EXPRESSION OF CD38 IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

M. ORCIANI, O. TRUBIANI[1], G. CAVALETTI[2], S. GUARNIERI[3], E. SALVOLINI, G. TREDICI[2] and R. DI PRIMIO

Department of Molecular Pathology and Innovative Therapies, Histology Section, Marche Polytechnic University, Ancona; [1]Department of Oral Science, University "G. D'Annunzio", Chieti-Pescsara; [2]Department of Neuroscience and Biomedical Technologies, University of Milano Bicocca, Monza; [3]Department of Basic and Applied Medical Science, Institute of Myology, University "G. D'Annunzio", Chieti-Pescara, Italy

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Human CD38 antigen is a 42-45 kDa type II transmembrane glycoprotein with a short N-terminal cytoplasmic domain and a long C-terminal extracellular region. It is widely expressed in different cell types including thymocytes, activated T cells, and terminally differentiated B cells (plasma cells) and it is involved in cellular proliferation and adhesion. CD38 acts as an ectocyclase that converts NAD^+ to the Ca^{2+}-releasing second messenger cyclic ADP-ribose (cADPR). It has been also demonstrated that increased extracellular levels of NAD^+ and cADPR are involved in inflammatory diseases and in cellular damage, such as ischemia. In the present study, we have characterized the expression of CD38 in human neuroblastoma SH-SY5Y cell line. All-trans-retinoic acid (ATRA) treatment was used to induce cell differentiation. Our results indicate that: a) even if SH-SY5Y cells have a negative phenotype express CD38 at nuclear level, ATRA treatment does not influence this pattern; b) CD38 localizing to the nucleus may co-localize with p80-coilin positive nuclear-coiled bodies; c) purified nuclei, by Western blot determinations using anti-CD38 antibodies, display a band with a molecular mass of ~42 kDa; d) SH-SY5Y cells show nuclear ADP-ribosyl cyclase due to CD38 activity; e) the basal level of CD38 mRNA shows a time-dependent increase after treatment with ATRA. These results suggest that the presence of constitutive fully functional CD38 in the SH-SY5Y nucleus has some important implications for intracellular generation of cADP-ribose and subsequent nucleoplasmic calcium release.

CD38 is a type II glycoprotein of 42-45 kDa that acts as a complex ecto-enzyme with NAD^+ glycohydrolase and ADP-ribosyl cyclase activities (1). Cyclic ADP-ribose (cADPR), the product of cyclase activity, is a second messenger that regulates intracellular Ca^{++} release (2) from ryanodine receptor (RyR) regulated stores (3); these stores are distinct from those controlled by inositol trisphosphate receptors (IP_3R) (4). CD38 is also a surface receptor involved in transmembrane signaling in different cell types, including activation, differentiation and adhesion (5). Different agents have been demonstrated to modulate the expression of CD38 along cell differentiation, one of which is all-trans-retinoic acid (ATRA) (6). ATRA is a potent and highly specific inducer of CD38 expression in human promyelocytic leukemia cells through a direct transcriptional control via activation of a retinoic

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Mailing address: Prof. Roberto Di Primio,
Department of Molecular Pathology
and Innovative Therapies-Histology,
Marche Polytechnic University,
Via Tronto 10/A, 60020 Ancona, Italy
Tel: ++39 071 2206076 Fax: ++39 071 2206078
e-mail: r.diprimio@univpm.it

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acid-alpha receptor (RAR alpha) located in the first intron of CD38 gene (7).

