Ligation of the CD44 adhesion molecule inhibits drug-induced apoptosis in human myeloid leukemia cells

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Adhesion molecules can improve hematopoietic cell survival; however, their role in leukemic cell resistance to drug-induced apoptosis is poorly documented. The CD44 adhesion molecule is strongly expressed on acute myeloid leukemia (AML) blasts. Using 2 myeloid cell lines, HL60 and NB4, evidence is presented that prior incubation with the CD44-specific monoclonal antibody (mAb) A3D8, reported to induce differentiation of AML blasts, significantly decreases apoptosis induced by 3 drugs used in AML chemotherapy: daunorubicin (DNR), mitoxantrone, and etoposide. In addition, in HL60 cells, CD44 ligation with A3D8 mAb fully abrogates the DNR-triggered generation of ceramide, a lipid second messenger involved in the DNR apoptotic signaling pathway. Moreover, results show that the A3D8 mAb and Bcl-2 additively inhibit DNR-induced apoptosis in HL60 cells overexpressing Bcl-2. These results suggest that, to eradicate AML blasts, the differentiation-inducing anti-CD44 mAb A3D8 should not be administered prior to apoptosis-inducing drugs. (Blood. 2000; 96:1187-1190)

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Introduction

Chemoresistance, a major cause of treatment failure in acute myeloid leukemia (AML), is a complex process involving various mechanisms. Among them, inhibition of drug-induced apoptosis is likely to be involved because chemoresistance of AML frequently correlates with increased expression of antiapoptotic molecules, such as Bcl-2.2 Interestingly, it was recently shown that AML cell apoptosis could be inhibited via integrins,3 suggesting a role for adhesive receptors in AML blasts chemoresistance.

The CD44 adhesion molecule, a receptor for hyaluronan,4 is strongly expressed on AML blasts.5 Recent reports suggest that CD44 can inhibit apoptosis; CD44 ligation with activating monoclonal antibodies (mAbs) or hyaluronan abrogates apoptosis of T lymphocytes and promotes survival of B lymphocytes.6,7 Therefore, we investigated whether CD44 ligation could decrease drug-induced apoptosis of AML blasts.

We used 2 human myeloid cell lines, HL60 and NB4, and independently ligated CD44 with 2 activating mAbs.8,9 Interestingly, A3D8 mAb induces differentiation of fresh AML blasts, whereas J173 does not.10 Hyaluronan was not assayed because of its low affinity for CD44 expressed on HL60 and NB4.11 After CD44 ligation, apoptosis was induced by 3 drugs used in AML chemotherapy: daunorubicine (DNR), mitoxantrone, or etoposide. Our results show that preincubation with A3D8 inhibits apoptosis induced by all drugs tested, in both cell lines. Moreover, A3D8-mediated antiapoptotic effect is additive to that of Bcl-2, in HL60 cells overexpressing Bcl-2. These findings may have implications for designing treatments potentially associating CD44-targeted differentiation and chemotherapy.

Study design

Reagents and cells

The A3D8 (Sigma Chemical, St. Louis, MO) and J173 (Coulter-Immunotech, Marseille, France) antihuman CD44 mAbs were purchased uncoupled or as fluorescein isothiocyanate (FITC) conjugates. Murine IgG1 (isotype control) and FITC-conjugated goat antimurine IgG (GAM-FITC) were from Coulter-Immunotech. Daunorubicin (Cerubidine) was obtained from Laboratoire Roger Bellon (Neuilly-sur-Seine, France) and mitoxantrone (Novantrone) from Lederle Parenteralss (Carolina, Puerto Rico). Methyl-[3H]-thymidine (79 Ci/mmol) and 9,10(n)-[3H]-palmitic acid (53 Ci/mmol) were purchased from Amersham (Les Ulis, France), annexinV-FITC from Roche (Meylan, France), and other reagents from Sigma.

Cells were cultured in RPMI 1640 containing 10% fetal calf serum, 2 mmol/L L-glutamine, 100 µg/mL streptomycin, and 200 U/mL penicillin (GIBCO, Grand Island, NY). HL60 was purchased from ATCC (CCL240; Rockville, MD). Bcl-2 overexpressing HL60/Bcl-2, HL60/Neo cells, and Western blotting for Bcl-2 detection have been described.12,13 NB4 cells were derived from an acute promyelocytic leukemia.14

Cross-blocking of mAbs

Cells were preincubated with 20 µg/mL uncoupled A3D8, J173, or mouse IgG1, then stained with 2 µg/mL FITC-conjugated A3D8 or J173. Fluorescence was analyzed on a Profile II flow cytometer (Coulter Immunology, Hialeah, FL) equipped with a 15-mW argon laser (488 nm) on 3000 to 5000 cells.

