The Involvement of Protein Kinase D in T Cell-Induced Mast Cell Activation

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Abstract

Background: It has recently been reported that mast cells (MC) can be activated to degranulate and release certain cytokines in response to direct physical contact with activated but not resting T cells or their membranes. The MAPK family members ERK and p38 were found to participate. In this work, we further characterize the signaling events involved in this novel pathway of activation. **Methods:** Human MC were stimulated by activated T cell membranes (T*m). Phosphorylation of kinases was assessed by Western blotting. Protein kinase D (PKD) translocation was visualized by confocal microscopy. Degranulation was assessed by β -hexosaminidase release and cytokine production by ELISA. **Results:** Stimulation of human MC by activated T*m resulted in the activation of PKD. PKD inhibition by the specific pharmacological inhibitor Gö6976 resulted in a reduction in the phosphorylation of p38 but not ERK. Gö6976 also inhibited degranulation and cytokine release. **Conclusions:** MC stimulation by physical contact with T cells results in PKD activation, leading to the phosphorylation of p38, degranulation and release of cytokines. Understanding the molecular events associated with T cell-induced MC activation might lead to therapeutic approaches for controlling T cell-mediated inflammatory processes in which MC participate.

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Introduction

Mast cells (MC) are central effector cells in the elicitation of early- and late-phase allergic inflammatory reactions. The close physical proximity between MC and T cells seen in inflamed tissues has led investigators to propose a bidirectional interaction between these 2 cell populations [1]. We have previously shown that direct contact with activated but not resting T cells, or their membranes or microvesicles, resulted in MC degranulation and the production of various cytokines such as IL-8, oncostatin M (OSM) and IL-24 [1–4]. This pathway is also associated with the activation of ERK and p38 [5].

In this study, we further explored the signal transduction pathway of T cell-induced MC activation.

Materials and Methods

Antibodies

The following antibodies were used for this study: anti-total protein kinase D (PKD) and anti-phosphorylated PKD (S916) (Abcam®, Cambridge, UK), PKD/PKC μ (C-20) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti-phosphorylated p38 and ERK p44/42 (Cell Signaling Technology, Danvers, MA, USA), anti-tubulin (Sigma-Aldrich, St. Louis, MO, USA), HRP- and Alexa Fluor® 594-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Cells

Reagents for cell culture were purchased from Biological Industries (Beit Haemek, Israel). Jurkat T cell lymphoma cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 12.5 U/mL nystatin. The human LAD2 MC were maintained in StemPro-34[®] SFM (GIBCO[™] Invitrogen Corp., Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 50 µg/mL streptomycin, 100 IU/mL penicillin, and 100 ng/mL recombinant human stem cell factor (SCF, PeproTech Asia, Rehovot, Israel) as previously described [6]. Primary cultured human cord blood MC were derived from human cord blood mononuclear cells in the presence of SCF, IL-6, and prostaglandin E₂, and used as previously described [4].

Preparation of T Cell Membranes

Jurkat T cell membranes were isolated by a method described previously [1]. Briefly, cells were either not activated, or activated with 75 ng/mL PMA (Calbiochem, San Diego, CA, USA) at 2×10^6 /mL for 60 min at 37°C. At the end of incubation, the cells were washed with PBS and resuspended in ice-cold TKMS lysis buffer, comprising 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM MgCl₂, 0.25 M sucrose, 1 mM PMSF, and Complete[™], a mixture of protease inhibitors (Roche Applied Science, Boehringer, Mannheim, Germany). The cells were kept on ice for 20 min, and lysed by 5 cycles of freezing and thawing following centrifugation at 800 g for 5 min at 4°C. The supernatants were collected and subjected to centrifugation at 100,000 g for 60 min at 4°C. The pellets were suspended in PBS and stored at -70°C.

