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Hemin-Dependent Induction and Internalization of CD38 in K562 Cells

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Abstract The cell surface antigen, CD38, is a bifunctional ecto-enzyme, which is predominantly expressed on hematopoietic cells during differentiation. In the present study, it is shown that hemin treatment of K562 cells gives rise to induction of enzymatic activities inherent to CD38. GDP-ribosyl cyclase activity, an indicator of CD38, increased initially in response to hemin in a time-dependent manner, reached a maximum level on the 5th day and, thereafter, declined sharply to the initial level. The increase in NAD^+ glycohydrolase and ADP-ribose uptake activities followed a similar time course. However, the decline in the latter activities after the 5th day of induction appeared to be rather slow in contrast to GDP-ribosyl cyclase activity. The time course of these changes was well correlated with the FACScan findings obtained by use of anti-CD38 monoclonal antibody. SDS–PAGE and Western blot analyses by use of the monoclonal antibody OKT10 revealed a transient hemin-dependent appearence of a 43 kDa membrane protein with maximum signal intensity on the first 4 days of incubation. There was subsequently a gradual decrease on the 5th day, concomitant with a reciprocal increase in activity of the internalized protein fraction. The results together indicated that hemin-induced expression of CD38 was followed by its down-regulation. J. Cell. Biochem. 90: 379–386, 2003.

Key words: CD38; internalization; cGDP-ribose; NAD glycohydrolase; ADP-ribose uptake

Human CD38, a surface antigen of 45 kDa, is a type II transmembrane glycoprotein with a short N-terminal cytoplasmic domain and a long C-terminal extracellular domain. Its expression is widely used as a phenotypic marker of differentiation and activation in T and B lymphocytes [Jackson and Bell, 1990; Summerhill et al., 1993; Malavasi et al., 1994; Kukimoto et al., 1996] and also a number of other cell types including erythrocytes [Lee et al., 1993; Zocchi et al., 1993]. CD38 has been shown to be a bifunctional enzyme with NAD^+ glycohydrolase and ADP-ribosyl cyclase activities [Howard et al., 1993; Takasawa et al., 1993; Zocchi et al., 1993; Inageda et al., 1995]. cADP-ribose, the product of cyclase activity, has gained considerable interest as an inositol 1,4,5-trisphosphate (IP-3)-independent Ca^{+2} mobilizer [Galione, 1992; Lee, 1994]; in addition, CD38 has been demonstrated to catalyze a base exchange reaction in which $NADP^+$ in the presence of nicotinic acid is converted to an additional calcium mobilizer, i.e., $NAADP^+$ [Aarhus et al., 1995; Lee, 2001]. The NAADP-dependent Ca^{+2}-release mechanism is not inhibited by 8-amino cADPR, a specific antagonist of the cADPR, indicating that it is a cADPR-independent pathway, and the importance of the NAADP-dependent pathway remains unknown in many systems. How cADPR production by CD38, an ectoenzyme, regulates the mobilization of Ca^{+2} from intracellular stores is unclear. One possible mechanism is that CD38 undergoes internalization in response to some external stimuli and/or interaction with an as

Abbreviations used: NAD, nicotinamide adenine dinucleotide; NGD, nicotinamide guanin dinucleotide; cGDPR, cyclic GDP-ribose; FACS, fluorescence activated cell sortes; FITC, fluorescein isothiocyanate; TBST, tris buffered saline & Tween 20.

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yet unknown cellular ligand followed by generation of cADPR intracellularly.

In this study, the time course of the induction of CD38 expression was followed along with hemin-induced differentiation of K562 cells. These cells, which can be induced towards erythroid lineages by different agent provided suitable model system for studying human erythrocyte [Rutherford et al., 1979]. To determine this effect, changes in CD38-specific enzymatic activities, ADP-ribosyl cyclase and NAD glycohydrolase, were investigated. The expression of CD38 was also determined by flow cytometry as well as SDS–PAGE and immunoblot analysis.

MATERIALS AND METHODS

Materials

NGD, cGDPR, protein standards, bovine serum albumin, NAD glycohydrolase from pig brain, and all chemicals of analytical grade were purchased from the Sigma Chemical Co. (St. Louis, MO). [Adenine-^{14}C]NAD, specific activity 534 Ci/mol, and [carbonyl-^{14}C]NAD, specific activity 55 Ci/mol, were obtained from DuPont NEN (Boston, MA). The CD38 specific monoclonal antibody, OKT10, was isolated from the culture of the mouse hybridoma cell line (ATCC CRL 8022) by ammonium sulfate precipitation [Harlow and Lane, 1988].