CD38 is expressed on the surface of monocytes, platelets, NK cells, myeloid cells and vascular endothelium, and also in tissues such as brain, cardiac and skeletal muscle, spleen, heart, liver, prostate, and kidney (8-9). Moreover, apart from different cell surface expression, a dynamic movement of CD38 including, shedding and internalization by a receptor-mediate endocytosis mechanism, has been proved(10-11). Several studies have recently established that CD38 is not only expressed on the plasma membrane but is also found in various sub-cellular compartments, including the nucleus where it is localized to the inner nuclear membrane (12-14). The nucleus of the mammalian cell is the site of many different functions required for the correct expression, storage, and replication of the genes within it. A number of discrete subnuclear structures has been documented including nucleolus, coiled bodies, interchromatin granules and interchromatin granule-associated zones, PML bodies and gems (15). In particular the coiled body (CB), which is a round-to-oval organelle with a diameter of 0.5-1 μm observed in many eukaryotic nuclei, ranging from human to plant. Despite the ubiquity of the CB in eukaryotic cells, little is known about its nature and function (16). A human autoantigen called p80 coilin has been identified and shown to be a marker protein for the CB (17).

In adult human brain the expression of CD38 has been demonstrated (18) but its distribution and function in the nervous system remains unclear. Polyclonal antibodies raised against selected peptide fragments of human CD38 used in immunochemical studies indicated a specific immunoreactivity into the perikaryal and dendritic cytoplasm of neurons. Interestingly, CD38 was also found in the neurofibrillary tangles, the pathologic hallmark of Alzheimer's disease that occurs in the neuronal perikarya and proximal dendrites (19).

SH-SY5Y cells, a clonal derivative of the human neuroblastoma SK-N-SH cell line which expresses high levels of RAR, can differentiate into mature neurone upon ATRA treatment (20). Treatment of SH-SY5Y cells with ATRA results in the disaggregation of cells, the inhibition of cell growth, and the elongation of multiple neuritic processes on cells (21). In this study we investigate the expression of CD38 in the human neuroblastoma SH-SY5Y cell line, before and after ATRA treatment, according to the notion that CD38 regulates the level of cADPR that mobilizes intracellular Ca^{2+} and thereby controls brain functions, such as neuronal plasticity. It is conceivable that alterations in the cADPR-mediated second messenger system occur in degenerating CNS neurons in certain pathologic conditions.

MATERIALS AND METHODS

Cell culture

The human SH-SY5Y cell line was maintained in continuous culture in F12 medium supplemented with 10% foetal bovine serum, 4 mm l-Glutamine, 100 mm Na-pyruvate and 25 mm Hepes. Cells were grown with more than 98% viability as determined by trypan blue exclusion test. During the log growth phase, the cells were treated with 50 μM of ATRA. After 5 and 10 days, samples were immediately processed for morphological and biochemical analyses.

Nuclear preparation

Purified nuclei were obtained essentially as described previously by Trubiani et al (22). Briefly, growing cells were washed with cold PBS, incubated for 10 min at 10°C in presence of 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM Tris-HCl, pH 7.4, and then treated with 0.3% Triton X-100. The cell suspension was passed twice through a syringe and $MgCl_2$ was adjusted to a final concentration of 5 mM. The partially lysed cells were washed twice in a solution of 5 mM $MgCl_2$, 0.1 mM PMSF, 10 mM Tris-HCl, pH 7.4 and resuspended in cold hypotonic lysis buffer containing 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 mM PMSF, 10 mM leupeptin and 10 mg/ml soybean trypsin inhibitor. The protein concentration was determined by the Bio-Rad assay.

FACScan analysis

Flow cytometry analysis was performed with a Becton Dickinson FACScan instrument equipped with an argon ion laser tuned at 488 nm. Data acquisition was done with CellQUEST software (Becton Dickinson).

Cells were gated according to their scatter light, and fluorescence intensity distribution was analyzed in histograms. Whole cells and isolated nuclei from ATRA-induced and control SH-SY5Y cells ($2x10^6$/ sample) were incubated with anti-CD38 antibody (SUN4B7, 2 μg/ml) or with isotype-matched control mouse IgG_1(negative control) for 30 min, washed with PBS+ 0.2% BSA and further incubated for 30 min at 4°C with FITC-

labeled goat anti-mouse secondary antibody. An aliquot of untreated cells was permeabilized using 0.1% Triton X-100 before incubation with the primary antibody. Anti-CD38 SUN4B7 was a kind gift from F. Malavasi; FITC-conjugated goat anti–mouse was purchased from ImmunoTools (Cat. No 22339913).