Mast Cell Activation

LAD2 cells (5×10^5) were untreated or incubated with 20 µg/mL of activated (T^{*}m) or nonactivated (Tm) Jurkat T cell membranes, for the indicated time periods. For IgE-mediated activation, cells were sensitized overnight with 500 ng/mL human myeloma IgE (Calbiochem), washed, and then stimulated with 10 µg/mL anti-human IgE (Dako, Glostrup, Denmark). In some experiments, various concentrations of the inhibitor Gö6976 (Calbiochem) were introduced to the LAD2 cells 30 min before the addition of stimulation. Cell viability was determined at the end of incubation period by Trypan blue staining. In all experiments, viability was found to be >95%.

β-Hexosaminidase Release

β-hexosaminidase release was determined by incubating 20-µL aliquots of supernatants and cell lysates for 90 min at 37°C with 50 µL of substrate solution consisting of 1.3 mg/mL p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) in 0.1 M citrate (pH 4.5). Reactions were stopped by the addition of 150 µL of 0.2 M glycine pH 10.7. Optical density was read at 405 nm. Results (mean ± SE) are expressed as the percentage of total β-hexosaminidase activity present in the cells.

Human Cytokine Assay

Supernatants of activated MC, as indicated above, were examined for released CCL2/MCP-1 and OSM by using a commercial ELISA kit, according to the manufacturer's instructions (DuoSet; R&D Systems, Minneapolis, MN, USA). IL-8 release was determined by using a commercial ELISA kit as per the manufacturer's instructions (development kit; PeproTech Asia).

SDS-PAGE and Immunoblotting

Cellular extracts were separated by SDS-PAGE with 10% polyacrylamide gels, transferred to PVDF filters, and processed for immunoblotting, as previously described [1]. Immunoreactive bands were visualized with the LAS-3000 imaging system (Fujifilm Corp., Tokyo, Japan).

Immunofluorescence

Cytospins of untreated MC or incubated with T^{*}m for the indicated time periods were fixed in 2% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked in 5.5% goat serum, and incubated with anti-PKD/PKC μ (Santa Cruz). Binding was detected by Alexa Fluor[®] 594-conjugated goat anti-rabbit fluorescent secondary antibody (Jackson ImmunoResearch Laboratories). Cells were analyzed by using a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical Analysis

Results are presented as means ± SE. Statistical analysis was performed with the paired Student *t* test. *p* ≤ 0.05 was considered statistically significant.

Results

Stimulation of Human MC by T^{*}m Leads to PKD Activation

We have previously shown that stimulation of MC by T^{*}m leads to degranulation and release of certain cytokines [1–3], and is associated with the phosphorylation of ERK and p38 [5]. The PKD family has been implicated in diverse cellular functions [7]. Furthermore, PKD is activated in murine bone marrow-derived MC (BMMC) stimulated through TLR2, Kit, or FcεRI [8]. Based on these observations, and in order to further explore the downstream events associated with T cell-induced MC activation, LAD2 MC were incubated with Tm or T^{*}m, and the cell extracts were analyzed for PKD activation. Tm induced a low level of PKD phosphorylation (Fig. 1a, left panels). In contrast, stimulation of LAD2 cells by T^{*}m induced high levels of PKD phosphorylation within 5 min, to a constant level over time, as shown by densitometry (Fig. 1b, left panel). Stimulation of MC with specific IgE and antigen also resulted in the phosphorylation of PKD, peaking within 5 min, but declining over time (Fig. 1a, b, right panels). The expression of PKD was also analyzed in mature (aged 9–10 weeks) human cord blood-derived primary MC (CBMC). A marked increase in PKD phosphorylation was observed in response to the incubation of these primary MC with T^{*}m but not with Tm (Fig. 1c), thus supporting the validity of LAD2 as a human MC model.

PKD has been shown to translocate from the cytosol to the plasma membrane following antigen receptor stim-

Fig. 1. PKD expression and activation in human MC. **a** LAD2 cells were untreated (med) or stimulated for the indicated time periods with Tm, T*m, or by cross-linking the FcεRI (IgE + Ag). The pPKD levels were analyzed by immunoblotting. **b** Densitometry of pPKD in T*m- or FcεRI cross-linking-stimulated LAD2 cells. **c** PKD phosphorylation in human CBMC incubated with either Tm or T*m for 5 min. **d** Immunofluorescence staining of PKD in untreated, Tm- or T*m-stimulated LAD2 cells. Representative results from 3 independent experiments are shown. OD, optical density.

ulation in the RBL 2H3 cell line [9]. Therefore, we investigated the localization of PKD in LAD2 cells. In resting MC and Tm-stimulated MC, the confocal images revealed that PKD is distributed in the cytoplasm (Fig. 1d, left and middle panels) whereas in T*m-stimulated cells, it was partially translocated to the plasma membrane (Fig. 1d, right panel). This could be observed within 5–30 min of stimulation.

