Evidence for a Causal Role of CD38 Expression in Granulocytic Differentiation of Human HL-60 Cells*

Received for publication, September 11, 2002, and in revised form, October 15, 2002
Published, JBC Papers in Press, October 16, 2002, DOI 10.1074/jbc.M209313200

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Granulocytic differentiation of human HL-60 cells can be induced by retinoic acid and is accompanied by a massive expression of CD38, a multi-functional enzyme responsible for metabolizing cyclic ADP-ribose (cADPR), a Ca2+ messenger. Immunofluorescence staining showed that CD38 was expressed not only on the surface of intact HL-60 cells but also intracellularly, which was revealed after permeabilization with Triton. Concomitant with CD38 expression was the accumulation of cADPR, and both time courses preceded the onset of differentiation, suggesting a causal role for CD38. Consistently, treatment of HL-60 cells with a permeant inhibitor of CD38, nicotinamide, inhibited both the CD38 activity and differentiation. More specific blockade of CD38 expression was achieved by using morpholino antisense oligonucleotides targeting its mRNA, which produced a corresponding inhibition of differentiation as well. Similar inhibitory effects were observed when CD38 expression was reduced by the RNA interference technique targeting two separate regions of the coding sequence of CD38. Further support came from transfecting HL-60 cells with a Tet-On expression vector containing a full-length CD38. Subsequent treatments with doxycycline induced both CD38 expression and differentiation in the absence of retinoic acid. These results provide the first evidence that CD38 expression and the consequential accumulation of cADPR play a causal role in mediating cellular differentiation.

CD38, first defined by monoclonal antibody typing as an antigen (1), has been widely used as a marker for lymphocyte differentiation. Sequence comparison reveals that it shares about 30% sequence identity with the Aplysia ADP-ribosyl cyclase, indicating that it is a mammalian homolog (2). This is later confirmed by studies showing CD38 can indeed catalyze the cyclization of NAD to produce cyclic ADP-ribose (cADPR)1 (reviewed in Refs. 3 and 4), a cyclic nucleotide messenger active in mediating Ca2+ signaling in a wide variety of cells spanning three biological kingdoms: protist, plant, and animal (Refs. 5–7; reviewed in Refs. 8 and 9). More remarkably, CD38 is, in fact, a multi-functional enzyme capable of using a different substrate, NADP, to catalyze a base exchange reaction (10) to produce nicotinic acid adenine dinucleotide phosphate, another general Ca2+ messenger with a totally distinct structure and a separate mechanism of action (Ref. 11; reviewed in Refs. 9 and 12). It is now known that CD38 is not specific for lymphocytes but is ubiquitously expressed in many tissues and cells (reviewed in Ref. 3). The physiological functions that involve CD38 are equally widespread and include, for example, cell proliferation (13) and expansion of human hemopoietic progenitors (14, 15). CD38 knockout mice exhibit defects in neutrophil chemotaxis (16), insulin secretion (reviewed in Ref. 17), and aberrant muscarinic Ca2+ signaling in pancreatic acinar cells (18), indicating the importance of CD38 in regulating functions in vivo as well as in vitro.

A dramatic increase in CD38 expression accompanies granulocytic differentiation induced by retinoic acid in human HL-60 cells (19–21). The cell line is derived from a patient with acute promyelocytic leukemia and can be induced to differentiate in vitro to a number of different cell types, such as granulocytes, monocytes, or macrophages (22). It is a widely used model system for elucidating hemopoietic differentiation (22, 23). In this study we present evidence that the CD38 expression may play a causal role in mediating the differentiation process in HL-60 cells.

EXPERIMENTAL PROCEDURES

Materials—ADP-ribosyl cyclase was prepared by a yeast expression system as described previously (24). Alcohol dehydrogenase from yeast (suitable for cycling), activated charcoal, diaphorase from Clostridium kluyveri, NAD+, nicotinamide, nucleotide pyrophosphatase from Crota- lus atrox venom, NADase from Neurospora crassa, NBT, phorbol 12-myristate 13-acetate, resazurin, tri-n-octylamine Tris, and 1,1,2-trichlorotrifluoroethane were from Sigma. Alkaline phosphatase from bovine intestine was obtained from Roche Molecular Biochemicals. Centricrin filters and Immobilon filter plates were from Millipore (Bedford, MA). Alexa Fluor goat anti-mouse IgG was obtained from Molecular Probes.