Confocal laser scanning microscope (CLSM) analysis of CD38 and Colin co-localization

To confirm the nuclear localization of CD38, isolated nuclei from treated and untreated SH-SY5Y were observed under laser scanning microscope and, subsequently, double immuno-staining experiments were arranged. Briefly, purified nuclei were washed in Cacodylate buffer 0.5 M pH 7.4, fixed for 1 h at 4°C with 2% paraformaldehyde in the same buffer and then incubated with 10% goat serum in PBS for 20 min, followed by incubation with the primary antibody anti-CD38 (SUN4B7, 2 μg/ml) for 4 h. Samples were washed and then incubated for 60 min with FITC-conjugated goat anti–mouse. Subsequently PBS washed samples were stained with a 0.1% PI solution for 10 min. For double immuno-staining experiments, samples were contemporary incubated with SUN4B7 and with the primary antibody anti- p-80 coilin (rabbit anti-p80 coilin polyclonal serum, 1 μg/ml) and then with the two secondary FITC-conjugated goat anti–mouse and Texas red–conjugated goat anti–rabbit antibodies. After washing, cells were mounted with 0.5% *p*-phenylenediamine in 20 mM Tris, pH 8.8, 90% glycerol, sealed, and left to dry before examination. Images were collected through a ZEISS LSM510 META (Jena, Germany), confocal system, equipped with an argon laser and helium-neon source. Optical sections were obtained with a 0.2 μm increment on the *z*-axis. To separate emissions of the two fluorochromes HTF 488/543 and NTF 545 primary and secondary dichroic mirrors respectively were used. Detector bandpass filters were set over 505-530 and 565-615 ranges for the green (FITC) and red (Texas red) emissions and simultaneously recorded. Cells had a mean thickness of 2 μm and the image series obtained from the Texas red and FITC signals were electronically merged and pseudo stained. The green and red digital images, respectively, were merged to detect any overlapping distribution of the two proteins (yellow). Anti p80-coilin rabbit polyclonal IgG (sc-32860) and the goat anti-rabbit IgG-TR (sc-2780) secondary antibody were from Santa Cruz Biotechnology.

Western blot assay

Total cellular lysates and purified nuclei from treated and untreated SH-SY5Y cells were separated on 12% polyacrylamide-SDS gels and transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with a 5% skim milk in TBST solution for 3 h at room temperature followed by incubation at 4°C over-night with the anti-CD38 monoclonal antibody (SUN4B7) at the final concentration of 3 μg/ml. The membrane was washed with TBST buffer for 1 h and then probed with horseradish peroxidase-labeled goat anti-mouse IgG antibodies (purchased from Santa Cruz) diluted (1:10000) in TBST at room temperature for 1 h. After washing thrice for 10 min with TBST, the membrane was visualized using a chemiluminescence system. A prestained SDS-PAGE standard protein marker (Sigma) was used to calibrate molecular mass.

Ecto-GDPR cyclase activity

The ecto-cyclase activity of CD38 was evaluated by incubating with NGD^+ (Sigma, N5131), an NAD^+ analog which is converted by ADP-ribosyl cyclases such as CD38 to cyclic GDPR which, unlike cADPR, is a fluorescent and cell impermeant end-product which can be detected in cell supernatants. Briefly, treated and untreated cells were resuspended at 3 x 10^5 cells/ml in NGT buffer (0.15 M NaCl, 5 mM glucose, 10 mM Tris-HCl, pH 7.4) containing or not containing Triton 0.1%. To the cell suspensions 10 μl 10 mM NGD^+ in 20 mM Tris-HCl, pH 7.4 or 10 μl buffer (control) was added. After 30 min at 37°C, supernatants were collected by brief centrifugation. To evaluate conversion of NGD^+ to the fluorescent end-product cGDPR, supernatants were analyzed by fluorescence spectrometer (Perkin-Elmer, Boston, MA) set at excitation 310 nm and emission at 400 nm.

