Physiological activation of human neutrophils down-regulates CD53 cell surface antigen

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Abstract: Tetraspanin transmembrane proteins have a metastasis suppressor effect by acting as cell motility brakes in tumor cells. CD53 is a panleukocyte antigen that belongs to the tetra-span superfamily. Human neutrophils express high levels of CD53. We tested the hypothesis that this antigen level changes when cells are activated. Treatment of human neutrophils with their physiological activators, tumor necrosis factor α or platelet-activating factor, resulted in down-regulation of this antigen from the cell surface, as assessed by immunofluorescence flow cytometry. Similar responses were observed when neutrophils were stimulated with chemotactic *N*-formyl-methionyl-leucyl-phenylalanine, phorbol ester, or the calcium ionophore ionomycin. The CD53 antigen down-regulation upon neutrophil stimulation was further confirmed by immunoblotting analysis and was not correlated with a change in the level of CD53 transcripts. This CD53 antigen down-regulation paralleled that of CD43 and CD44 antigens in these cells, despite their different protein structure. The down-regulation of the three antigens CD53, CD43, and CD44 could be inhibited by phenylmethylsulfonyl fluoride, suggesting that CD53 antigen down-regulation is the result of the activation of a proteolytic mechanism. Down-regulation of CD53 antigen level, as a result of cellular stimulation, might play a role in the different aspects of neutrophil biology, by modulating its interactions on the cell surface. *J. Leukoc. Biol.* 63: 699–706; 1998.

Key Words: cell surface molecule · human, tetraspanin antigens · tumor necrosis factor · platelet-activating factor · phorbol ester · CD44 · CD43

INTRODUCTION

The tetraspanin family is a novel group of membrane proteins that has been implicated in several important biological processes, but their physiological role in cell biology is not yet known [1–3]. Therefore, this protein family is still defined by the common structural features of its members [3]. They have four transmembrane domains, their amino and carboxy ends are facing the cytoplasm, and have two, small and large, extracellu-

lar loops. The large extracellular loop accounts for most of the differences found among the distinct members of this group, it is glycosylated in most cases and appears to be implicated in binding to an unknown ligand [3]. Tetra-span transmembrane proteins include several tumor markers as well as positive and negative regulators of cell proliferation and of cell motility. Among the tetra-span transmembrane proteins [2, 3] are CD37, a B cell marker; CD53/OX44, which modulates growth properties; CD81/TAPA1, which has antiproliferative properties; CD63/ME491, a melanoma antigen; CD9/MRP1, which affects cell motility; and CD82/IA4/C33/KAI1, which inhibits syncytium formation in HTLV-I-infected cells [4].

Some tetra-span transmembrane proteins, such as CD9/MRP1 and CD82/KAI1, have been shown to exert a metastasis suppressor phenotype in pancreatic and prostate cancer [5, 6]. This phenotype appears to be the result of a braking effect on cell motility, thus low levels will facilitate cell motility and result in an increased capacity to form metastasis. In five types of cancer, melanoma [7], non small-cell lung cancer [8, 9], breast [10], prostate [11], and pancreas [12], the level of tetra-span antigens supports the correlation of low level expression with poor prognosis.

Several tetraspanin antigens are known to promote cellular adhesion. This has been demonstrated for CD81 [13], CD9 [14], and CD53 [15, 16]. These three antigens are also known to coprecipitate together, suggesting they are forming a complex on the cell membrane which is physically associated with integrins on the cell surface and modulates their biological functions [17–19].

We reasoned that the braking action is a phenotypic manifestation of the biological effect of tetra-span proteins, which can modulate integrin function [17–19]. In normal cells where migration plays an important role, such as neutrophil response to infection, which requires mobilization of cells to the site of

Abbreviations: TM4SF, transmembrane-4-superfamily; PAF, platelet-activating factor; PKC, protein kinase C; DIFP, diisopropyl fluorophosphate; TNF- α , tumor necrosis factor α ; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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local infection, these proteins could modulate their levels depending on the activation state. Thus, it could be envisaged that when cells are stimulated with chemotactic agents there should be a reduction in the cell surface level of tetraspanin proteins. We tested this hypothesis using human neutrophils and determining the changes of CD53 antigen levels in response to different types of stimuli.

