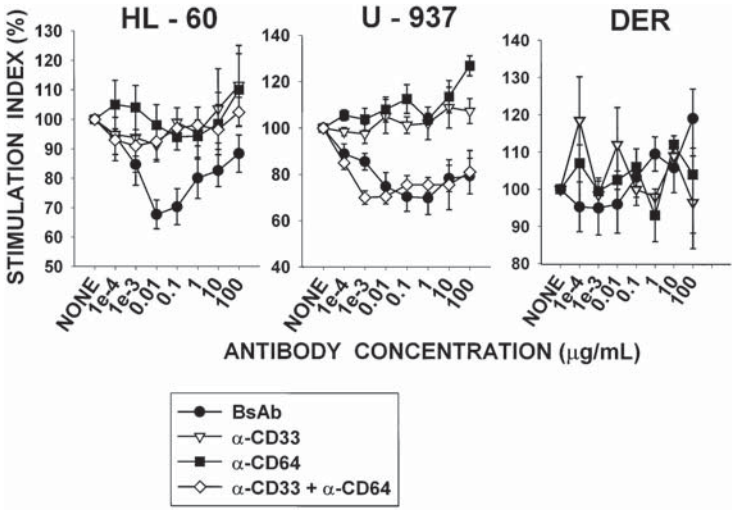

Fig. 1. Effect of BsAb on proliferation of leukemia cells. Leukemia cells were cultured for 4 h in triplicate for each condition in tissue culture media supplemented with 0.5% FBS in the presence of various concentrations of antibodies. ^{3}H-thymidine was added at the initiation of culture. The basal level of cell proliferation was considered as 100%, and the percentage of basal stimulation was calculated for each condition. Each histogram represents results of five independent experiments. Error bars indicate the standard error about the mean (S.E.M.).

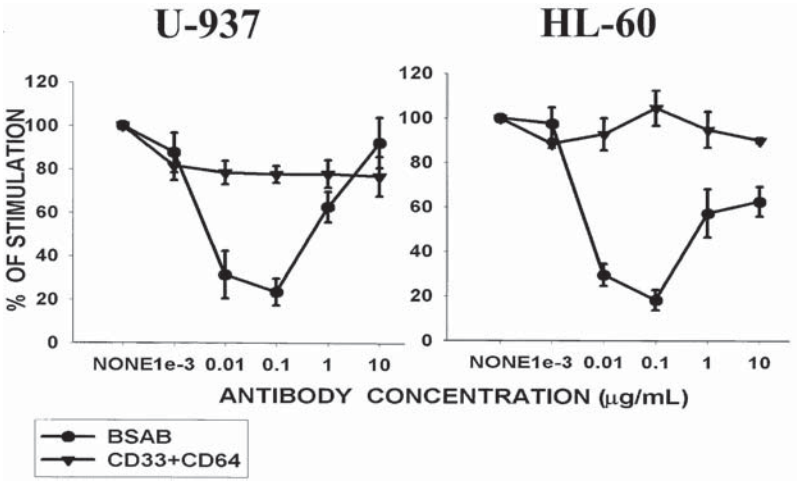

Fig. 2. Effect of BsAb on leukemia cells colony formation. Leukemia cells were cultured in 0.3% agarose in triplicate for each condition in the presence of various concentrations of antibodies. After 14 days of culture, the number of colonies was enumerated in each dish. The basal level of colony formation was considered as 100%, and the percentage of stimulation was calculated for each condition. Each histogram represents results of four independent experiments. Error bars indicate S.E.M.

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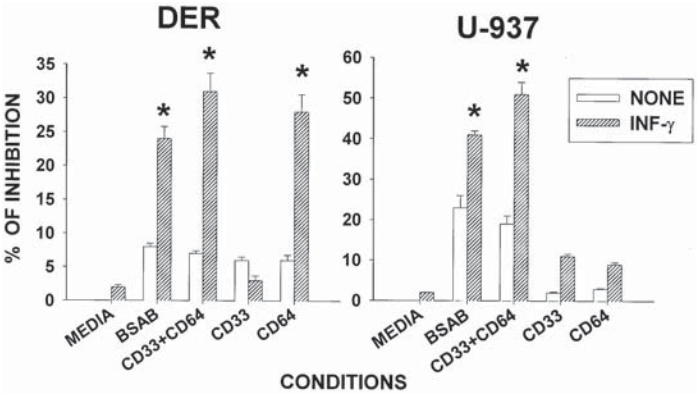