RNA isolation and RT-PCR analysis

Total RNA was isolated from 5 x 10^6 treated and untreated cells using the "SV Total RNA Isolation Systems" (Promega) according to the manufacturer's instructions. The integrity of isolated RNA was evaluated by visualizing 28S and 18S ribosomal RNA on a 1% agarose gel.

1.5 μg of total RNA of each sample was reverse-transcribed in a total volume of 40 μl with 1.25 μM of oligo $d(T)_{16}$ using ImProm-II™ Reverse Transcriptase (Promega).

PCR amplifications of cDNA, using intron-spanning primers (Invitrogen) to avoid genomic DNA contamination, were performed in a DNA thermal cycler starting with an initial denaturation step at 94° for 4' followed by a different number of cycles, each consisting of denaturation at 94° for 15 s, annealing for 30 s at 61°, and annealing at 72° for 20 s. Previously, for each primer pair we determined the right PCR cycle number (26 for β-actin and 38 for CD38) to prevent plateau effects. β-

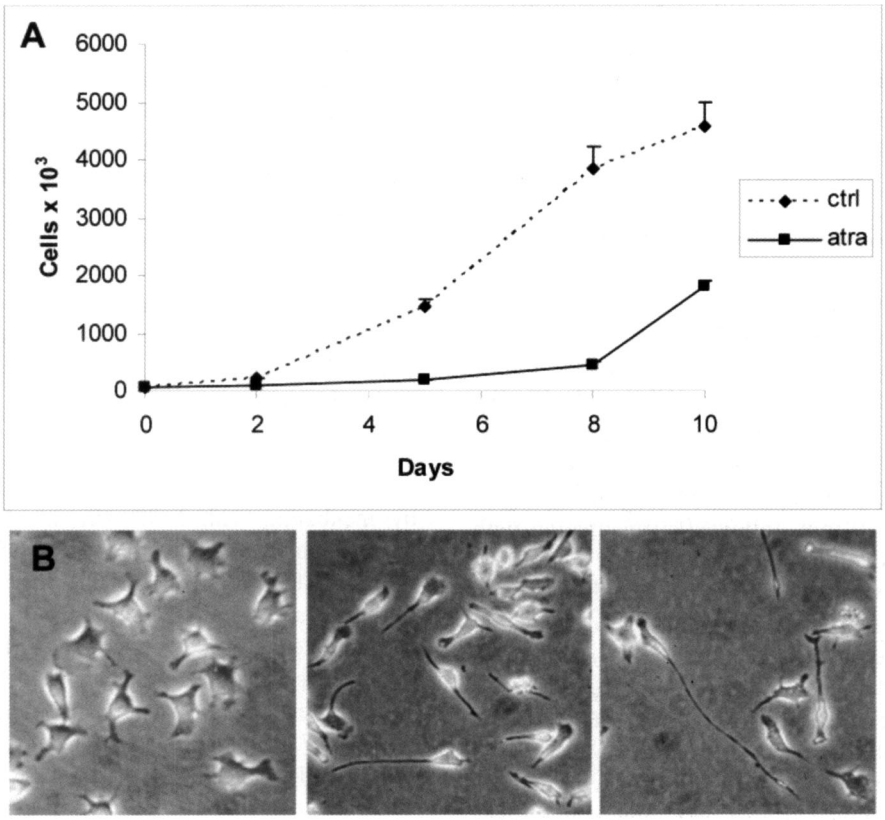


Fig. 1. *Growth inhibitory effect of ATRA in SH-SY5Y cell line. (A) SH-SY5Y cells were seeded in 10 flasks at a density of 50 x10³ cells/flask and cultured in 10%FBS supplemented medium. After 4 h, cells of 5 flasks were treated with 50 μM ATRA for 10 days. Every two days, one flask of treated (continued line) and one of untreated cells (dotted line) was used for the cellular count. Each point represents the mean with the standard deviation (SD) of three counts. (B) Control cells (left image) and ATRA-induced morphological changes (medium and right images) with axonal elongation*

actin was used as an internal standard. Each amplification product was run on a 2% agarose gel stained with ethidium bromide and quantified by densitometry using KODAK EDAS 290 system (densitometer) and "KODAK 1D Image Analysis Software". The expression of each specific gene is referred to as ratio of the intensities of specific transcripts to that of β-actin.