The human CD53 antigen [3] is a tetraspanin panleukocyte antigen that is detected in cells of lymphoid and myeloid lineages, including B and T lymphocytes, macrophages, neutrophils, and natural killer (NK) cells, but not in erythroid cells [1, 2]. CD53 has been associated with immunocompetence and positive selection in the thymus [20, 21]. CD53 is not present in cell lineages from other origins, differing in this regard from other tetraspanin proteins that are also present in cells with an epithelial origin, including several types of carcinoma [3]. The widespread distribution of CD53 in all leukocytes suggests that it might have a similar biological role in all of them. CD53 cross-linking, in rat macrophages, activates a cellular response that is mediated by protein kinase C (PKC) and releases nitric oxide as a consequence of inducing expression of nitric oxide synthase [22].

Neutrophil biology requires the participation of several types of membrane proteins to carry out different functions, like attachment and detachment to endothelium and target cells as well as cellular migration to places of local infection. These processes require selective and regulated neutrophil-endothelium and cell-cell interactions, which are mediated by a large array of membrane proteins, as well as regulation of cell motility [23–25]. Neutrophil activation in response to different signals, such as chemotactic peptides or cytokines, involves the modulation of the cell surface expression of several proteins, some are down-regulated and others are up-regulated. This change in the levels of cell surface proteins results in neutrophil detachment, migration, rolling, extravasation, and accumulation at the site of injury [25–29]. In this context, proteolytic modulation of membrane proteins is becoming an important regulatory mechanism in cell biology. This proteolytic down-regulation has been shown on an increasing number of proteins, including some cytokine receptors such as tumor necrosis factor α (TNF- α) receptor and interleukin-6 (IL-6) receptor [30], Fas ligand [31], adhesion molecules such as L-selectin [32, 33], ICAM-3 [34], CD43, and CD44 [35]. Specific proteases are present on the cell surface to specifically activate the thrombin and plasminogen systems [36, 37]. Cell surface proteolytic activity has also been implicated in human neutrophil activation [38, 39].

We have found that the cell surface expression of the CD53 antigen is down-regulated on neutrophil activation. This antigen down-regulation is triggered by several physiological neutrophil activating agents, and correlates well with the previously reported proteolytic down-regulation of other glycoproteins, CD43 and CD44 [35].

MATERIALS AND METHODS

Chemicals and reagents

Cell culture reagents were from Flow Laboratories (Irvine, CA). Phorbol 12-myristate 13-acetate (PMA), fMLP, cytochalasin B, phenylmethylsulfonyl

fluoride (PMSF), diisopropyl fluorophosphate (DIFP), Bdtellin, and antitrypsin inhibitor were from Sigma (St. Louis, MO). Ficoll-Hypaque was from Pharmacia (Uppsala, Sweden). Ionomycin was from Calbiochem (San Diego, CA). Acrylamide, bisacrylamide, ammonium persulfate, TEMED, and prestained molecular weight markers were from Bio-Rad (Hercules, CA). All other chemicals were from Merck (Darmstadt, Germany) or Sigma.

Monoclonal antibodies

mAb were used as hybridoma culture supernatants. MEM53 (anti-human CD53) was a gift of Dr. Vaclav Horejsi (Czech Academy of Sciences, Prague, Czech Republic) and was also obtained from Serotec (Oxford, UK). The mAb Bear-1, recognizing CD11b, was kindly provided by Dr. J. E. De Vries (Unicet, Lyon, France) [40]. Anti-CD43 (mAb TP1/36.1), anti-CD44 (mAb HP2/9), and mAb W6/32 (specific for a monomorphic determinant on HLA-A,B molecules) [35, 41] were kindly provided by Dr. F. Sánchez-Madrid (Hospital de La Princesa, Madrid, Spain). P3X63 myeloma culture supernatant was used as negative control.

Neutrophil isolation

Human neutrophils were obtained from fresh peripheral blood by dextran sedimentation and centrifugation on Ficoll-Hypaque followed by hypotonic lysis of residual erythrocytes as previously described [42]. The final cell preparation contained more than 98% neutrophils, as assessed by Giemsa-Wright staining.

Immunofluorescence flow cytometry

Immunofluorescence flow cytometry analysis was performed in a FACStar-Plus flow cytometer (Becton-Dickinson, San Jose, CA) as previously described [43]. Cells were incubated at 4°C with the corresponding mAb followed by washing and labeling with a fluorescein isothiocyanate (FITC)-labeled-goat anti-mouse IgG antibody (DAKO, Glostrup, Denmark). Linear and logarithmic immunofluorescence values were obtained in each experiment and the fluorescence produced by the myeloma P3X63 supernatant was considered as background. Specific linear fluorescence was obtained by subtracting the control fluorescence in which the mAb was substituted by the myeloma P3X63.