Treatment of K562 Cells

The human erythroleukemia cells K562 were propagated in DMEM (Sigma Chemical Co.) containing 10% fetal calf serum (Sigma Chemical Co.) and 0.1% penicillin/streptomycine and treated with hemin (20 μM) for 1, 2, 3, 4, 5, 6, and 7 days or left untreated (control). After treatment, the cells (10^8) were washed twice with phosphate buffered saline pH:7.4 and sedimented by centrifugation at 1,000*g* for 10 min.

Benzidine Staining

The benzidine stock solution contained 0.2%w/v benzidine hydrochloride in 0.5 M acetic acid. Cells (10^5) were washed twice with ice-cold phosphate-buffered saline. The cell pellets were resuspended in ice-cold phosphate-buffered saline (27 μl). After addition of the benzidine solution (3 μl) containing hydrogen peroxide (final concentration, 0.0012%), the cell suspension was incubated for 10 min at room temperature. Benzidine-positive cells were quantitated by light microscopy. At least 100 cells were counted in triplicate for each sample [Lam et al., 2000].

Assay for ADP-Ribosyl Cyclase Activity

The activity of ADP-ribosyl cyclase was assayed by using NGD^+ as substrate and measuring the production of cGDPR as an increase in fluorescence. Cyclic GDP-ribose (cGDPR), the guanine nucleotide equivalent to cADPR, is (in contrast to cADPR) resistant to hydrolysis [Graeff et al., 1994a] and also fluorescent, allowing continuous monitoring of the reaction fluorimetrically. K562 cells (5×10^6 cells) were incubated for 1 h at 37°C in 1.5 ml PBS containing 50 μM NGD^+ and 20 mM Tris-HCl, pH:7.4. The excitation wavelength was set at 300 nm and the emission was measured at 410 nm (PTI, QuantaMaster spectrofluorometer). The amount of cGDPR produced was determined by comparing the fluorescence intensity with that of cGDPR standards.

NAD^+ Glycohydrolase Activity

NAD^+ glycohydrolase activity was determined by the release of [carbonyl-^{14}C] nicotinamide from NAD^+. The reaction mixture (50 μl) contained 10^6 cells in PBS and 10 μM [carbonyl-^{14}C] NAD^+. After incubation for 30 min at 37°C, the reaction was terminated by the addition of 0.1% SDS. The samples were applied to BioRad AGX4 columns and eluted with H_2O [Kim et al., 1993]. The radioactivity in the eluant was counted in a liquid scintillation analyzer (Packard, Tri-Carb 1000 TK, Meriden, CT).

ADP-Ribose Uptake

Uptake of ADP-ribose by K562 cells was assayed by incubation of 5×10^6 cells with 5 μM [^{14}C]NAD in a volume of 120 μl in PBS at 37°C for 30 min. Following incubation, the reaction mixtures were layered over 0.15 ml of 250 mM sucrose in PBS in 1.5 ml tubes and centrifuged in a microfuge (Heraeus Biofuge B, Hanau, Germany) for 30 s at 16,000*g*. The pellets were resuspended in 100 μl of distilled water and lysed by freezing–thawing. Total radioactivity in the lysates was determined by application of aliquots on GF/A filters and determination of radioactivity as described [Ustundag et al., 1997].

FACScan Analysis

Control and hemin-treated K562 cells (10^6 in each sample) were incubated with FITC-

conjugated anti CD38 antibody (DAKO, Glostrup, Denmark) for 10 min and washed with PBS at 1,500*g* for 5 min. The cells were fixed with 1% paraformaldehyde in PBS and analyzed in a FACScan analyzer (Becton Dickinson, San Jose, CA, Software CellQuest).