RESULTS

SH-SY5Y cells were treated with ATRA for 5 and 10 days; ATRA-induced differentiation was assessed by cell growth inhibition (Fig. 1, section A) and morphological changes in treated cells (Fig. 1, section B).

Whole cells and isolated nuclei from untreated and treated SH-SY5Y cells were analyzed on a FACScan after incubating with a primary anti-CD38 antibody followed by a FITC-labeled goat anti-mouse secondary antibody (Fig. 2, panel A, 1-2-3). Intact cells, independently of ATRA treatment, showed a negative immunophenotype for surface CD38 (white histograms), while isolated nuclei (red histograms) displayed CD38 expression; this pattern was never influenced by ATRA treatment. In addition, untreated cells were permeabilized by TritonX-100 before immunostaining and, as expected, they gave a positive signal for the presence of CD38 (blue histogram, panel A, 4). An irrelevant isotype was used, instead of anti-CD38, as negative control experiments (green histogram, shown only in panel A, 1). These data suggest the presence of a constitutive, non-inducible, nuclear CD38. Nuclei from treated (data not shown) and untreated cells were also analyzed at confocal laser scanning microscope. PI red staining allows to observe the DNA arrangement and the nuclear cyto-organization

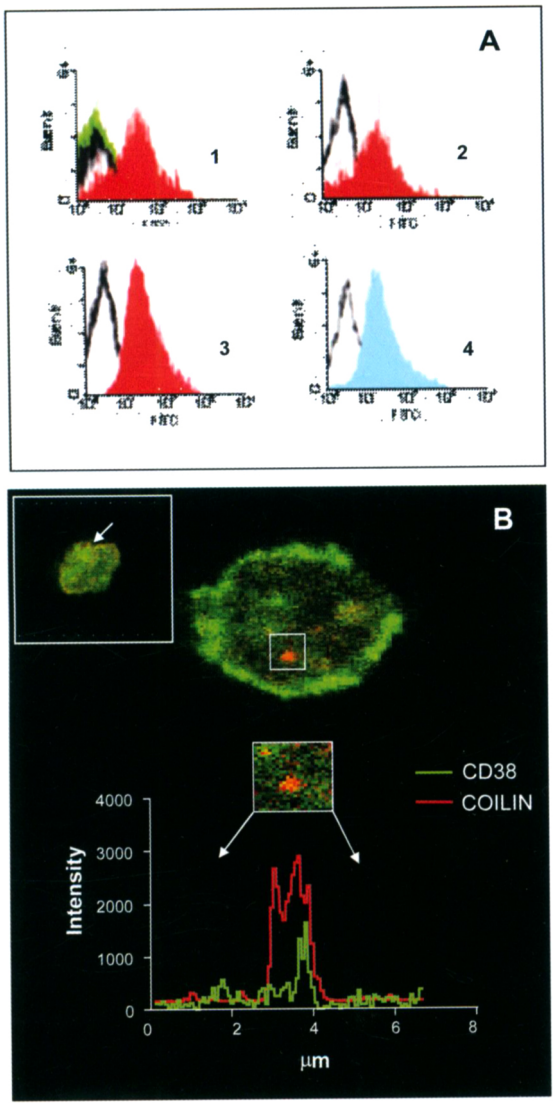


Fig. 2. *(A) FACScan analysis of the expression of CD38 in untreated cells (panel 1), 5 days ATRA treatment (panel 2), 10 days ATRA treatment (panel 3). Green histogram, shown only in panel 1, is referred to intact cells labeled with an isotype control (IgG1) followed by incubation with FITC-labeled goat anti-mouse secondary antibody; in all panels, dark and red histograms are referred respectively to intact cells and isolated nuclei labeled with anti-CD38 antibody (IB4) and then with FITC-labeled goat anti-mouse secondary antibody. In panel 4, CD38 staining of untreated SH-SY5Y cells before (dark histogram) and after (blue histogram) permeabilization by Triton X-100. (B) CD38 immunolocalization by confocal laser microscope analysis (CLSM) and double immunostaining of CD38 and p-80 coilin in untreated SH-SY5Y cells. The insert reports the merged picture of green FITC-CD38 and red PI staining in isolated nuclei. Arrow shows nuclear green-iperstained roundest bodies. To prove the localization of CD38 in Cajal bodies, isolated nuclei were processed for double immunostaining, using anti-CD38 (FITC-labeled) and anti-p80 coilin (Red Texas-labeled). The graph at the bottom refers to the intensity profile of CD38 and coilin spots traced over the Cajal body enlarged in the box.*

