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Small Rho GTPases Mediate Tumor-Induced Inhibition of Endocytic Activity of Dendritic Cells

Irina L. Tourkova,* Galina V. Shurin,* Sheng Wei, ‡ and Michael R. Shurin2*†

The generation, maturation, and function of dendritic cells (DC) have been shown to be markedly compromised in the tumor microenvironment in animals and humans. However, the molecular mechanisms and intracellular pathways involved in the regulation of the DC system in cancer are not yet fully understood. Recently, we have reported on the role of the small Rho GTPase family members Cdc42, Rac1, and RhoA in regulating DC adherence, motility, and Ag presentation. To investigate involvement of small Rho GTPases in dysregulation of DC function by tumors, we next evaluated how Cdc42, Rac1, and RhoA regulated endocytic activity of DC in the tumor microenvironment. We revealed a decreased uptake of dextran 40 and polystyrene beads by DC generated in the presence of different tumor cell lines, including RM1 prostate, MC38 colon, 3LL lung, and B7E3 oral squamous cell carcinomas in vitro and by DC prepared from tumor-bearing mice ex vivo. Impaired endocytic activity of DC cocultured with tumor cells was associated with decreased levels of active Cdc42 and Rac1. Transduction of DC with the dominant negative Cdc42 and Rac1 genes also led to reduced phagocytosis and receptor-mediated endocytosis. Furthermore, transduction of DC with the constitutively active Cdc42 and Rac1 genes restored endocytic activity of DC that was inhibited by the tumors. Thus, our results suggest that tumor-induced dysregulation of endocytic activity of DC is mediated by reduced activity of several members of the small Rho GTPase family, which might serve as new targets for improving the efficacy of DC vaccines. The Journal of Immunology, 2007, 178: 7787–7793.

Dendritic cells (DC) perform an essential role in the initiation and regulation of adaptive immune responses (1). Immature DCs constantly sample their environment for Ags by phagocytosis, macropinocytosis, and highly efficient receptor-mediated endocytosis. In the presence of appropriate danger signals, immature DCs undergo maturation characterized by the up-regulation of expression of MHC and costimulatory molecules and the down-regulation of endocytic activity. Subsequent migration to lymphoid tissue results in efficient presentation of optimally processed Ags to T cells (2, 3). These specialized functions of DC, specifically endocytosis, migration, and Ag presentation, permit the generation and maintenance of adaptive immune responses, including an antitumor response. It is therefore not surprising that many tumors have evolved mechanisms that alter the function and/or survival of DC. Different tumors could suppress DC generation, differentiation, maturation, and subsequently DC function, including Ag uptake, processing, and presentation (4–8). However, molecular mechanisms and intracellular pathways that are involved in the misbalance of the DC system in cancer have not yet been fully understood.

A number of laboratories over the past few years revealed that small Rho GTPases (a subgroup of the Ras superfamily of 20–30-kDa GTP-binding proteins) play a crucial role in diverse cellular events such as membrane trafficking, transcriptional regulation, cell growth control, differentiation, and apoptosis (9–14). Like the most members of the Ras superfamily, the small Rho GTPases function as molecular switches cycling between an inactive GDP-bound state and an active GTP-bound state. The ratio of the GTP- to the GDP-bound forms is regulated by the opposing effect of guanine nucleotide exchange factors, which enhance the exchange of bound GDP for GTP, and the GTPase-activating proteins, which increase the intrinsic rate of hydrolysis of bound GTP. An additional level of regulation is provided by guanine nucleotide dissociation inhibitors, which can suppress both the exchange of GTP and the hydrolysis of bound GTP (15, 16). Activation of small Rho GTPases is induced by the specific stimuli from the upstream cell surface receptors, including growth factor receptors, cytokine receptors, G-protein-coupled receptors, and adhesion receptors. The activated Rho-related proteins transfer the signals from the external stimuli to the downstream effectors, resulting in diverse functional responses (17, 18). Although much is known about the Rho-type GTPase structure and signal transduction, little was reported about their regulation and function within immune cells, and no data are available on their role in DCs functioning in the tumor microenvironment.

It has been recently reported that Rac1 and Cdc42 control the levels of endocytosis during DC differentiation and maturation (19–22). The functional role of Rac1 and Cdc42 for the morphology and Ag-presenting capacity of DCs was described for DCs grown from hemopoietic stem cells (23) and PBMC-derived DC (24, 25). Selective Rac1 inhibition in DCs was shown to diminish apoptotic cell uptake and cross-presentation in...
transgenic mice (26). Recently, we have reported that Cdc42, RhoA, and Rac1 regulate murine bone marrow-derived DC functions that are critical for DC-induced immune response, such as adherence, Ag presentation, motility, and chemotaxis (27). In this study, for the first time we have investigated the role of small Rho GTPases in the regulation of DC endocytic activity in the tumor microenvironment. We revealed that tumor cells inhibited receptor-mediated endocytosis and phagocytosis in DCs cocultured with tumor cells in vitro or prepared from tumor-bearing mice. This suppression was associated with a down-regulation of Rac1 and Cdc42 activity and might be restored by the genetic modification of tumor-treated DCs using \textit{Vaccinia} virus (VV)-based constructs that encode the constitutively active forms of Cdc42 and Rac1 genes.