Neutrophil activation

Cells were resuspended at 3×10^6 neutrophils/mL in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/glucose buffer (150 mM NaCl, 5 mM KCl, 10 mM HEPES, 1.2 mM MgCl₂, 1.3 mM CaCl₂, 5.5 mM glucose, pH 7.5) and incubated in the absence or presence of distinct stimuli at 37°C for the times indicated in the respective legends. Subsequently, cells were sedimented by centrifugation at 300 *g* for 10 min. Supernatants were saved and lyophilized to determine release of proteins, and the pelleted cells were saved to determine the content of different cell surface antigens by flow cytometry. Also, sedimented cells were used to obtain membranes for immunoblot analysis. Cells were disrupted by freeze-thaw, and membranes were sedimented by centrifugation at 100,000 *g* for 90 min at 4°C in a Beckman TLA rotor [44]. Membrane pellets were resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM PMSF and stored at –20°C until use.

Immunoblot analysis

Total membrane proteins (40 μ g) from resting and activated human neutrophils were separated through sodium dodecyl sulfate (SDS), 10 or 15% polyacrylamide gel electrophoresis under reducing conditions, in the presence of 2-mercaptoethanol, transferred to nitrocellulose filters, and subjected to immunological detection as previously described [45]. After electroblotting and blocking for 1–2 h in 2% (w/v) powdered defatted milk in TBS (50 mM Tris-HCl, pH 8.0, 150 mM NaCl), the nitrocellulose filters were incubated overnight with the specific anti-CD53 mAb at a dilution of 1/400 in TBS buffer containing 0.05% (v/v) Tween 20. Signal was developed using the biotin-streptavidin-horseradish peroxidase system and detected with the ECL kit (Amersham, Little Chalfont, UK) according to the manufacturer's instructions.

Fig. 1. Effect of TNF- α on the cell surface antigen expression in human neutrophils. (A) Neutrophils were incubated for 15 min at 37°C in the absence of any stimulus (solid lines) or in the presence of 500 U/mL TNF- α (dashed lines). (B) Neutrophils were incubated for 15 min with 500 U/mL TNF- α in the presence of 2 mM PMSF (solid lines). Cells were stained for CD53, CD11b, HLA-A,B, CD43, and CD44 antigens and analyzed by immunofluorescence flow cytometry as described in Materials and Methods. Staining with negative control antibody P3X63 is also shown (A, dotted line). One representative experiment out of four is shown.

Northern blot analysis

RNA was extracted from the cells according to standard protocols using the acid guanidinium-phenol-chloroform extraction method. Ten micrograms of total RNA were electrophoresed on 0.9% agarose-formaldehyde gels and then transferred to Hybond-N nylon membranes (Amersham) and hybridized to the appropriate probe following standard protocols [46]. We used the human cDNA clone, ph15CD53, containing the complete CD53 coding region [P. A. Lazo, unpublished results] as a probe for CD53. For internal controls a cDNA probe against glyceraldehyde-3-phosphate dehydrogenase was used.

RESULTS

Human neutrophils are physiologically activated by the cytokine TNF- α and PAF, a potent phospholipid mediator. Both molecules play important regulatory roles in neutrophil recruitment to sites of injury. We found that CD53 was down-regulated after neutrophil stimulation with 500 U/mL of TNF- α (**Fig. 1**). TNF- α is also known to down-regulate sialophorin (CD43) and the hyaluronic acid receptor (CD44) during neutrophil activation by a proteolytic mechanism that is inhibited by PMSF [35]. We determined these two antigens as down-regulation controls in all experiments. As additional control antigens we used CD11b, an integrin that is up-regulated in neutrophil activation [44], and HLA-A,B, which is not affected by cell stimulation.

As shown in Figure 1, CD53 antigen, as well as CD43 and CD44, were down-regulated, whereas the cell surface expression of CD11b was up-regulated, and that of HLA-A,B was unaffected. We conclude from these data that CD53 antigen down-regulation is also part of the human neutrophil response to TNF- α (**Fig. 1**, **Table 1**). All TNF- α -induced down-regulation reactions were inhibited by the addition of 2 mM PMSF. The stimulation of human neutrophils with 1 μ M PAF for 15 min also triggered a reduction in CD53, CD43, and CD44 antigen levels, which were partially inhibited by the addition of PMSF (**Table 1**). It is interesting to note that PMSF did not affect the overexpression of CD11b (**Table 1**), thus ruling out the possibility that PMSF could block the overall neutrophil activation mechanism.