while FITC-CD38 green staining displays the site of immunocomplexes. PI-CD38 immuno-staining in merged image is reported in Fig. 2 (top left insert panel B) and shows that CD38 appears distributed within all nucleus and also arranged in more evident circular bodies, which for size may correspond to Cajal bodies. These organelles contain a marker protein, p80 coilin.

To characterize the precise nuclear localization of CD38 inside CBs, we carried out double immunostaining experiments with anti-CD38 and anti-p80coilin in isolated nuclei. As reported in Fig. 2, panel B, in purified nuclei from untreated cells the immuno-staining of the two proteins coincides, indicating the localization of CD38 in Cajal bodies. The co-localization is high evidenced by the graph that displays the overlapped intensity profile along the dotted line traced over the p80-coilin spot. These experiments of double immunostaining were performed also in nuclei from treated cells, and the results coincide (data not shown), proving evidence that ATRA treatment does not influence CD38 subnuclear expression. To understand the functional feature of CD38 protein in normal and ATRA-differentiated SH-SY5Y cells, we analyzed the ecto-cyclase activity. Cells were incubated with NGD and the enzymatic activity was measured fluorometrically as production of cGDPR. As reported in Fig. 3, there is not a detectable cyclase activity in whole cells, independently of ATRA treatment. After permeabilization, the substrate is accessible to the intracellular enzyme and an increase in fluorescence is observed, indicating that the nuclear CD38 is functionally active. ATRA treatment did not improve cyclase activity, as demonstrated by the similar fluorescent levels detected in treated samples. The amount of CD38 protein in purified nuclei and total cell lysates from treated and untreated cells was then analyzed by Western blot. Under reducing conditions an immunoreactive protein band of 43 kDa was detected in all samples (Fig. 4, panel A).