Materials and Methods

Animals

Male C57BL/6 mice, 6–8 wk old, were obtained from Taconic. Animals were maintained at Central Animal Facility at the University of Pittsburgh (Pittsburgh, PA) according to the standard guidelines and Institutional Animal Care and Use Committee-approved protocols.

Cell preparation and treatments

Tumor cell lines RM1, MC38, 3LL, and B7E3 and nonmalignant fibroblast cell line 3T6 were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin sulfate, 10 mM HEPES, 10% FBS, 10 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen Life Technologies), referred to as complete medium (CM). RM1 prostate carcinoma was a gift from Dr. Timothy C. Thompson (Baylor College of Medicine, Houston, TX), MC38 colon carcinoma and 3LL lung carcinoma were gifts from Dr. Michael T. Lotze (University of Pittsburgh Cancer Institute, Pittsburgh, PA), B7E3 oral squamous cell carcinoma was a gift from Dr. Christine Milcarek (University of Pittsburgh Cancer Institute, Pittsburgh, PA), and murine fibroblast cell line 3T6 was a gift from Dr. Christian Milcarek (Department of Immunology, University of Pittsburgh, Pittsburgh, PA). A single-cell suspension of tumor cells (5 × 10^6 RM1 cells in 0.1 ml of HBSS) was injected s.c. into the right flank of syngeneic C57BL/6 mice. Injection of HBSS served as a control. Three weeks later, bone marrow was harvested for DC preparation.

Murine DCs were generated from tumor-bearing and tumor-free mice as described previously (28). Briefly, hemopoietic progenitors were isolated from bone marrow and depleted of RBC with lysing buffer (Sigma-Aldrich). Murine fibroblast cell line 3T6 was transfected with pEGFP-C1 (Clontech). DC precursors were incubated with four different tumor cell lines (10^6 cells/well) separated by membrane inserts with 0.4-μm pore size (Transwell system; Falcon) in CM for the first 3 days in cultures. Murine fibroblast cell line 3T6 (10^6 cells/well) or CM alone served as controls. At day 6, DCs were transduced with \textit{Vaccinia} virus encoding dominant negative (dn) or constitutively active (ca) forms of Rho GTPases. Six hours later, DCs were collected and used for further analyses.

**Endocytosis assay**

The regulation of endocytic activity of DC by the Rho-family GTPases was assessed by FITC-dextran (40 mg/ml; Molecular Probes) uptake characterizing receptor-mediated endocytosis, and latex bead uptake reflecting phagocytic activity of cells. Control non-treated DC, DC generated from tumor-bearing mice, DC cocultured with tumor cell lines, DC cocultured with nonmalignant cells (fibroblast cell line 3T6), and VV/Rho GTPase-transduced DC were used in our studies. Active uptake of dextran 40 was measured for 40 min at 37°C, whereas background diffusion was determined at 4°C. Cells were washed four times with cold PBS containing 0.1% BSA and 0.1% NaN₃, and analyzed by FACSscan (BD Biosciences). Data analysis was performed using CellQuest software (BD Biosciences), and the results were expressed as the mean fluorescent intensity (MFI) and percentage of positive cells.

DC phagocytosis was evaluated using fluorescent latex beads (1 μm mean particle size) (Sigma-Aldrich). Tumor-treated DCs, VV/Rho GTPase-transduced DCs, and control non-treated, treated with nonmalignant cells, or control-transduced DCs were collected, washed in RPMI 1640 and incubated (1 × 10⁶ cells/ml) with beads (~100 beads/cell) for 1 h at 37°C. Phagocytosis was stopped, and nonbound beads were separated by washing the cells twice in ice-cold RPMI 1640. DC were cytospun (100 x g, 5 min), air-dried, and fixed for 10 min with 4% formaldehyde-PBS. Slides were washed in PBS, covered, and photographed with an Olympus Provis AX-70 fluorescence microscope (×40 phase objective) equipped with a color camera. Phagocytosis (percent) was quantified by counting the number of DCs with two or more beads as a proportion of the total number of cells in five randomly selected imaging fields (40–50 cells/field).