Neutrophils can be activated by stimulation with fMLP, a short chemotactic peptide that induces changes in the cell surface expression levels of several antigens [47]. fMLP stimulates neutrophils through a PKC-dependent pathway [48, 49]. We have determined the fate of CD53 antigen in human neutrophils that have been stimulated with fMLP (**Fig. 2**, **Table 1**). The addition of 1 μ M fMLP for 15 min induced a reduction in the cell surface CD53 antigen level on these cells (**Fig. 2**). This CD53 down-regulation was also observed after 5 min incubation with fMLP (data not shown). The fMLP-induced

TABLE 1. Effect of Distinct Physiological Stimuli on the Cell-Surface Expression of Several Leukocyte Antigens in Human Neutrophils

Cell surface antigen	37°C	TNF- α	TNF- α + PMSF	PAF	PAF + PMSF	fMLP	fMLP + PMSF
CD53 MEM53	46 $\pm$ 7 (100)	26 $\pm$ 4 (56)	41 $\pm$ 5 (90)	30 $\pm$ 4 (65)	46 $\pm$ 2 (100)	19 $\pm$ 5 (41)	40 $\pm$ 3 (87)
CD43 sialophorin	87 $\pm$ 7 (100)	51 $\pm$ 7 (58)	97 $\pm$ 9 (111)	56 $\pm$ 8 (64)	98 $\pm$ 9 (113)	33 $\pm$ 6 (38)	86 $\pm$ 3 (99)
CD44 hyaluronate receptor	96 $\pm$ 8 (100)	58 $\pm$ 8 (60)	102 $\pm$ 9 (106)	63 $\pm$ 7 (66)	97 $\pm$ 8 (101)	31 $\pm$ 5 (32)	92 $\pm$ 7 (96)
CD11b Mac1, Mo1	216 $\pm$ 15 (100)	289 $\pm$ 13 (133)	301 $\pm$ 21 (139)	298 $\pm$ 10 (138)	296 $\pm$ 12 (137)	396 $\pm$ 27 (183)	331 $\pm$ 24 (153)
HLA-A,B	48 $\pm$ 7 (100)	43 $\pm$ 2 (90)	45 $\pm$ 4 (94)	44 $\pm$ 1 (92)	46 $\pm$ 4 (96)	41 $\pm$ 6 (85)	43 $\pm$ 3 (90)

Neutrophils were incubated for 15 min at 37°C either in the absence or in the presence of the following stimuli: 500 U/mL TNF- α , 1 μ M PAF, 1 μ M fMLP. When indicated, cells were preincubated for 5 min with 2 mM PMSF. Cells were analyzed by immunofluorescence flow cytometry. Data are expressed as means $\pm$ SD of specific linear fluorescence intensity values of four independent experiments. The values in parentheses indicate the percentage of cell surface antigen expression with respect to that of untreated control cells at 37°C with no additions. The fMLP experiments were carried out in the presence of 5 μ g/mL cytochalasin B.

CD53 down-regulation was blocked by addition of PMSF (Fig. 2; Table 1). The fMLP-induced CD53 down-regulation paralleled the responses of CD43 and CD44, used as control antigens for down-regulation (Table 1). In contrast, CD11b was up-regulated, and HLA-A,B was not affected (Table 1).

Signaling mediated by PKC, such as stimulation with phorbol esters or Ca^{2+} [50], can also activate human neutrophils. Therefore, we tested the effects of PMA and the Ca^{2+} ionophore, ionomycin, on CD53 surface level. Phorbol esters and Ca^{2+} ionophores are potent activators of neutrophil functions. Shortly after the addition of 100 ng/mL PMA or 3 μM ionomycin to neutrophils there was a decrease in the CD53 antigen cell surface level (Fig. 2, Table 2). The PMA-induced CD53 down-regulation was very rapid, and similar to the fMLP-induced down-regulation (Fig. 2). Down-regulation could be detected following PMA treatment as rapidly as 5 min (data not shown). This mechanism appears to be mediated by PKC signaling, as preincubation of neutrophils with either 50 μM H7 or 150 nM staurosporine completely abrogated CD53 down-regulation (data not shown). CD53 (Fig. 3A) responded to PMA in the same way as CD43 (Fig. 3C) and CD44 (Fig. 3D). The use of serine protease inhibitors, such as PMSF, prevented the PMA or ionomycin-induced down-regulation of CD53 cell surface expression (Fig. 2, Table 2), as well as that of CD43 and CD44