SDS–PAGE and Immunoblotting

K562 cell lysates were fractioned by centrifugation for 10 min at 10,000*g*. The supernatant was used as internalized protein fraction. The pellet, purified by repeated washes in PBS, was used as ectocellular fraction [Zocchi et al., 1996; Chidambaram and Chang, 1999]. Cell lysates, ectocellular fraction, or internalized protein fraction (50 μg each in Tris buffer, pH: 8.0) were subjected to SDS–PAGE [Laemmli, 1970] and then transferred onto nitrocellulose membranes. The blots were blocked with TBS–Tween 20 (TBST, 0.5% BSA, 1 h), followed by incubation with the CD38-specific monoclonal antibody OKT10. The immunoblots were finally developed using horseradish peroxidase-conjugated anti-mouse antibody (TBST 0.05%, 1:1000,1 h) and 3-amino-9-ethyl-carbazole substrate solution [Gershoni and Palade, 1983].

RESULTS

Figure 1 illustrates the increase in differentiation of K562 cells following hemin treatment, as revealed by benzidine staining. The relative number of benzidine-positive cells increased gradually upon induction with hemin chloride and attained after 5 days a constant value of around 60%. Concomitantly, expression of CD38 was implicated by the increase in

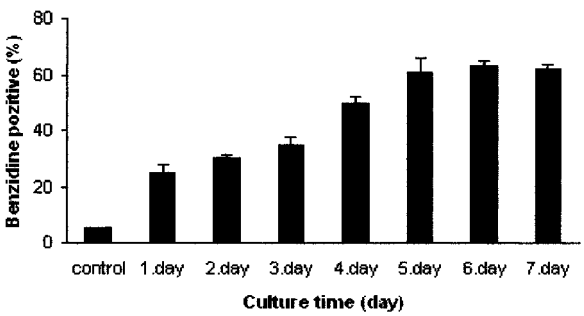

Fig. 1. Effect of hemin on differentiation. Differentiation was measured by benzidine labeling as described in the Materials and Methods. The cells were diluted to an initial concentration of 10^5 cells/ml and incubated at 37°C with hemin at 20 μM for the indicated times. Differentiation was measured by a benzidine oxidation test each day. The values represent the mean from three separate experiments.

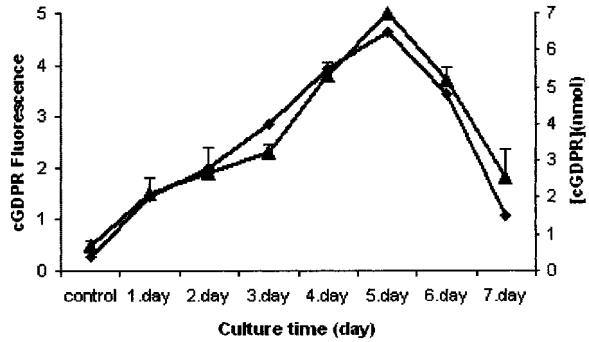

Fig. 2. Fluorometric assay of GDP-ribosyl cyclase activity. K562 cells (5×10^6 cells) with or without hemin were incubated with 50 μM NGD^+ for 1 h and the fluorescence of cGDPR (▲) was monitored. The fluorescence intensity of samples was converted to cGDPR concentration (■) by comparing to cGDPR standards. The control cells incubated similarly produced no increase in cGDPR fluorescence.

GDP-ribosyl cyclase activity in hemin-induced cells. This activity which was nearly negligible before induction, increased in parallel with the differentiation of the cells and reached a