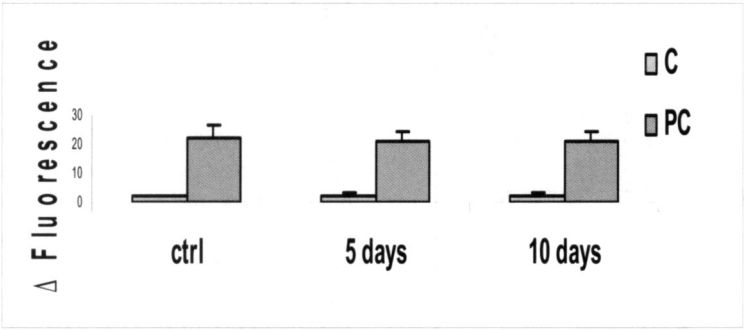


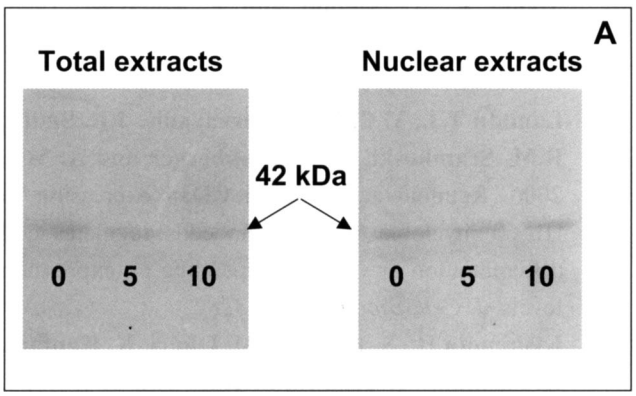
Fig. 3. *Cyclase activity in treated and untreated SH-SY5Y cells. The ADP-ribosyl cyclase activity was measured fluorometrically as the production of cGDPR from the substrate NGD⁺. Grey histograms are referred to intact cells and any enzymatic activity is detectable. After permeabilization (striped histograms), the substrate may reach the functional CD38 located in the nuclear compartment and the presence of ecto-cyclase activity is observed; ATRA treatment does not influence this activity. For each preparation (C: whole cells; PC: permeabilized cells) of treated and untreated cells, we measured the fluorescence of control samples (only with buffer) and this value was subtracted to that observed in presence NGD⁺. Results shown are representative of three separate experiments. Y axis = Δ fluorescence emission 410 nm. The data shown represent the average (±SD) of three independent observations; X axis: days of ATRA treatment.*

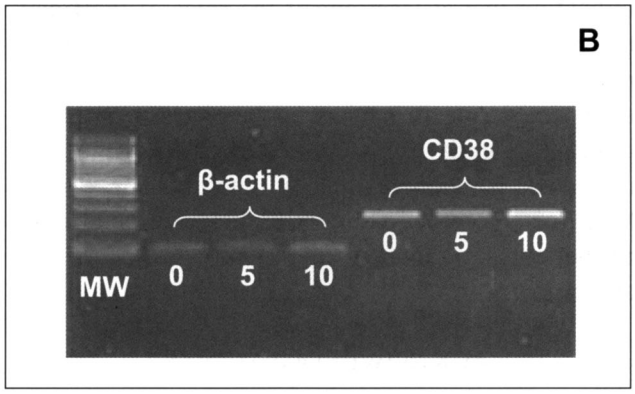
The signals, visualized using a chemiluminescence system, showed the same intensities in isolated nuclei as in total cells confirming that CD38 was expressed only at nuclear level. The presence of the transmembrane form would be revealed by more evident immunosignal. In nuclear and cellular lysates referred to treated cells, the intensity of the bands remained the same as the untreated ones, reproving that ATRA treatment had no effects on the protein expression. To verify whether ATRA treatment produces any effects at mRNA level, semi-quantitative RT-PCR was performed to evaluate transcript expression in SH-SY5Y cells before and after ATRA exposure. CD38 mRNA was detected in treated and untreated cells after 38 cycles of PCR amplification (Fig. 4, panel B). The expression, calculated for each sample as ratio of the intensities of CD38 amplification band to that of β-actin, indicated that there was a basal level of CD38 mRNA that showed an increase after treatment with ATRA in a time-dependent manner (Fig. 4, panel C). After 5 days of ATRA, the level of CD38 was double in respect to the control and it had increased 4-fold at the end of ATRA induction.

DISCUSSION

The cell surface antigen, CD38, is a bifunctional ecto-enzyme which is widely distributed in hemopoietic cells as well as in kidney, cardiac, pancreatic, brain, spleen, lung, and liver cells. It is known for its involvement in the metabolism of cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate, two nucleotides with calcium mobilizing activity independent of inositol trisphosphate. In brain, cADPR has been shown to be abundant (23) and to be implicated in many neural processes, such as synaptic transmission, neurotransmitter release and calcium events of rhythmic bursting (24).