Fig. 2. Down-regulation of CD53 antigen on cell stimulation with PMA or fMLP and its prevention by PMSF in human neutrophils. (A) Neutrophils were incubated for 15 min at 4°C (solid line) or 37°C (dashed line) in the absence of any stimulus. (B) Neutrophils were incubated for 15 min at 37°C in the presence of 100 ng/mL of PMA (solid line) or 1 μM fMLP (dashed line). (C) Neutrophils were incubated as in (B) but in the presence of 2 mM PMSF during the activation process. Cells were stained for CD53 antigen and analyzed by immunofluorescence flow cytometry. Staining with the negative control P3X63 is also shown (A, dotted line). One representative experiment out of five is shown.

Fig. 3. Effect of PMA on the cell-surface expression of CD53, CD11b, CD43, and CD44 antigens in human neutrophils. Neutrophils were incubated in the absence of any stimulus or in the presence of 100 ng/mL PMA for 15 min at 37°C. Resting (solid line) and stimulated (dashed) cells were stained for CD53 (A), CD11b (B), CD43 (C), and CD44 (D) antigens and analyzed by immunofluorescence flow cytometry as described in Materials and Methods. Cells were also incubated with PMA in the presence of 2 mM PMSF (dotted dashed line) and examined for the above antigens. Staining with the negative control P3X63 is also shown (A, dotted line). One representative experiment out of four is shown.

(Fig. 3, Table 2). The control CD11b up-regulation was also observed in our assays (Fig. 3B), but the increase in this antigen level was smaller in the presence of PMSF (Table 2). In an attempt to further characterize the protease implicated in CD53 down-regulation we used additional protease inhibitors. DIFP, another serine protease inhibitor, prevented CD53 down-regulation, whereas Bdeelin, an inhibitor of metalloproteinases, leupeptin, and anti-trypsin inhibitor did not prevent CD53 down-regulation (data not shown). Based on these observations, we suggest that a signal mediated by PKC can proteolytically down-regulate CD53 antigen.

Time course studies of CD53 down-regulation on PMA or TNF- α treatment showed that CD53 down-regulation was almost complete after 1 h incubation (Fig. 4).

The loss of CD53 antigen could be due to internalization by endocytosis as a consequence of neutrophil stimulation. Therefore, we performed experiments in the presence of cytochalasin B, a microfilament disrupting agent, which has been used as an inhibitor of endocytosis [51]. Cytochalasin B did not prevent down-regulation of CD53 antigen (Table 2). Cytochalasin B has been widely used to enhance neutrophil responses, increasing exocytosis. This is clearly observed in Table 2, where the up-regulation of CD11b is highly increased as a result of the mobilization of internal CD11b pools in intracellular granules [44].

Immunoblotting of CD53 antigen from neutrophil membranes clearly showed a diminished surface expression of this molecule on activated, compared with resting neutrophils (Fig. 5A), in accordance with the immunofluorescence flow cytometry data. The human CD53 antigen can be N-glycosylated and both forms, glycosylated and non-glycosylated, are present on the cellular membrane of different cell types [52]. In human

TABLE 2. Effect of PMA and ionomycin on the cell surface expression of several leukocyte antigens in human neutrophils and inhibition by PMSF

Antigen/CD	4°C	37°C	PMA	PMA + PMSF	Ionomycin	Ionomycin + PMSF	Ionomycin + cytochalasin B + PMA
MEM53 CD53	45 ± 5	46 ± 7 (100)	16 ± 5 (34)	33 ± 9 (72)	23 ± 5 (51)	38 ± 2 (83)	19 ± 22 (42)
Sialophorin CD43	91 ± 8	87 ± 7 (100)	27 ± 4 (31)	80 ± 9 (91)	20 ± 1 (23)	105 ± 8 (120)	N.D.
Hyaluronate receptor CD44	97 ± 9	96 ± 8 (100)	40 ± 6 (41)	94 ± 7 (97)	21 ± 3 (21)	87 ± 7 (90)	N.D.
Mac1, Mo1 CD11b	181 ± 11	216 ± 15 (100)	481 ± 25 (222)	320 ± 41 (148)	484 ± 31 (224)	301 ± 48 (139)	545 ± 35 (301)
HLA-A,B	46 ± 3	48 ± 7 (100)	38 ± 6 (83)	42 ± 6 (91)	39 ± 5 (81)	41 ± 4 (90)	47 ± 4 (98)