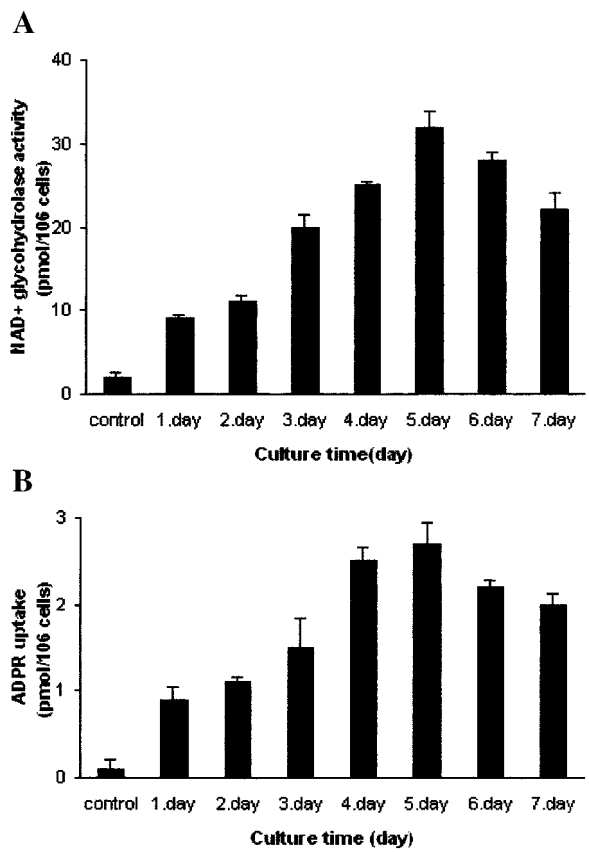

Fig. 3. Induction of NADase activity and ADP-ribose uptake on the cell surface of hemin–treated K562 cells. K526 cells were cultured with 20 μM hemin for the indicated days. NADase activity (**Panel A**) and ADP-ribose uptake (**Panel B**) were determined as described under Materials and Methods.

maximum value by the 5th day after hemin treatment. However, GDP-ribosyl cyclase activity declined on the subsequent two days sharply to below the level of the first day post induction (Fig. 2).

The time course of hemin-induced increase in NAD^+ glycohydrolase activity is illustrated in Figure 3A. This activity attained also a maximum value on the fifth day after hemin induction, but unlike the cyclase activity, declined only slightly (<20%) on the following day. The time course of the hemin-induced increase in ADP-ribose uptake activity was essentially similar to that of NAD glycohydrolase activity (Fig. 3B).

The results of FACScan analyses revealed a resemblance with those of enzymatic assays. They indicated a biphasic cell surface expression of CD38 after hemin treatment, with an increase in CD38-specific signals up to day 5, followed by their disappearance by day seven (Fig. 4).

An apparent de novo synthesis of CD38 upon hemin treatment was shown also by SDS–