Culture of HL-60 Cells and Induction of Differentiation—HL-60 cells were obtained from the American Type Culture Collection. The cells were maintained in suspension in RPMI medium supplemented with 10% fetal calf bovine serum and kept at 37 °C in a 5% CO2 atmosphere. The cells were passaged by dilution in fresh medium to a density of 1 106 cells/ml. Prior to induction of differentiation by retinoic acid (RA), the cells were maintained at a logarithmic growth rate and seeded at a density of 0.2 106 cells/ml. RA was added at a final concentration of 1 μM by dilution from a 10 μM stock solution prepared in Me2SO. Control cells were treated with a similar dilution of Me2SO, which was found to have no effect on the differentiation or the rate of cell division. At the indicated times of continuous exposure to RA, the cells were pelleted by centrifugation at 700 × g for 5 min. Differentiation of HL-60 cells was measured by adding 1 ml of cell suspension (0.5–2 106 cells) to a solution containing 2 mg/ml of NBT and 20 ng/ml of phorbol myristate acetate in phosphate-buffered saline. The incubation was allowed to proceed for 1 h at 37 °C and was stopped by the addition of 0.4 ml of cold 2 M HCl. The formazan product was obtained by centrifugation of the sample at 700 × g for 10 min. The supernatant was discarded, and the formazan was dissolved in 1 ml of Me2SO. The

1 This work was supported by National Institutes of Health Grants GM60333 and GM61568 (to H. C. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: cADPR, cyclic ADP-ribose; NGD, nicotinamide guanine dinucleotide; cGDP, cyclic GDP-ribose; FACS, fluorescence-activated cell sorter; NBT, nitro blue tetrazolium; RA, retinoic acid; RNAi, RNA interference; siRNA, small interfering RNA.
fluorescence-activated Cell Sorter (FACS) Analyses of CD38 Expression—Following treatment of HL-60 cells with RA, 1.5–2 × 10^6 cells were pelleted by centrifugation at 700 × g for 5 min. The cells were resuspended in 1 ml of FACS buffer (phosphate-buffered saline containing 2.5% fetal bovine serum and 0.02% NaN3) and washed once by centrifugation step at 14,000 × g for 10 min in a microcentrifuge and resuspended in 125 μl of FACS buffer. The cells were incubated with the primary monoclonal antibody, IB4 (1:100), for 30 min on ice and washed once in 1 ml of FACS buffer and resuspended in 200 μl. The secondary antibody, Alexa Fluor goat anti-mouse IgG, was added at a dilution of 1:200, and the sample was incubated on ice after mixing and kept on ice until the plate was ready for staining. Stainings were then performed, and the samples were resuspended in 1 ml of FACS buffer containing 1% paraformaldehyde. The samples were sorted on a FACScalibur instrument, and data from 10,000 cells were collected and analyzed by the CELLQuest Pro software.

Measurements of Endogenous cADPR—Acid extracts were prepared for 5–20 × 10^6 cells after centrifugation and the addition of 0.5–1 ml of cold 0.6 M perchloric acid, which could be stored at −80 °C until processing. After thawing, the acid was removed by extraction with a solution (3:1) of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine on ice as described (25). The neutral extracts were supplemented with 20 mM sodium phosphate, pH 8, and treated with an enzyme mixture consisting of 0.44 unit/nucleotide pyrophosphatase, 0.0625 unit/ml NADase, 12.5 units/ml alkaline phosphatase, and 2 mg MgCl₂ for 15–18 h at 37 °C as described (25), to effectively remove interfering nucleotides, such as NAD, without degrading cADPR. Subsequently, the enzymes were removed by ultrafiltration with Centricon filters or 96-well Immobilon-P plates. The cADPR in the extracts was measured by an enzyme cycling assay as described previously (25). For comparison, cADPR standards were prepared in 20 mM sodium phosphate, pH 8, and processed in a manner identical to that of the cell extracts. This parallel processing of cADPR standards allowed for adjustment of losses of cADPR resulting from the procedure, which was typically about 30%. The enzyme cycling assay was performed in 96- or 384-well plates, and volumes of 100 or 4 μl, respectively, were used. The cADPR content was determined from the slopes of fluorescence increase of samples and compared with those produced by cADPR standards (25). The assay of cADPR was linear in the concentration range from 0 to 25 nmol of cADPR formed per 10^6 cells.