In this study, we provide evidence of the presence of nuclear active CD38 in the human neuroblastoma cell line SH-SY5Y by means of morphological, molecular and enzymatic experiments. ATRA treatment has been used to induce cell differentiation. ATRA treatment acts only on the level of CD38 mRNA, which shows a time-dependent increase after treatment with ATRA. Protein amount and cyclase activity are not influenced by differentiative treatments. This apparent contradiction between mRNA and protein after ATRA treatment might be explained by the known feature of human CD38 gene to give rise to several mRNA transcripts. Different previous works (25-26) found five major transcripts in various T and B-lymphoblastoid cell lines. Another alternative splice product was described by Nata et al. (27) in many human tissues. It is not known if any of the currently existing antibodies





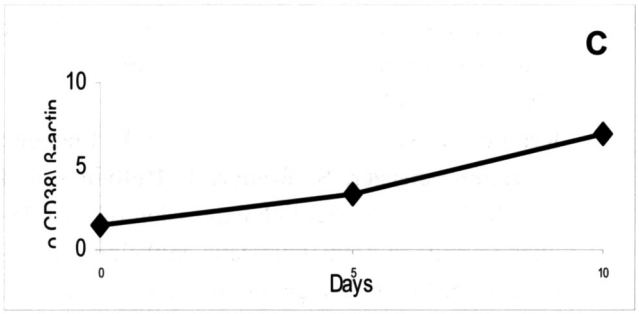


Fig. 4. *(A) Western blot analysis of cytoplasmic and nuclear protein extracts purified from control cells (0) and 5 and 10 days ATRA-treated cells (5; 10). Differentiated cells do not show alterations in CD38 expression and distribution. An immuno-reactive protein band of 42 kDa was detected in nuclear and total extracts of control and treated cells with the same intensity. (B) Ethidium bromide-stained 2% agarose gel showing PCR amplification of β-actin (loading control) and CD38. MW: molecular weight. (C) Effect of ATRA treatment on the mRNA expression levels of CD38 in SH-SY5Y cell line as measured by semiquantitative PCR. Expression is indicated as the ratio of amplicon density of the target genes over that of β-actin. Data are the mean values obtained from four separate experiments; error bars represent the SD.*

recognize these alternative products. There are two possible explanations: i) increasing mRNA observed after ATRA treatment is the product of alternative splicing, and the resulting protein presents different epitopes not recognized by our antibodies; ii) there is a post-transcriptional control that blocks the production of the protein. Considering that also cyclase activity does not show any increments after treatment and does not request anticorpal recognition, it is more probable that there is a post-transcriptional control that stops the protein production, even if the product of alternative splicing described by Nata did not show any enzymatic activity. The fate and the identity of CD38 mRNA after ATRA treatment still remain unclear, but the presence of active and constitutive CD38 in the nucleus evokes some important implications for intracellular production of cADPR and nucleoplasmic calcium release.

As shown also in previous studies, the catalytic site of CD38 lies within the nucleoplasm, indicating that the enzyme may catalyse the intranuclear cyclization of NAD^+ to cADPR, which then activates nuclear-membrane RyRs to trigger Ca^{2+} release.

RyRs are located in the inner nuclear membrane and exhibit its agonist-binding sites towards the nucleoplasm, but it is still not clear how cADPR, that is its antagonist, can cross the inner nuclear membrane freely to access binding sites on the receptors.

Thus, CD38 may play a role in the intranuclear generation of cADPR and the following activation of inner-nuclear-membrane RyRs and nucleoplasmic Ca^{2+} release into and out of the nucleoplasm at the nuclear envelope. Our morphological data also prove a dynamic association of CD38 with p80 coilin which is the greatest marker for identifying the coiled bodies. Moreover, recent studies have provided evidence that Cajal bodies may play a role in the transport and/or maturation of both splicing small nuclear snRNPs and nucleolar snoRNPs,

indicating that at least one of the roles of the Cajal body likely involves a functional interaction with nucleoli. In this respect CD38 may assume an active unexpected role in nuclear metabolism. Continued efforts to identify the activities of CD38 as nuclear molecular components and to determine its kinetic behavior in living cells are likely to contribute further clues to this new enigma in nuclear function and organization.

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