The role of proteinase-activated receptor 3 (PAR_3) in mouse hybridoma studied with monoclonal antibody generated against thrombin cleavage site

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Abstract

Thrombin produced upon tissue damage provides a link between inflammation/blood coagulation and immune reactions through proteinase-activated receptor (PAR) types 1, 3 and 4. In contrast to PAR_1 and PAR_4, functions of PAR_3 in cells other than platelets are poorly understood. We generated mAb 8E8 against fragment 31-47, which includes the thrombin cleavage site of human PAR_3. MAb 8E8 bound mouse platelets and delayed their aggregation induced by thrombin. This antibody also specifically stained B-lymphocyte-derived hybridoma in flow cytometry. Thrombin dose-dependently inhibited hybridoma cell proliferation and so did mAb 8E8 and PAR_3 31-47 fragment, but not the PAR_4 activating peptide. It is concluded that mAb 8E8 recognizes PAR_3 expressed in hybridoma and mimics the inhibitory effect of thrombin on hybridoma proliferation mediated by PAR_3. The data obtained also suggest that, unlike in platelets, targeting PAR_3 in mouse B lymphocyte-derived cells doesn't involve PAR_4.

Key words: *proteinase-activated receptor, thrombin, monoclonal antibody, hybridoma, proliferation.*

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Introduction

Inflammation is a critical event preceding and affecting the specific immune response. Correspondingly, lymphocytes express receptors for a variety of inflammatory substances and cytokines. Thrombin produced upon tissue damage and inflammation exerts its cellular effects through proteinase-activated receptors (PARs), which mediate cell activation upon receptor cleavage. The cleavage unmasks a peptide motif to function as a tethered ligand for the receptor. Three members of PAR family, PAR_1, PAR_3 and PAR_4, are activated by thrombin, while PAR_2 is cleaved by trypsin and tryptase [1]. Human T lymphocytes were shown to express PARs 1, 2 and 3 [2-3], PAR_1 being functionally connected with T cell antigen receptor [4]. The roles of PAR_2 and PAR_3 in lymphocytes are currently unknown. A consensus of data regards PAR_3 as a cofactor for PAR_4 in mouse platelets [5]. However, a differential expression of PAR_3 and PAR_4 genes in mouse tissues assumes that PAR_3 can play an independent physiological role [6]. Unlike other members of the PAR family, PAR_3 was not found to be activated by tethered ligand-derived peptide [1]; therefore the development of alternative tools, such as functionally active antibodies is of great importance.

The aim of our experiments was to generate monoclonal antibodies to the fragment 31-47 of human PAR_3, which contains the thrombin cleavage site 38-39. Difficulties met upon mAb generation encouraged us studying the expression and function of PAR_3 in mouse hybridoma cells.

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Results

Mice immunized with hemocyanin-conjugated PAR_3 (31-47) peptide produced both IgG and IgM serum peptide-specific antibodies. However, generation of corresponding monoclonal antibodies appeared problematic. Almost all PAR_3 peptide-specific hybridoma clones produced IgM, were unstable and lost their activity after several passages in culture. We succeeded to select a single stable clone 8E8 producing mAb of IgG2b subclass, which specifically bound PAR_3 peptide with high affinity (Figure 1). This mAb also bound mouse platelets previously reported for PAR_3 expression [7] and slightly delayed their aggregation stimulated with thrombin (Figure 2). The latter finding agreed with the results obtained using rabbit PAR_3-specific antibodies [7] and $PAR_3^{-/-}$ mice [8] and indicated that