Neutrophils were held at 4°C or treated for 15 min at 37°C either in the absence of any stimulus or in the presence of the following stimuli: 100 ng/mL PMA, 3 μ M ionomycin. When indicated, cells were preincubated for 5 min with 2 mM PMSF or 5 μ g/mL cytochalasin B. Cells were analyzed by immunofluorescence flow cytometry. Data are expressed as means $\pm$ SD of specific linear fluorescence intensity values. Mean values were obtained from four independent cells experiments. In parentheses are the values represented as percentage of the cell surface antigen expression with respect to that found in untreated control cells determined at 37°C. N.D., not determined.

neutrophils, we detected by immunoblotting two bands of approximately 35 and 21 kDa, corresponding to both forms of CD53. Treatment with PMA resulted in the loss of both CD53 forms and the addition of PMSF inhibited this proteolytic down-regulation. These observations confirmed that glycosylated and non-glycosylated CD53 were down-regulated, likely by proteolysis. As control antigen in these experiments we used CD43, which is also lost after stimulation with PMA, and this response is also inhibited by addition of PMSF (Fig. 5A). The proteolytic attack could have two effects, either solubilizing the CD53 antigen or simply destroying the only accessible loop to the antibody and thus losing immunoreactivity. We were unable to detect soluble CD53 antigen (data not shown). These results suggest that the proteolytic attack could affect the extracellular loop where the antigenic epitope is located.

We also determined whether, as a result of PMA stimulation, there were changes in the level of mRNA coding for CD53 (Fig. 5B). The drop in CD53 cell surface expression after neutrophil activation suggested that the synthesis of new antigen might also be turned off as a result of down-regulation. Total RNA from neutrophils prepared at different time points before and after different types of stimulation were analyzed by Northern blot. The blot was hybridized to a human CD53 cDNA probe. As shown in Figure 5B, there were no changes in the CD53 mRNA level despite the reduction in membrane antigen levels.

Fig. 4. Time-course assay of the effect of PMA and TNF- α on CD53 antigen surface level in human neutrophils. Neutrophils were incubated at 37°C in the absence (solid line) or in the presence of 100 ng/mL PMA (A) or 500 U/mL (B) for 15 min (dashed line with plus signs), 30 min (dotted dashed line), and 60 min (dashed line). Then cells were stained for CD53 antigen and analyzed by immunofluorescence flow cytometry. Staining with the negative control P3X63 is also shown (dotted line). One representative experiment out of three is shown.

DISCUSSION

Tetraspanin transmembrane proteins in tumor cells appear to function as metastasis suppressors, by acting as brakes of cell motility [5, 6]. Thus low levels of these proteins have been

Fig. 5. Western blot and Northern blot analysis of CD53 in resting and activated human neutrophils. (A) Western blot analysis of membrane fractions from resting and activated human neutrophils. Blots contained 40 μ g of membrane proteins from control untreated neutrophils, neutrophils treated with PMA for 30 min, and neutrophils treated for 30 min with PMA in the presence of PMSF. Blots were analyzed with anti-CD53 mAb (top panel) or with anti-CD43 mAb (bottom panel). (B) Northern blot analysis of mRNA levels of CD53 in human neutrophils after stimulation with PMA, fMLP, or TNF- α for different incubation times. The Northern blot was hybridized to a human complete cDNA probe for CD53. Basal control levels in untreated neutrophils are also shown. Autoradiograph was developed after 24-h exposure.

correlated with an increased metastatic potential, and thereby poor prognosis [7–11].

In human neutrophils, as a result of activation, there is an increase in cell motility in order to migrate to the site of injury. Therefore we postulated that if the CD53 panleukocyte antigen was having an inhibitory effect on cell motility in this cell type, it should reduce its surface level after physiological stimulation.