Inhibition of CD38 Expression by Antisense Oligonucleotides—Morpholino antisense oligonucleotides have been recently designed to overcome some of the known limitations of regular antisense oligonucleotides. Morpholinos are assembled from four subunits, each of which contains one of the four bases linked to a six-membered morpholino ring, which are joined in a stop-oxidation rodiaminate linkages. Applications of morpholino antisense oligonucleotides in different species indicate that it has improved specificity and stability against nucleases (reviewed in Refs. 27 and 28). Morpholino antisense and sense oligonucleotides were synthesized by Gene Tools (Philomath, OR) to target a sequence residing in the 69 base pair (bp) region of human CD38 mRNA. The DNA and liposome dilutions were combined in equal volumes and incubated at room temperature for 20 min. HL-60 cells (8 × 10^6) in 500 μl of OPTIMEM-I medium was added and incubated at 37 °C for 4 h. Afterward, 5 ml of RPMI 1640 (with 10% fetal calf serum) was added, and the transfected cells were incubated at 37 °C. After two cell divisions (48 h), the cells were resuspended in 5 ml of RPMI 1640 (with 10% fetal calf serum) and incubated at 37 °C for 24 h. RA was added to a final concentration of 1.0 μM, and the cells were harvested 72 h later for analyses.

Silencing of CD38 by Small Interfering RNA (siRNA)—Down-regulation of CD38 was facilitated by using the following primers targeting two separate coding regions of CD38 mRNA: region 3 sense primer, 5′-cGAGACTGAGGC-3′; region 27 sense primer, 5′-TACAGCAGCCAAGACAGATTCTgtctc-3′; region 27 sense primer, 5′-AGGACTGACGAAACACCCTTctgtctc-3′; and region 27 antisense primer, 5′-GGGTTTGTGCTGACCTTCTTctgtctc-3′. The two regions start 70 and 533 bases downstream of ATG, respectively. Bases indicated by capital letters correspond to the region in the CD38 mRNA, while the 8-nucleotide stretch at the 3′ end, in lowercase letters, is required for the T7 promoter primer sequence. 5′-TAATACGACT-ACATATAGgagacagg-3′, to hybridize to the sense and antisense primers for transcription. The two thymidines, in italics, are needed for the stability of the siRNA (30). The HiScript RNAi transcription kit from New England BioLabs was used to synthesize the double-stranded siRNA. The siRNA was purified twice by ethanol precipitation and dissolved in sterile RNase-free water.

Transfection of HL60 cells was facilitated by the TransIT-TKO Transfection Reagent (Mirus Corporation, Madison, WI). 24 μl of the TKO reagent was first incubated with 100 μl of OPTIMEM-I medium (Invitrogen) at room temperature for 15 min before siRNA was added. After another 15 min at room temperature, 500 μl of RPMI 1640 (with 10% calf serum) containing 0.8 × 10^6 cells was added. The final siRNA concentration was 125 nm, and the incubation proceeded for 4 h at 37 °C and 5% CO₂, after which 3.5 ml of RPMI 1640 (with 10% calf serum) was added, and the incubation continued at 37 °C for 24 h. RA was added to a final concentration of 1.0 μM, and the cells were harvested 72 h later for analyses.