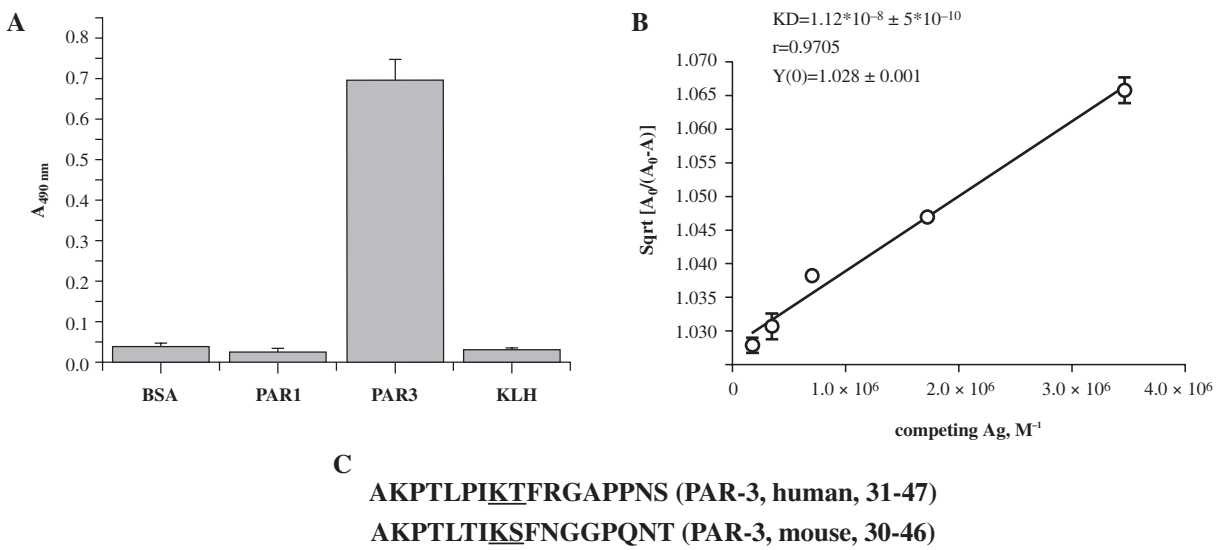

Fig. 1. Binding of mAb 8E8 to human PAR_3 (31-47); human PAR_1 (30-99); hemocyanin (KLH) and BSA in ELISA (**A**) and dissociation constant (K_d) calculated according to [10] with PAR_3 (31-47)-BSA conjugate as competing antigen (**B**). $A_0$and A – optical density in the absence or presence of competing antigen, respectively; Sqrt – square root. **C** – amino acid sequences of human PAR_3 (31-47) and corresponding mouse PAR_3 fragment. The thrombin cleavage sites are underlined