CD44 ligation and induction of apoptosis

Cells (5 × 10⁵ cells/mL) were preincubated at 37°C for 16 hours with 2.5 µg/mL A3D8, J173, mouse IgG1, or culture medium. Subsequently, DNR...
(0.5 µmol/L), mitoxantrone (0.3 µmol/L), or etoposide (10 µmol/L) was added, during 6 or 24 hours. Cell viability was evaluated by trypan blue dye exclusion.

Quantification of apoptosis

Apoptosis was quantified by 3 previously described methods: flow cytometry on cells labeled with annexin V-FITC and propidium iodide, microscopical examination of smears stained with May-Grünwald-Giemsa stain, and quantification of fragmented DNA, based on an [3H]-thymidine release assay.

Ceramide quantitation

Cells were metabolically labeled for 48 hours with 1 µCi/mL 9,10(9)-[3H]-palmitic acid. A3D8 mAb was added during the last 16 hours of labeling. Total cellular ceramide content was measured for 0 to 15 minutes after addition of 0.5 µmol/L DNR, as described.

Statistical analysis

A paired Student t test was used to assess differences between treated and control groups. A P value less than .05 was considered significant.

Results and discussion

A3D8 anti-CD44 mAb inhibits drug-induced apoptosis

The HL60 and NB4 cells were preincubated with A3D8 or J173 mAbs, or culture medium, then treated with DNR, mitoxantrone, or etoposide. Apoptotic cells, labeled with annexin V-FITC and excluding propidium iodide, were quantified by flow cytometry. Drug treatment alone induced apoptosis of 24% to 34% HL60 and 34% to 40% NB4 cells (Figure 1A,B). Preincubation with A3D8 mAb reduced apoptosis induced by all 3 drugs by over 50% in HL60 (Figure 1A) and by 75% to 100% in NB4 cells (Figure 1B). In contrast, J173 did not modulate drug-induced apoptosis in these cells.

These results were confirmed by cell morphology analysis and DNA fragmentation. Indeed, preincubation of HL60 with A3D8 significantly decreased DNR-induced apoptosis (from 62.9 ± 2.8% to 41.5 ± 1.7%, P < .01, Figure 1C) and DNA fragmentation (from 42.5 ± 1.6% to 23.1 ± 1.5%, P < .001, Figure 1D). Apoptosis inhibition was specifically mediated by A3D8 anti-CD44 mAb, because both a control isotype (IgG1) and another anti-CD44 mAb (J173) were inactive (Figure 1C,D). Note that culture medium or antibodies alone induced less than 2% apoptosis (Figure 1A-C).

To determine whether A3D8 antiapoptotic effect was associated with increased cell viability, we compared cell survival in DNR-treated cultures preincubated with A3D8, J173, or culture medium. Only A3D8 improved survival of HL60, HL60/Bcl-2, and NB4 cells to DNR treatment (Figure 1E). However, no viable cells were recovered after 5 days.

Inactivity of J173 was not due to poor binding because it bound CD44 as strongly as A3D8 (Figure 1F, left panels). Moreover, J173 and A3D8 did not cross-block each other’s binding (Figure 1F), suggesting that they recognize distinct epitopes of CD44. Because of its inactivity, J173 subsequently served as surface-binding, isotype-matched control.

A3D8 inhibits proliferation of myeloid leukemia cells

To investigate the mechanisms of A3D8 antiapoptotic activity, we first examined its effect on cell viability. We noticed that, in DNR-free cultures, A3D8 inhibited proliferation of HL60 (Figure 1). To investigate the mechanisms of A3D8 antiapoptotic activity, we first examined its effect on cell viability. We noticed that, in DNR-free cultures, A3D8 inhibited proliferation of HL60 (Figure 1).