Cationic liposomes were used for transfecting HL60 cells with the pTet-On plasmid. DC-Cholesterol, l,α-dioleoyl phosphatidylethanolamine, and diphtyranol phosphatidylethanolamine were purchased from Avanti Polar Lipids. Formulations used were 1:1 and 1:3 (molar ratio) of l,α-dioleoyl phosphatidylethanolamine/DC-cholesterol. 5 mg of total lipid of each formulation were dried down and resuspended in 250 μl of distilled H₂O, followed by 5 min of sonication. 250 μl of 2% phosphate-buffered saline, pH 7.4, was added, and the liposomes were further sonicated for 3 min.

5 μl of the liposomal suspension was diluted in 50 μl of OPTIMEM-I medium and incubated at room temperature for 5 min. 1.2 μg of the SacI-digested pTet-On plasmid was diluted in 50 μl of OPTIMEM-I medium, and the DNA and liposome dilutions were mixed by vortexing and sonicated in equal volumes and incubated at room temperature for 20 min. HL-60 cells (8 × 10^6) in 500 μl of OPTIMEM-I medium was added and incubated at 37 °C for 4 h. Afterward, 5 ml of RPMI 1640 (with 10% fetal calf serum) was added, and the transfected cells were incubated at 37 °C. After two cell divisions (48 h), the cells were resuspended in 5 ml of RPMI 1640 (with 10% fetal calf serum) and incubated at 37 °C. The cells stably expressing reverse tetracyclin-controlled transactivator were isolated by selection with 200 μg/ml G418 in the medium. The medium was replaced every 5 days. The cells stably expressing reverse tetracyclin-controlled transactivator were obtained after repeated selection with G418. Over the next 9 weeks the cells were passaged 12 times until there were virtually no dead cells in the cultures. Control experiments show that these stably transfected cells differentiate in each monolayer culture into a portion of the cells was frozen, and the remaining cells were used for the next transfection. All of the subsequent incubations were carried out in the presence of 380 μg/ml G418 in the medium.

A pBl plasmid containing both green fluorescent protein and a full-length version of CD38 was used to transfect HL60 cells in order to determine the efficiency of rTet-On control.
CD38 and Differentiation of HL-60 Cells

Fig. 1. Immunofluorescence staining of HL-60 cells. Following treatment of HL-60 cells with 1 μM RA, 1.5–2 × 10⁶ cells were pelleted by centrifugation at 700 × g for 5 min. The cells were resuspended in 1 ml of FACS buffer (see "Experimental Procedures") and washed once by centrifugation at 14,000 × g for 20 s in a microcentrifuge. The aliquots were treated without (− Triton) or with (+ Triton) 0.1% Triton for permeabilization and subsequently incubated with primary (IB4) and secondary (fluorescein-labeled anti-mouse IgG) antibodies as described under "Experimental Procedures." The cells were fixed with 1% paraformaldehyde in FACS buffer and added to polylysine-coated glass coverslips before viewing with a fluorescence confocal microscope. The left side shows bright field images corresponding to the fluorescence images on the right.

RESULTS

Treatment of HL-60 cells with RA induces differentiation into granulocytes (22), which possess many of the functional characteristics of normal peripheral blood granulocytes, including phagocytosis and chemotaxis. The underlying mechanism is largely unknown. During phagocytosis, rapid generation of superoxide anion occurs, which can be conveniently monitored by NBT. It is a water-soluble dye, which is converted to insoluble intracellular blue formazan by phagocytizing neutrophils, a reaction mediated by superoxide (31, 32). Differentiated cells that are phagocytizing are thus stained blue and black, whereas undifferentiated cells are not stained. The NBT reaction can also be monitored in cell suspensions by measuring the increase in absorbance at 590 nm. Differentiated cells produce greatly increased NBT reaction as compared with control cells. Either the absorbance changes or direct counting of NBT staining cells was used for quantifying granulocytic differentiation.

We and others have shown that accompanying differentiation, RA also induces expression of CD38 in HL-60 cells (19–21), which can be conveniently measured by using FACS analyses or by measuring the ADP-ribosyl cyclase activity of CD38 in cell extracts using the NGD technique (21, 26). CD38 cyclizes NGD, a nonfluorescent substrate analog of NAD, to cADPR, a fluorescent product, which can be measured fluorimetrically.