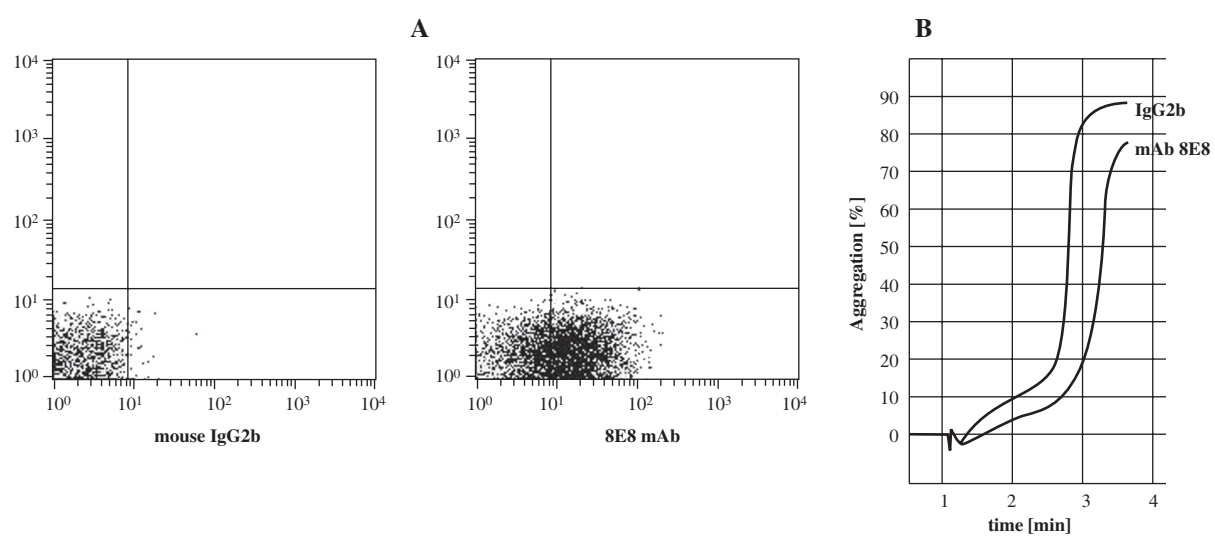

Fig. 2. Flow cytometry dot-plots of mouse platelets stained with mAb 8E8 or non-specific IgG2b (**A**, no antibody was applied along the ordinate) and the curves of platelet aggregation induced by 3 nM thrombin in the presence of either non-specific IgG2b or mAb 8E8 (**B**)

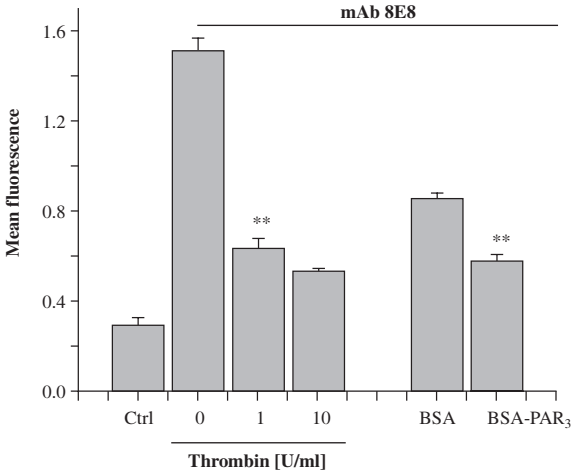

Fig. 3. Staining of hybridoma 1D6 with mAb 8E8 in flow cytometry. Cells were pre-treated with thrombin for 15 min prior to the mAb addition or mAb was pre-incubated with either BSA or PAR-3 (31-47)-BSA conjugate overnight (6.5 μg per 1 μg mAb). Shown are the data of one experiment out of three performed. ** – $p<0.005$ compared to mAb 8E8 alone (for thrombin) or to PAR-3-BSA compared to BSA. Ctrl – cells non-treated with mAb 8E8. Binding of non-specific IgG was negligible

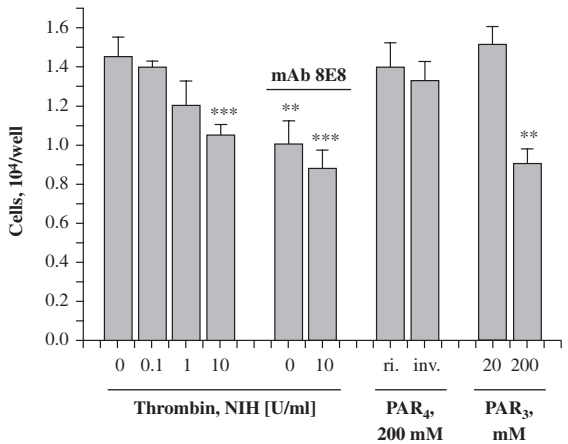

Fig. 4. Numbers of hybridoma 1D6 cells cultured for 3 days in the presence of thrombin and/or mAb 8E8 (20 μg/ml), PAR_4 (right and inverted) or PAR_3 peptides. Shown are the data of one experiment out of three performed. ** – $p<0.005$; *** – $p<0.0005$ compared to the non-treated culture (the first column)

mAb 8E8 recognized the native receptor and could interfere with the thrombin effect.

The difficulties in generating mAbs made us suggesting that PAR_3 is expressed in hybridoma and can be involved in regulating its vital functions. To test this we studied mAb 8E8 interaction with unrelated hybridoma 1D6. As shown in Figure 3, mAb 8E8 stained hybridoma in flow cytometry, the binding being inhibited by either pre-treating cells with thrombin or pre-incubating the mAb with BSA-coupled PAR_3-peptide. The apparent inhibitory effect of BSA alone was due to partial inactivation of mAb in the course of overnight incubation necessary to establish equilibrium in the mAb-peptide binding; however, the effect of peptide-BSA conjugate was significantly stronger than that of BSA.