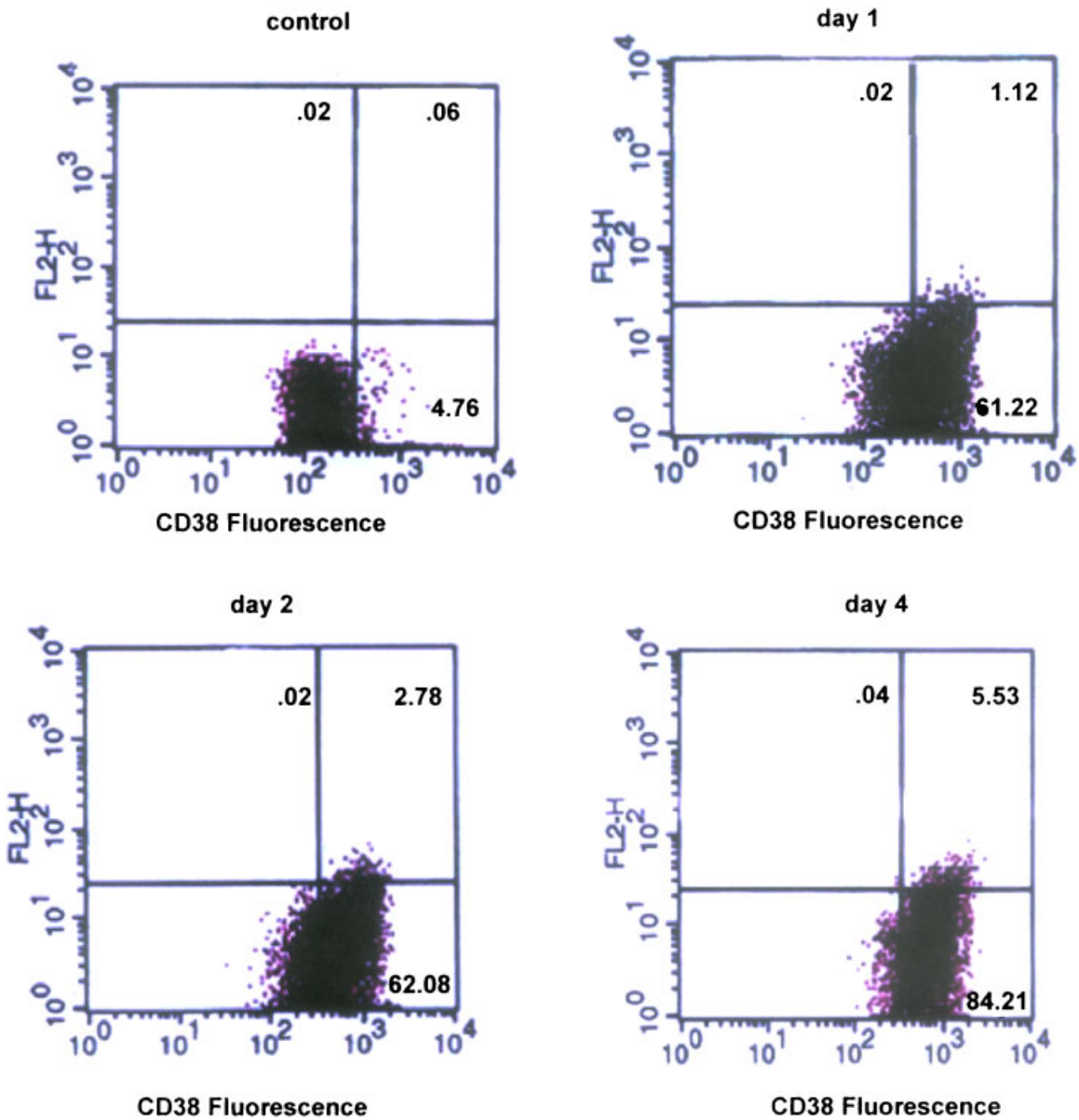

Fig. 4. Expression of CD38 in the control and hemin-induced K562 cells. The cells were single stained as described under Materials and Methods. Quadrants on the right show that CD38 are prevalently expressed in the hemin-induced K562 cells. Representative data from three experiments is shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