We herein report a significant down-regulation in the levels of CD53 antigen on the cell surface during human neutrophil activation by different activators, such as TNF- α , PAF, and fMLP, signaling through a receptor-mediated pathway, and PMA and ionomycin, signaling via PKC, through a non-receptor route. The regulatory effects of TNF- α and PAF on CD53 cell expression were weaker than those exerted by PMA or ionomycin. However, on 1-h incubation with either PMA or TNF- α , CD53 surface antigen expression was almost completely reduced (Fig. 4). The differences in the CD53 cell surface levels between resting and TNF- α -activated neutrophils are similar to those detected between cells with low and high metastatic potential [8–12]. This change in the cell surface antigen level occurred rapidly and was already detectable after 5 min stimulation. This rapid response is not accompanied by changes at the mRNA level. The mechanism of this change in CD53 levels is not the result of internalization by endocytosis, as shown by the lack of effect of cytochalasin B. The fact that the protease inhibitor PMSF blocks the down-regulation of CD53, but not the up-regulation of the cell surface expression of CD11b induced by TNF- α or PAF may indicate that activation of a specific protease is the mechanism involved in the modulation of CD53. Furthermore, DIFP, another serine protease inhibitor, also prevents CD53 down-regulation. These data agree with a previous report showing that PMSF prevented the CD43 and CD44 proteolytic down-regulation with no effect on CD11b up-regulation when neutrophils were activated with TNF- α [35]. There is evidence showing that several protease inhibitors can affect different neutrophil functions [38, 39], which is consistent with our observation that in the presence of PMSF the up-regulation of CD11b was smaller when triggered by non-membrane receptor stimuli (Table 2). Thus, protease activation could mediate several aspects of neutrophil biology.

The CD53 down-regulation herein reported was similar to that of CD43 and CD44 [35], and was equally inhibited by PMSF in the three antigens, which suggests that proteolysis is a physiological regulator of neutrophil membrane-associated functions. There is no apparent homology among the three down-regulated antigens herein studied, CD53, CD43, and CD44. Thus, either there is a family of proteases, one for each target, or there is a protease that recognizes a three-dimensional structure common to apparently unrelated proteins. This putative protease must already be present in an inactive state in the resting cell, because its activation takes place immediately after cell stimulation. The presence of serine protease activity on cell membranes has long been known, like in the case of plasminogen activator [37], and in the activation of human neutrophils [38, 39].

In many cases, the consequence of a protease action is the release of a soluble form of a growth factor, as is the case for

IL-6, TNF- α , and FasL. In others, there is no solubilization, just a loss of antigenic reactivity, as is the case with CD43 and CD44 [35]. This latter process is likely to be occurring in the case of CD53, as its very hydrophobic nature with four transmembrane domains makes very unlikely that a proteolytic cleavage could solubilize the protein, particularly when this proteolytic attack appears to occur in the loop of the protein containing the epitope [53]. This is consistent with the lack of detection of CD53 antigen in soluble form upon cell activation, and with the reduction of the antigen level that remains detectable by Western blot on cellular membranes.

Because CD53 antigen is present on the surface of human neutrophils, which have specific interactions in different cellular environments, its cell surface expression modulation might be a mechanism that will allow binding to alternative molecules. In this context, it could be envisaged that neutrophils can detach as a consequence of CD53 down-regulation, and migrate toward a different location, where the cells could bind to a, perhaps, distinct ligand molecule. This issue will only be settled when the specific ligands for CD53 and other tetra-span transmembrane proteins are identified. This is important for two major reasons. First, several tetra-span proteins have been identified as tumor antigens, and their levels have been correlated with tumor prognosis in several types of cancer [7–12]. Second, several tetraspanin antigens are known to be implicated in adhesion processes, such as CD9 [14], CD81 [13], and CD53 [15], and these three antigens interact with integrins and are modulators of integrin functions [54].

The observations reported in this study further call attention to the biological importance of selective proteolytic cleavage in regulation of cell function. The characterization of the specific proteases involved, as well as the identification of specific inhibitors, could open up a field of great biological and pharmacological relevance in the control of specific pathologies. CD53 physiological function remains to be elucidated, but this antigen is a membrane protein that has been implicated in signal transduction [21, 55–57]. The proteolytic down-regulation of CD53, a prototype tetraspanin transmembrane protein, parallels that of other antigens that are also down-regulated in neutrophil activation, suggesting that tetraspanin proteins are a component of the neutrophil cellular interactions needed for some unknown aspects of their biology and, by analogy to other neutrophil adhesion proteins, their alteration might have clinical consequences, like recurrent infections. In this regard we have recently reported a family with recurrent infectious diseases that has as the only identified alteration a deficiency of CD53 antigen [58].

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