Thrombin inhibited hybridoma 1D6 proliferation and so did mAb 8E8 by its own (Figure 4). The effect of mAb 8E8 was additional to that of thrombin upon low, but not high thrombin concentrations indicating that thrombin and mAb 8E8 bound the same receptor. Neither PAR_4 activating peptide AYPGKF [9] nor its partly inverted analogue YAPGKF influenced hybridoma proliferation, whereas PAR_3 antigenic peptide did it similarly to thrombin and mAb 8E8.

Discussion

The results presented demonstrate that mAb 8E8 generated against human PAR_3 peptide recognized mouse PAR_3. Corresponding human and mouse PAR_3 fragments are identical in the N-terminal portion (Figure 1), therefore, the epitope recognized by mAb 8E8 seems to include the AKPTL sequence close to thrombin cleavage site. Such fine specificity underlies the mAb 8E8 thrombin-like activity found. The ligand-like effect of receptor-specific antibodies, which either cross-link receptors [10] or even cleave target proteins [11] is documented. There is no direct data whether mAb 8E8 cleaved or not PAR_3 in hybridoma cells. However, some evidence on the mechanism of PAR_3/mAb 8E8 functioning was obtained using $PAR_{3/4}$ peptides.

The thrombin effect on hybridoma proliferation could be mimicked by PAR_3 (31-47) peptide, but not by PAR_4 activating peptide. Therefore, unlike in platelets, PAR_4 was not involved in PAR_3-mediated effect in hybridoma cells, while PAR_3 tethered ligand (TFRGAP) contained within PAR_3 (31-47) was. This fragment was shown to activate PAR_1 or PAR_2 on human T lymphocytes [2-3]. If it is the case in hybridoma cells, mAb 8E8 should be catalytic to cleave PAR_3 and to generate the tethered ligand. If mAb 8E8 is not catalytic, it should be recognized that PAR_3 aggregation on hybridoma cell surface is sufficient to trigger receptor signaling. Additional experiments are required to distinguish between these two hypotheses.

In conclusion, we succeeded in generating functionally active PAR_3-specific mAb 8E8, which can be used to study

PAR_3 functions in human and mouse cells. Our data for the first time demonstrate the presence of PAR_3 in mouse B lymphocyte-derived hybridoma and show that this receptor mediates the inhibitory effect of thrombin on cell proliferation. Such finding explains difficulties in generating PAR_3-specific hybridomas and suggests that thrombin is able to influence the B lymphocyte-dependent branch of immunity.

Material and Methods

Animals

BALB/c mice used for hybridoma generation were kept in the animal facility of Palladin Institute of Biochemistry, Kyiv. SV129 mice used for platelet isolation were born and bred at DLAR facility at SUNY, Stony Brook, NY. For retroorbital bleeding mice were anesthetized with Metofane, while for spleen removal they were sacrificed by cervical dislocation in accordance with approved protocols of SUNY and Palladin Institute of Biochemistry IACUC.

Cell lines

Mouse hybridoma 1D6 specific to α3 subunit of nicotinic acetylcholine receptor was generated by us previously [12]. The SP-2/0 hybridoma used as a fusion partner was from the stocks of Palladin Institute of Biochemistry. Cell lines including newly obtained hybridomas were grown in RPMI 1640 medium supplemented with 20 mM HEPES, 20 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 40 μg/ml gentamicin and 5-10% fetal calf serum (Atlanta, USA) at 37°C.

Thrombin and synthetic peptides

The bovine thrombin preparation ("Sigma", T4648) with the established proteolytic activity of 50 to 175 NIH units/mg has been used. The thrombin activity of 0.1, 1 and 10 NIH units/ml approximately corresponded to 1, 10 and 100 nM of thrombin.