It is generally believed that CD38 is an antigen and is mainly expressed on the cell surface. Fig. 1A shows immunofluorescence localization of CD38 in the differentiated cells. Intact cells (− Triton) showed ring-like immunostaining as revealed by confocal fluorescence microscopy, consistent with surface expression. Permeabilization with a detergent, Triton, before staining allowed internal access and resulted in even more intense staining that exhibited prominent intracellular structures (Fig. 1B). The RA-induced expression of CD38 is thus not limited to the cell surface but intracellularly throughout the cells as well, appropriate for a signaling role.

This is supported by measuring the cellular accumulation of its enzymatic product, cADPR, as shown in Fig. 2. At various times after RA (1 μM) was added to HL-60 cells, the cell extracts were prepared as described under "Experimental Procedures." A, CD38 expression was measured as cGDP4 production from NGD. The values shown are the averages of four determinations, and the standard deviation (S.D.) is partly hidden by the symbols. B, the cellular content of cADPR was measured in acid extracts from control and RA-treated cells as described under "Experimental Procedures." The values of cADPR content represent the averages ± S.D. of three or four determinations. C, differentiation of HL-60 cells was determined by the increase in NBT absorbance at 590 nm/10⁶ cells. The values are the averages ± S.D. of three determinations.
prepared, the observed inhibition actually reflected the reduction of CD38 expression. This was surprising, but the results were confirmed by using FACS analyses as shown in the inset in Fig. 3A. Treatment with nicotinamide (20 mM) during the RA induction resulted in most cells exhibiting less CD38 fluorescence.

The cellular cADPR levels exhibited similar changes to those of the CD38 activity as shown in Fig. 3B. The extent of reduction in cADPR levels appeared more pronounced than the CD38 activity, and the levels were reduced close to the basal levels of the control cells without the RA treatment. This is likely to be due to the combined effects of the inhibition of the cyclase activity of CD38 by nicotinamide as well as the actual reduction in CD38 expression in the cells. Parallel to the reduction in cADPR and CD38, there was corresponding inhibition of cellular differentiation as shown in Fig. 3C. The inhibitory effect of nicotinamide was specific for differentiation because the treated cells were not only viable throughout the 72 h of incubation but also proliferated equally well as compared with control cells not treated with the inhibitor. Furthermore, the NAD content of the cells actually doubled, from 0.35 ± 0.05 to 0.70 ± 0.01 nmol/10⁶ cells, after 72 h of treatment with nicotinamide, indicative of the treated cell being in an energetically favorable state.

A common method for suppressing expression of a protein is to use antisense oligonucleotides. Morpholino oligonucleotides represent a recent improvement of the technique and offer better specificity and stability against nucleases than regular oligonucleotides (reviewed in Refs. 27 and 28). Fig. 4 shows that preincubation with the antisense oligonucleotides reduced both the expression of CD38 and cellular differentiation to levels similar to the control cells not treated with RA. Preincubation with the sense oligonucleotides or with just the carrier (ethoxylated polyethyleneimine) affected neither the expression

**Fig. 3.** Effect of nicotinamide on CD38 expression, cADPR content, and differentiation of HL-60 cells. The cells were treated with 1 μM RA for 24 h, and 20 mM nicotinamide was added at 24 and 48 h (indicated by arrows). A, CD38 expression was measured as GDP-ribosyl cyclase activity in cell extracts. The values are the averages of triplicates ± S.D. There was no detectable activity in control extracts. The inset shows the FACS analyses of CD38 expression. The expression during the RA induction was inhibited by nicotinamide (20 mM), resulting in most cells exhibiting less CD38 fluorescence. B, the cADPR content of control and RA-treated cells was measured in perchloric acid extracts and represent the average of four values ± S.D. C, the differentiation of HL-60 cells induced by RA was determined by NBT absorbance at 590 nm/10⁶ cells. The data represent the averages ± S.D. of three determinations.