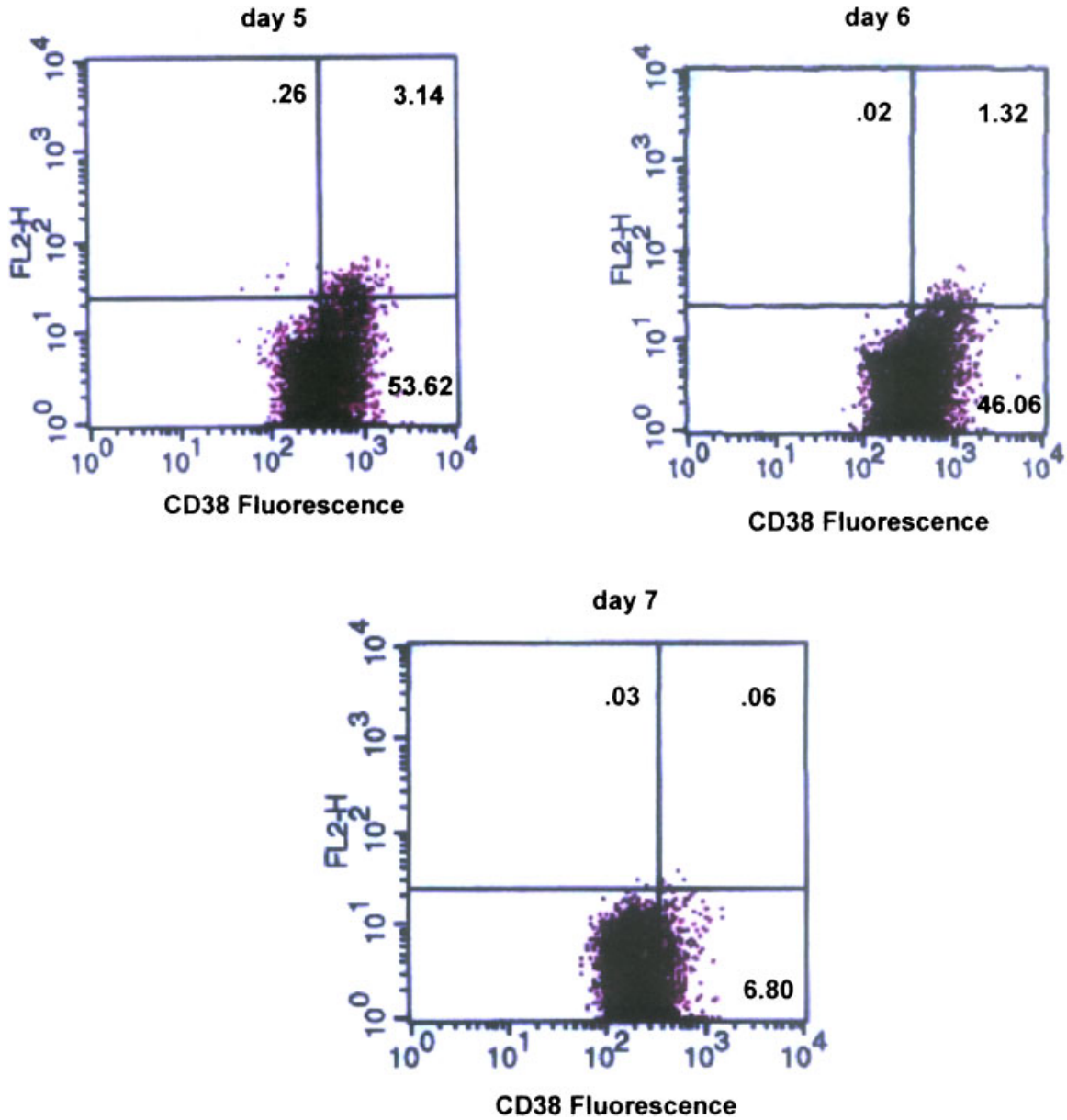

Fig. 4. (*Continued*)

PAGE and Western blot analyses (Fig. 5). By use of CD38-specific monoclonal antibody OKT10, a membrane protein of about 43 kDa was detected in hemin treated K562 cell lysates, ectocellular fraction, or/and internalized protein fraction. The OKT10-specific signal in the lysate fraction was apparent already on the first day post induction and, thereafter, remained at a constant maximum level throughout the incubation (Fig. 5A). The signal intensity of this band which was not discernible before induction increased transiently in the ectocellular fraction, with a maximum on the 1st to 4th days of induction and a subsequent decrease on the following 2 days (Fig. 5B). The changes in signal intensity followed concomitantly a reciprocal time course with gradual increase on the days 5–7 in the internalized fraction (Fig. 5C).

DISCUSSION

In this study, the time course of CD38 expression was followed along with hemin-induced differentiation of K562 cells. We first measured CD38-specific ADP-ribosyl cyclase activity in K562 cells during cell differentiation using NGD^+ as substrate. It was observed that an