The peptides Ac-AKPTLPIKTFRGAPPNS-amide corresponding to 31-47 fragment of human PAR_3, AYPGKF and YAPGKF, corresponding to activating PAR_4 peptide and its inverted analogue, respectively, and ESKATNATLDPRSFLLRNPNDKYEPFWEDEEKNESGLTEYRLVSINKSSPLQKQLPAFISEDASGYLTSS corresponding to 30-99 fragment of human PAR_1 were synthesized at CASM facility at SUNY, Stony Brook. For immunizations and antibody testing PAR_3 peptide was coupled to keyhole limpet hemocyanin or BSA using glutaraldehyde as described elsewhere [12].

Hybridoma production and mAb characterization

BALB/c mice were immunized intraperitoneally with 50 μg of PAR_3 peptide conjugated to hemocyanin in complete Freund's adjuvant. The second immunization was performed one month later with the same dose of the antigen emulsified in incomplete Freund's adjuvant. The sera were tested by ELISA. Mice having high antibody titers were boosted with 50 μg of the antigen without adjuvant four days before the spleen removal.

Hybridomas were generated by fusing the splenocytes of immunized mice with SP-2/0 cells as described [13] and were re-cloned three times by limiting dilutions. The antibodies were purified from the hybridoma culture medium by affinity chromatography on Protein A-agarose (Pharmacia, Sweden) and were biotinylated by a standard procedure using N-hydroxysuccinimido-biotin (Sigma) as a coupling reagent.

The mAb specificity, affinity and isotype were tested by ELISA, the bound antibodies being revealed with goat anti-mouse Fab-specific peroxidase conjugate (Sigma) or with mouse isotype-specific goat antibodies (Sigma) followed by anti-goat peroxidase conjugate. The bound peroxidase activity was developed with o-phenylendiamine-containing substrate solution. Dissociation constants were calculated according to [14] with Stevens's correction for bivalent antibodies [15].

Flow cytometry

Platelets were purified from the citrated murine blood by centrifugation followed by gel filtration over a Sepharose 2B column equilibrated with HEPES-buffered modified Tyrodes buffer (138 mM NaCl, 2.7 mM KCl, 0.4 mM $NaHPO_4$, 12 mM $NaHCO_3$, 0.2% BSA, 0.1% dextrose, 10 mM HEPES, pH 7.5). The platelets eluted in void volume were diluted with the buffer to a count of 1.3×10^8/ml. Purified platelets or hybridoma 1D6 cells were stained with biotinylated mAb 8E8 followed by Streptavidin-Phycoerythrin conjugate (PharMingen BD). In competition experiments, either the cells were pre-treated with thrombin for 15 min, or mAb 8E8 was pre-incubated with PAR_3-BSA conjugate overnight prior to staining. Flow cytometry was performed on FACS Calibur (Beckton Dickinson) in Stony Brook and on EPICS XL (Beckman-Coulter) in Kyiv.

Platelet aggregation

The rested platelets were pre-incubated with mAb 8E8 or mouse IgG2b in a buffer supplemented with 60-100 μg/ml of human fibrinogen and 2 mM $MgCl_2$, and then placed in a whole blood aggregometer (Chronolog, model 570 with Aggrolink software). Aggregation was induced with a range of thrombin doses and was monitored optically at 37°C for 5 minutes. Control and test samples were run simultaneously for a direct comparison.

Testing hybridoma proliferation

The colorimetric methylthiasol tetrasolium assay [16] was used to study 1D6 hybridoma cell proliferation. Cells were seeded into 96-well culture plates at 5×10^3 cells per

well in the culture medium containing purified antibodies, peptides or thrombin. Methylthiasol tetrasolium solution was added 3 days after for a final concentration of 0.4 mg/ml and cells were incubated for 4 hours at 37°C. The resulting formazan crystals were dissolved in 100 µl/well of dimethyl sulphoxide. Then 0.1M glycine in 0.1M NaCl, pH 10.5, 25 µl/well was added for 2 min and the absorbance at 540 nm was measured using Stat Fax-2100 microplate reader (Awareness Technology INC). Cell numbers that corresponded to the measured absorbance values were calculated using a calibration curve.

Acknowledgements

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