PKD Inhibition Affects p38 in T*m-Activated Human MC

It has previously been shown that the PKD family is involved in the activation of MAPK [7]. We therefore investigated whether PKD is involved in p38 and ERK phosphorylation in T*m-activated human MC. By using Gö6976, a PKD inhibitor [10], introduced to LAD2 cells 30 min prior to the addition of T*m, we could inhibit

PKD and p38 phosphorylation in a dose-dependent manner, while ERK phosphorylation was not affected (Fig. 2a, b). In contrast, the phosphorylation of both p38 and ERK was inhibited dose-dependently by Gö6976 in MC activated via FcεRI-cross-linking (Fig. 2c, d).

PKD Involvement in Degranulation and Cytokine Release by T*m-Activated Human MC

PKD activation was found to be related to degranulation and the increase in CCL2/MCP-1 expression and release by stimulated BMMC [8]. Hence, we thought to examine the involvement of PKD in degranulation and cytokine release by T*m-stimulated MC. We first analyzed whether the stimulation of LAD2 cells by T*m would induce the release of this cytokine/chemokine as well. As presented in Figure 3a, activation of LAD2 cells by T*m resulted in a 14-fold release of CCL2/MCP-1 (62 and 870

Fig. 2. The involvement of PKD in MAPK activation. LAD2 cells were preincubated for 30 min with indicated concentrations of Gö6976. Subsequently, cells were either untreated (med) or stimulated for 5 min with T*m (a) or by FcεRI cross-linking (IgE + Ag; c). The levels of phosphorylated PKD, ERK, and p38 were analyzed by immunoblotting. **b** Densitometry of phosphorylated kinases in T*m-stimulated MC. **d** Densitometry of phosphorylated kinases in LAD2 cells stimulated by cross-linking the FcεRI. Representative results from 5 independent experiments are shown. Max., maximum; pP38, phosphorylated p38.

pg/mL, respectively). Thereafter, we explored whether PKD is involved in degranulation (by means of β -hexosaminidase release) and cytokine release. By using Gö6976, a significant dose-dependent inhibition of both β -hexosaminidase (Fig. 3b) and the 3 cytokines which were tested (Fig. 3c) could be observed, reaching maximal inhibitions of 70, 75, 76 and 65% for β -hexosaminidase, CCL2/MCP-1 and OSM, and IL-8, respectively. Taken together, these results indicate that PKD is involved in T*m-induced human MC degranulation and cytokine release.

Discussion

Recent studies have shown that heterotypic adhesion of MC to activated but not resting T cells induces MC degranulation and cytokine production in a pathway associated with the activation of Ras and the MAPK signaling pathway [1–5].

We now show that T cell-induced MC activation induces PKD activation as well. The LAD2 human MC line, as well as primary CBMC, express PKD protein constitutively, phosphorylated upon stimulation with T*m but

not with Tm, thus supporting the validity of LAD2 as a human MC model [11, 12]. FcεRI cross-linking stimulation of LAD2 human MC resulted in PKD phosphorylation as well (Fig. 1). Moreover, murine and rat MC were also found to constitutively express PKD, which was activated in these cells upon stimulation through FcεRI, the high-affinity receptor for IgE [8, 13]. In contrast, in an untreated HMC-1 human MC line, a low basal level of phosphorylated PKD could be noticed [14]. This discrepancy between LAD2 and HMC-1 human MC lines could be due to differences in the phenotype of the cells, with the former exhibiting a more mature phenotype [6].