**Fig. 4.** Inhibition of CD38 expression and differentiation in HL-60 cells by antisense oligonucleotide to CD38. Sense and antisense oligonucleotides in the vehicle ethoxylated polyethyleneimine were prepared as described under “Experimental Procedures” and incubated with HL-60 cells 24 h before the addition of 1 μM RA. The total RA treatment period was 48 h. The cell extracts were assayed for GDP-ribosyl cyclase activity (CD38 Activity) as a measure for CD38 expression. Differentiation was determined by NBT absorbance at 590 nm/10⁶ cells. The data are the averages ± S.D. of three determinations.

**Fig. 5.** Inhibition of CD38 expression and differentiation in HL-60 cells by RNA interference. HL60 cells were transfected with siRNA corresponding to two regions, 3 and 27, in the coding region of full-length CD38 cDNA (see “Experimental Procedures”). Top panel, CD38 activity shown as a percentage of nontransfected cells induced with RA. Transfection with siRNA regions 3 and 27 suppressed CD38 activity significantly. No appreciable reduction in CD38 activity was observed when the cells were mock incubated with the TKO transfection reagent alone. Bottom panel, differentiation of HL-60 cells measured by NBT staining, indicating that the levels of differentiation followed the same trend as the CD38 cyclizing activity, with region 27 siRNA having the most significant impact. The data shown are the averages of three independent cultures ± S.D.
Fig. 6. Expression of CD38 in Tet-On HL60 cells. A, FACS analysis of HL60 cells transfected with the Tet-On expression construct and induced with (Tet-On + Dox) or without (Tet-On – Dox) doxycycline (2 μg/ml). B, doxycycline induction of CD38 expression also induced differentiation in the absence of RA as measured by the NBT assay (Tet-On – RA + Dox). RA induced normal differentiation in Tet-On HL60 cells in the absence of doxycycline (Tet-On + RA – Dox) as compared with control nontransfected cells (Control + RA). Tet-On in the absence of both doxycycline and RA (Tet-On – RA – Dox) did not express CD38, and no differentiation was detected. The data shown are the averages of three independent cultures ± S.D.

Fig. 7. Correlation between CD38 activity and differentiation of HL 60 cells. CD38 activity and differentiation in HL60 cells were normalized to nontransfected cells induced by RA. The line shown here is the least squares fit.

of CD38 nor cellular differentiation induced by RA. Similar to that described above for nicotinamide, HL-60 cells proliferated equally well in the presence of the antisense oligonucleotides as compared with control cells not treated, indicating that its inhibitory effect was specific for cellular differentiation.

In addition to antisense oligonucleotide, the RNAi technique has recently been used for the same purpose (reviewed in Ref. 34). Two separate regions (regions 3 and 27; see “Experimental Procedures”) in the coding sequence of CD38 were targeted. The sequences of both regions are unique to CD38 as indicated by a sequence search. As shown in Fig. 5A, RNAi directed against region 27 produced about 40% inhibition of CD38 expression as compared with control cells treated with RA alone. Differentiation in these cells was also inhibited to a similar extent (Fig. 5B). RNAi directed against region 3 was less effective in inhibiting either the CD38 expression or differentiation. Mock incubation (Fig. 5B, TKO + RA) had essentially no effect. Compared with the antisense oligonucleotide technique (Fig. 4), the RNAi method appeared to be less effective. The reason for this is unknown but may be related to the fact that the RNAi technique relies on the endogenous RNA degradation pathway being fully functioning, which may not be the case in HL-60 cells. Alternatively, the two targeted regions on the mRNA, which were selected because of their unique sequence, may not be optimal for degradation. The exact cause for the inefficiency of the RNAi was not investigated further. Nevertheless, there appeared to be a remarkably good correlation between the extent of inhibition of CD38 expression and differentiation (detailed below).