Fig. 3. Effect of INF-γ on sensitivity of AML cells to antibody treatment. U-937 and DER cells were cultured in the presence or absence of 200 u/ml of INF-γ for 24 h under standard conditions. After two washings proliferation assays were performed in the presence of various antibodies as described in Fig. 1. Each bar represents the percentage of inhibition, calculated as the difference between the basal level of ^{3}H uptake (media alone) and the results of proliferation for each condition. Error bars indicate S.E.M.

integrate pre-stained *E. coli* particles in the presence of BSAB, unconjugated anti-CD33 and anti-CD64 antibodies or the combination of both mAb. We found that, in contrast to anti-CD33 mAb, anti-CD64 mAb as well as BsAb significantly increased ($P < 0.001$) the phagocytic activity of monocytes (Fig. 4). This effect was present when we treated cells with a combination of two unconjugated antibodies, but the level of phagocytosis was lower. Time-course experiments revealed that in all five samples tested, the level of BsAb-mediated phagocytosis after 3 h of treatment was higher compared to that at 1 h. Meanwhile, at both time points, the effect was dose-dependent.

3.4. Induction of tyrosine phosphorylation by BsAb ligation

In order to determine the mechanisms of the functional diversity between normal monocytes and AML cells, we analyzed the signaling cascade leading to leukemia cell growth inhibition. Since tyrosine phoshorylation is an early and obligatory event in cell activation, we examined protein phosphorylation induced by BsAb ligation by immunoblot analysis using an antibody to phosphotyrosine. After the addition of BsAb to normal monocytes or AML cells, in normal monocytes, we observed rapid phosphorylation of protein(s) that were of approximately 100–120 kDa (data not shown). In contrast, similar treatment of AML cells did not induce phosphorylation of additional proteins. However, the majority of proteins in AML cells were constitutively phosphorylated (data not shown). Time-course experiments showed that induced protein tyrosine phosphorylation could be detected as early as 1 min after BsAb ligation. Protein phosphorylation reached a peak level at approximately 2 min, but still could be detected for at least 1 h after stimulation (data not shown). This indicates that BsAb ligation induces rapid tyrosine phosphorylation of cytosolic proteins independent of other stimulatory signals and serves as a stimulatory factor that is sufficient to induce activation of phagocytosis in normal monocytes. How-

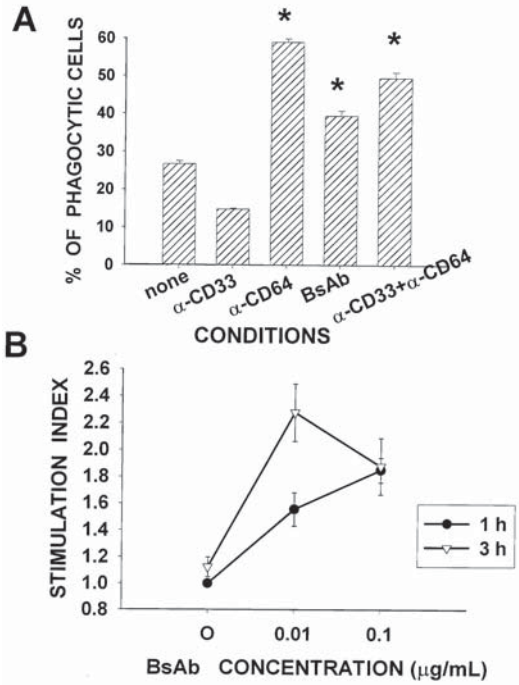

Fig. 4. Effect of BsAb on phagocytosis of normal monocytes. Normal monocytes were stained with CD14-FITC and mixed with pre-stained tetramethylrhodamine *E. coli* particles in the presence of various concentrations of antibodies. The numbers of double stained (phagocytic) cells were estimated for each condition under fluorescent microscope. Each bar indicates the percentage of phagocytic cells for each condition. Error bars indicate S.E.M. Bars marked with an asterisk have mean values that are significantly higher than that of the control condition ($P < 0.005$, Student's t-test).

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