In naïve and Tm-stimulated LAD2 cells, PKD was distributed in the cytoplasm whereas upon stimulation with T*m, it partially translocated to the plasma membrane (Fig. 1d). This could be observed for a relatively long time period of 30 min. However, in RBL 2H3 cells, stimulation via FcεRI resulted in rapid transient redistribution of GFP-PKD from the cytosol to the plasma membrane and vice versa, within 10 min of antigen receptor engagement [9]. We also documented rapid PKD phosphorylation, which peaked at 5 min, in human MC stimulated via FcεRI. This discrepancy in PKD translocation could be

Fig. 3. The involvement of PKD in degranulation and cytokine release from human MC. **a** LAD2 cells were incubated with T*m for overnight and CCL2/MCP-1 release was assayed by ELISA (* $p < 0.05$). LAD2 cells were preincubated for 30 min with the indicated concentrations of Gö6976. Subsequently, cells were incubated with T*m overnight. Supernatants were collected and analyzed for the

measurement of β -hexosaminidase (**b**) or CCL2/MCP-1, OSM, and IL-8 release (**c**). Data are expressed as percentage of maximal secretion. Results are shown as mean $\pm$ SE of 3 independent experiments done in duplicate. β -Hex, β -hexosaminidase; Max., maximum.

explained by the different activation pathway which was used. While our human MC were activated by T cells, the murine MC were activated through Fc ϵ RI cross-linking. Indeed, we have previously reported that activation of MC after contact with T cells induces an expression profile that is different from stimulation by Fc ϵ RI cross-linking, resulting in a different pattern of mediator release. In particular, the cytokines IL-8, OSM, and IL-24 are uniquely secreted in the T cell-induced activation pathway [2–4].

Another difference between these 2 pathways of MC activation was noticed when we explored whether MAPKs are affected by PKD activation. While PKD phosphorylation in MC activated by T cells leads to the phosphorylation of p38, but not of ERK, activation of the cells by Fc ϵ RI cross-linking leads to PKD phosphorylation that results in both p38 and ERK activation. Therefore, we can pos-

tulate that in T cell-induced MC activation, PKD probably exerts its effects via p38, while in “classical” IgE receptor cross-linking, PKD influences both p38 and ERK MAPK. Indeed, a variation between these 2 MC activation pathways was shown previously with regard to ERK activation. While activated T cells caused sustained ERK phosphorylation and IL-8 release by human MC, Fc ϵ RI cross-linking was followed by transient ERK activation and the preferential secretion of TNF- α [5].

PKD was found to exert its effect by using different MAPKs under various conditions. The involvement of PKD in activating p38 was found while exploring the TLR5 response to its ligand, flagellin, in the HEK 293T epithelial cell line. PKD mediated the phosphorylation of TLR5 in response to flagellin, which, in turn, contributed to the activation of the p38 and the release of IL-8. Fur-

thermore, this PKD involvement in the p38-mediated IL-8 response to flagellin was inhibited by using Gö6976 [15]. PKD was also found to be involved in the activation of ERK. In differentiated primary mouse keratinocytes, PKD has served as a major regulator of a proliferative response, probably by causing prolonged ERK activity [16].

In this study, we also show that PKD is associated with the degranulation and release of several cytokines and chemokines from T^{*}m-stimulated MC (Fig. 3), such as CCL2/ MCP-1, which is known to mediate the activation and recruitment of inflammatory cells and plays an important role in asthma [17]. CCL2/ MCP-1 production was also reported in BMDC activated by palmitoyl-3-cysteine-serine-lysine-4 (Pam₃CSK₄), SCF or FcεRI cross-linking; however, in contrast to our findings, Murphy et al. [8] did not find PKD to be involved in degranulation. The usage of human versus murine MC and the diverse stimuli used could explain these contradictory results.

PKD can be activated in a PKC-dependent way or by other pathways independently of PKC [18]. Future work is required to address this issue in T^{*}m-stimulated MC. In summary, we show for the first time that PKD is involved in T cell-induced MC activation. These findings may contribute to our understanding of the molecular events associated with this pathway of activation.

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Disclosure Statement

All authors confirm that they have no conflict of interests in relation to this work.

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