A more direct test for the causal role of CD38 is to enhance its expression artificially without activating the endogenous RA signaling pathway. This was achieved by transfecting HL-60 cells with the Tet-On expression construct containing full-length CD38. The expression system can then be activated by treating the transfected HL-60 cells with doxycycline. We first verified that doxycycline itself has no effect on either differentiation or CD38 expression of control HL-60 cells without the construct. Tet-On in the absence of both doxycycline and RA (Tet-On – RA – Dox) did not express CD38, and no differentiation was detected. The extent of increase in CD38 activity induced by doxycycline is about 40% of the activity of either control or transfected cells treated with RA. It is clear that even though the artificial Tet-On expression system was not as effective as the natural system activated by RA, its activation by doxycycline was able to induce differentiation in the transfected cell to an extent similar to that observed for the increase in CD38 activity.

That there is a direct correlation between the extent of CD38 expression and cellular differentiation is shown more clearly in Fig. 7, where the results of the four different treatments described above are normalized to that induced by RA alone and plotted together. Thus, the antisense oligonucleotide treatment, which blocked CD38 expression most effectively, also inhibited differentiation most effectively. The regression line shown has an r² value of 0.976, close to perfect linearity.

**DISCUSSION**

In this study four different treatments were used to block CD38 in HL-60 cells, which include nicotinamide, a chemical inhibitor, an RNAi technique targeting two separate regions of the coding sequence of CD38, and antisense morpholino oligonucleotides targeting the 5’ cap of the CD38 mRNA. All four treatments led to inhibition of granulocytic differentiation to varying degrees. Conversely, two different treatments that enhanced CD38 expression, namely, treatment with a natural inducer, RA, or artificially using doxycycline to activate the Tet-On expression system, both led to induction of differentiation. In fact, the correlation between CD38 expression and differentiation was close to perfect as shown in Fig. 7. The results of the nicotinamide treatment were not included in the plot because the chemical has dual effects of not only inhibiting CD38 expression but also blocking its enzymatic activity. The
regression line of the correlation extrapolated to an intercept of about 10% CD38 expression, suggesting that CD38 expression must surpass a threshold level before differentiation can be activated. That the nicotinamide treatment affected CD38 expression was unexpected but may suggest the existence of a positive feedback mechanism during the RA-induced differentiation, by which the increase in cADPR levels positively stimulate the expression.

The exact mechanism of how CD38 expression can induce differentiation remains to be elucidated but is likely to be related to the accumulation of cADPR, a Ca^{2+} messenger, and an enzymatic product of CD38. Consistent with this notion is the time course measurements (Fig. 2) showing cADPR accumulation lagged slightly behind CD38 expression but preceded prominently cellular differentiation (19). Further support comes from the results of the nicotinamide treatment, which showed that the inhibition of differentiation correlated better with cADPR levels than with CD38 expression (Fig. 3). That cADPR level may be the causal factor is also consistent with the observation that a threshold of CD38 expression appeared to be required (Fig. 7), because it is likely that cADPR production must exceed degradation before it can accumulate and exert its signaling function.

A wide range of physiological functions have now been shown to be mediated by the Ca^{2+} mobilizing activity of CD38 in cells spanning three biological kingdoms, from protist, to plant to animal (reviewed in Refs. 4, 8, and 9). Although the presence of cADPR-sensitive Ca^{2+} stores in HL-60 cells has not yet been reported, it is likely to be present because a similar cell type, the mouse neutrophil, has been shown to possess them, the mobilization of which is involved in the chemotactic response of the cells (16). Also, HL-60 cells have been shown to possess Ca^{2+}-sensitive stores to nicotinic acid adenine dinucleotide phosphate (35), another Ca^{2+} messenger that is also an enzymatic product of CD38 (10).

The fact that CD38, the enzymatic product of CD38, accumulates inside HL-60 cells indicates that CD38 is expressed intracellularly. This was directly shown using immunofluorescence staining and Fabio Malavasi (Universita di Torino, Italy) for generously providing the IB4 monoclonal antibody.

Acknowledgments—We thank Santina Bruzzone for help in immunofluorescence staining and Fabio Malavasi (Università di Torino, Italy) for generously providing the IB4 monoclonal antibody.

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