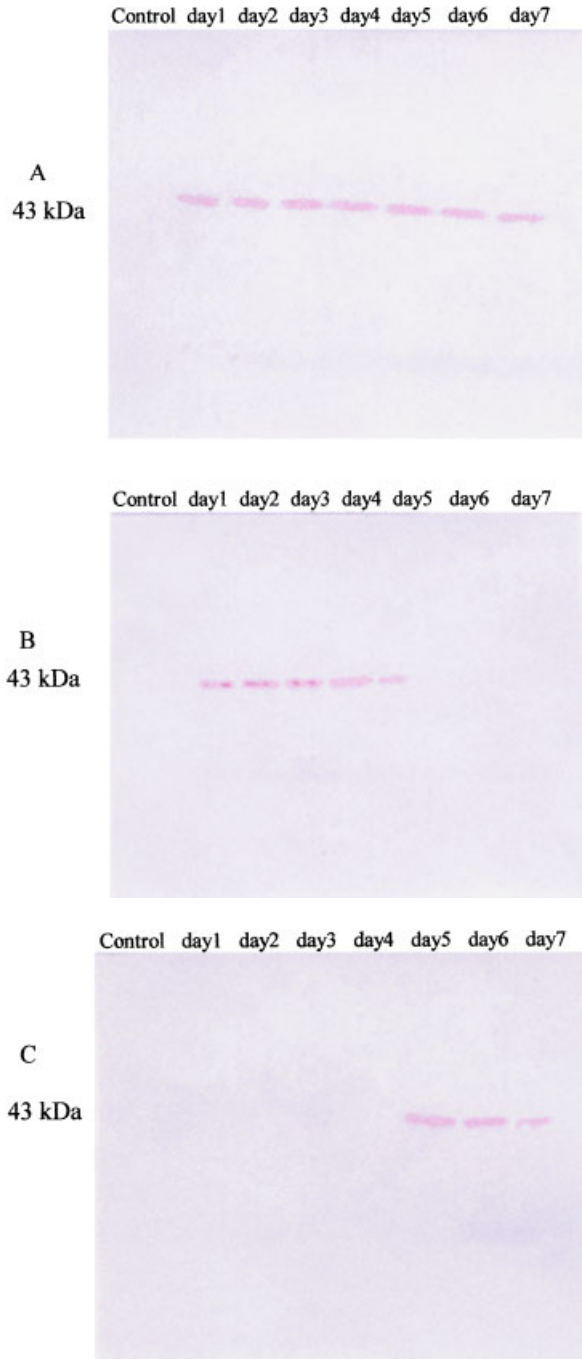

Fig. 5. Hemin-induced CD38 expression in K562 cells. Cell lysates (**A**), ectocellular fraction (**B**), and internalized protein fraction (**C**) from control or hemin-treated cells were analyzed by Western blotting with an anti-CD38 antibody. Results are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

increase in ADP (GDP)-ribosyl cyclase activity appeared during the first 5 days of incubation with hemin. However, with the prolongation of the incubation over the 6th and 7th days, the cyclase activity on the whole cells declined rather sharply. NAD glycohydrolase and ADP-ribose uptake activities had similarly rapid increases in K562 cells along with the differentiation. They reached the maximum level by the day 5 of the incubation with hemin and then declined slowly. This relatively slow decrease may indicate the existence of another type of NAD glycohydrolase other than CD38. The activity changes appeared to have close correlation with FACS analyses. Western blot data, by use of CD38-monoclonal antibody suggested, in turn, that a 43 kDa membrane protein was induced by hemin treatment and, thereafter, internalized in the course of prolonged incubation.

These results underline that changes in GDP-ribosyl cyclase and NAD glycohydrolase activities upon hemin-treatment in K562 cells might be attributed to expression of CD38 antigen. On the other hand, CD38 has been reported to exhibit a unique expression pattern in lymphoid and erythroid cells: it is highly expressed on immature cells, its expression declines during differentiation [Kontani et al., 1993; Graeff et al., 1994b].

Recent reports by different groups have suggested that CD38 expression, at least in myeloid cells, may be linked to the action of retinoids [Malavasi et al., 1992]. The results provide direct evidence that RA-induced expression of CD38 is mediated by direct transcriptional regulation via activation of a RAR/RXR heterodimer on a regulatory region called RARE (retinoic acid response element) located in the first intron of *CD38* gene [Kishimoto et al., 1998]. In the present study, it has been shown that CD38 expression can be achieved in K562 cells also by hemin treatment. However, it is not known whether hemin-induced expression of CD38 follows a similar mechanism.

The *aplysia cyclase* gene has been cloned and the three-dimensional structure of the recombinant cyclase determined [Prasad et al., 1996]. The 3-dimensional model predicts that the amino acid residues corresponding to cysteine 119 and cysteine 201 of human CD38 which have been shown by site-directed mutagenesis experiments to be crucial for cADPR hydrolase activity, i.e., for catalytic bifunctionality, are located on the surface of the enzyme and linked by a disulfide bond [Tohgo et al., 1994]. It was also shown that cysteine 119 and cysteine 201 of CD38 are essential sites for its internalization

[Han et al., 2002]. The differentiation of erythroid cells in vitro coincides with the accumulation of hemoglobin [Friend et al., 1971]. Enhanced hemoglobin levels might cause an increased intracellular O_2^- flux and glutathione could directly participate in the neutralization of free radicals. Hemin induced-K562 erythroleukemia cells reveal an increase in glutathione content during differentiation [Percival and Harris, 1991]. The primary biological function of glutathione is to act as a reducing agent to help keep cysteine thiol side chains in a reduced state on the surface of proteins [Leppa et al., 1997]. Breakage of the disulfide bond between cysteine 119 and cysteine 201 itself may result in a conformational change of CD38 to induce its internalization, as predicted previously [Tohgo et al., 1994]. As it is known, CD38 expression is high on immature precursor cells and declines once the cells differentiate and acquire a mature phenotype. It is also speculated that CD38 ligation on mature as compared to precursor cells might reflect different signal transduction pathways used in different stages of lymphocyte maturation. Indeed, cADPR has been reported to exert a mitogenic effect on mature, but not on immature lymphoid cells [Malavasi et al., 1992]. It would be of interest to investigate in the light of the results of this study of K562 cells whether internalization of CD38 may be a marker of maturation for differentiated K562 cells.

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