Figure 1. A3D8 anti-CD44 mAb inhibits drug-induced apoptosis. The inhibitory effect of CD44-specific A3D8 mAb on drug-induced apoptosis was determined in HL60, as shown in A, C, and D, and NB4 (B) myeloid cells. Cells were preincubated for 16 hours with 2.5 µg/mL anti-CD44 mAbs A3D8 or J173, control mouse IgG1, or culture medium, then treated with DNR (0.5 µmol/L), mitoxantrone (0.3 µmol/L), or etoposide (10 µmol/L) for 24 hours (A-C) or 6 hours (D). Apoptosis was evaluated by measuring the percentage of annexin V-FITC-positive, propidium iodide-negative cells, by flow cytometry (A and B); the percentage of apoptotic cells, by microscopical observation of May-Grünwald-Giemsa stained cytosmears (C); and the percentage of fragmented DNA, in which the spontaneous DNA fragmentation of untreated cells (5% DNA fragmentation) has been subtracted, by the [3H]-thymidine release assay (D), as described. Panels A and B show results from 1 representative experiment of 3. The mean ± SEM from triplicate samples are shown for panels C and D. (E) Analysis of cell survival in DNR-treated cultures preincubated with CD44-specific mAbs. HL60, HL60/Bcl-2, and NB4 cells were preincubated for 16 hours with culture medium, A3D8 or J173 mAbs, as indicated, then treated with 0.5 µmol/L DNR or culture medium alone (DNR-free control) for 24 hours. The total number of viable cells per milliliter was evaluated by trypan blue dye exclusion. For each preincubation, the percentage of surviving cells was calculated as follows: [number of viable cells/mL in DNR-treated culture] / [number of viable cells/mL in DNR-free culture] × 100. Results represent 1 of 2 independent experiments. (F) A3D8 and J173 anti-CD44 mAbs do not cross-block each other’s binding to HL60 cells. HL60 cells were preincubated with a saturating concentration (20 µg/mL) of A3D8 or J173 mAbs, or IgG1 control isotype, and subsequently stained with FITC-conjugated A3D8 or J173. Cell fluorescence (black histograms) was analyzed by flow cytometry. Nonspecific background fluorescence was determined on cells incubated with control IgG1, followed by GAM-FITC (gray histograms).
2A), HL60/Bcl-2 (Figure 2B), and NB4 cells (not shown). As expected, J173 was inactive.

**A3D8 abrogates the DNR-induced generation of ceramide**

We next examined how A3D8 could affect DNR-induced apoptotic signaling.17 We found that A3D8 totally abrogated the generation of ceramide, a lipid second messenger involved in the apoptotic signaling of cytotoxic agents18 (Figure 2C). Note that, like A3D8, protein kinase C (PKC) activators inhibit both DNR-induced ceramide generation and apoptosis in HL60 cells.19 Because CD44 ligation can activate PKC,20 this suggests that A3D8 might inhibit ceramide generation via PKC activation.

**CD44 and Bcl-2 additively inhibit DNR-induced apoptosis**

We previously demonstrated that, unlike A3D8, Bcl-2 inhibits DNR-induced apoptosis in HL60 cells without affecting ceramide generation,13 suggesting different antiapoptotic mechanisms. Using HL60/Bcl-2 cells, we first checked Bcl-2 anti-apoptotic activity; significant apoptosis inhibition was observed in HL60/Bcl-2, compared to HL60/Neo cells (26.8 ± 2.3% versus 64.8 ± 3.5% apoptotic cells, P < .001, Figure 2D). Strikingly, preincubation of HL60/Bcl-2 cells with A3D8 further decreased DNR-induced apoptosis in these cells (13.3 ± 3.2% versus 26.8 ± 2.3%, P < .01, Figure 2D), thus demonstrating additive anti-apoptotic effects of A3D8 and Bcl-2. As expected, J173 (Figure 2D) or control IgG1 (not shown) were inactive. These results suggest that A3D8 and Bcl-2 inhibit apoptosis signaling at different levels, upstream and downstream of ceramide, respectively. In agreement, we found that A3D8 did not inhibit apoptosis induced by exogenous, cell permeant ceramide in HL60 cells (data not shown).

In conclusion, we demonstrate that prior treatment of AML cell lines NB4 and HL60 with A3D8 anti-CD44 mAb significantly reduces drug-induced apoptosis. The A3D8 antiapoptotic effect may be related to its antiproliferative and differentiating activity on AML cells, as shown for patients’ blasts,10 and HL60 and NB4 cells (Charrad and coworkers, article in preparation). Indeed, several authors reported that differentiating agents inhibit drug-induced apoptosis of AML.21-23 Moreover, in agreement with our present experiments, prior administration of all-transretinoic acid in patients with acute promyelocytic leukemia decreased chemotherapy efficiency.24 Taken together, these considerations suggest that, in a therapy combining A3D8-induced differentiation and drug-induced apoptosis, A3D8 should not be administered first.

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**References**