**p21-activated kinase-l-and Rhotekin-based pull-down assay for the GTPase activity**

Affinity purification assays that monitor Cdc42, Rho, and Rac activation are based on the fact that these proteins act as molecular switches, cycling between inactive GDP-bound and active GTP-bound states (29). The Cdc42/Rac and Rho Activation Assay kits have been used to assess specific binding and precipitation of GTP-Cdc42/GTP-Rac and GTP-Rho in all of the lysates from the DC used in our experiments (Upstate Biotechnology). DC lysates (10⁷ cells/ml) were incubated for 1 h at 4°C with 10 μg of p21-binding domain (PBD) of p21-activated kinase-1 (PAK-1), which specifically binds to and precipitates the GTP-bound form of Rac and Cdc42. This allows the isolation of active Cdc42 and Rac proteins, whereas use of 30 μg of Rhotekin Rho-binding domain (Rhotekin RBD) causes the precipitation of the GTP-Rho complex. All binding domains were immobilized on agarose. Before the addition of agarose beads, equal aliquots of lysates were removed from all samples and run on a separate SDS-PAGE gel to serve as an equal loading control. As a positive control, DC lysates were incubated for 15 min with GTP•S (0.1 mM) before the incubation with PAK-1 PBD and Rhotekin RBD-agarose to transfer small Rho GTPases into an active state. Pellets were washed three times, and precipitated proteins were eluted with a sample buffer, separated on SDS-PAGE gels, and analyzed by Western blotting. Proteins were transferred to polyvinylidene difluoride membranes and probed with specific anti-Rac1, anti-Cdc42, and anti-RhoA Abs (Upstate Biotechnology and BD Transduction Laboratories). The secondary Abs were HRP conjugated (1/100,000; Pierce). The membrane was processed and treated with chemiluminescent reagents (Pierce), and the bands were visualized on Kodak film (Eastman Kodak). The bands corresponding to active Rho GTPases on the Western blots were quantified by densitometry and presented as a ratio to the equal loading control values.

**Statistical analysis**

Differences between groups were determined using the Student t test or the nonparametric Mann-Whitney U test after evaluation for normality. For all statistical analyses, the level of significance was set at a probability of 0.05. All experiments were repeated two to three times. Data are presented as the mean ± SEM.

**Results**

**Endocytic activity of DC was down-regulated in tumor-bearing mice**

In our previous studies, we have demonstrated that tumor-derived factors inhibit both in vivo and in vitro generation of DC from bone marrow precursors assessed by DC phenotyping, IL-12 production, and the stimulatory capacity to induce T cell proliferation...
To evaluate whether tumors influence the endocytic activity of DCs, we first analyzed receptor-mediated endocytosis and phagocytosis in bone marrow-derived DCs obtained from tumor-bearing mice. RM1 tumor cells were injected s.c.; 3 wk later, bone marrow precursors were used for DC generation from tumor-bearing and control tumor-free mice. The analysis of FITC-dextran 40 uptake by DC showed that receptor-mediated endocytosis in DC from tumor-bearing mice was reduced by up to 30%. For instance, it decreased from 237.7 ± 32.6 in tumor-free to 169.6 ± 41.9 in RM1-bearing mice, when expressed as the MFI (p < 0.05, n = 3). Phagocytic activity of DC from tumor-bearing and tumor-free mice was characterized by uptake of fluorescent-labeled latex beads. A significant reduction (up to 50%) in the number of cells with beads was observed when DCs were generated from RM1-bearing mice (p < 0.05, n = 3). For example, uptake of beads by DC was reduced from 53.3 ± 2.2% to 30.0 ± 3.4% when compared with tumor-free mice. Therefore, these results suggest that receptor-mediated endocytosis and phagocytosis in DC were inhibited in tumor-bearing mice compared with tumor-free mice.

**Tumor cells inhibited DC receptor-mediated endocytosis and phagocytosis in vitro**

To study the mechanisms of altered endocytic activity of DC in the tumor microenvironment, we next evaluated the effect of tumor cells on DC by analyzing FITC-dextran 40 uptake in DC that were preincubated with tumor cell lines (RM1, MC38, 3LL, and B7E3), nonmalignant cells (3T6), or CM in a Transwell system in vitro. The flow cytometry results showed that preincubation with 3T6 cells did not influence uptake of dextran 40 by DC compared with medium alone, whereas all examined tumor cell lines inhibited dextran 40 uptake by DCs (Fig. 1A).
FIGURE 2. RM1 tumor cells down-regulated active forms of Cdc42 and Rac1 in DCs. Murine bone marrow-derived DCs (10⁶ cells/well) were cocultured with RM1 prostate carcinoma cells (10⁶ cells/well) as described in the Fig. 1 legend. A, Control nontreated (C) and tumor-treated (Tu) DC lysates (from 10⁶ cells) were incubated for 1 h at 4°C with 10 μg of PDB of PAK-1. As a positive control (P), DC lysates were incubated for 15 min with GTPγS (0.1 mM) before the incubation with PAK-1 PBD. Pellets were washed and precipitated proteins were eluted, separated by SDS-PAGE and visualized by Western blotting. B, Before the addition of beads, equal aliquots of DC lysates were removed from each tube and run on a separate SDS-PAGE to serve as an equal loading control. For both A and B, proteins were transferred to polyvinylidene difluoride membranes and probed with specific anti-Rac and anti-Cdc42 Abs. The secondary Abs were HRP conjugated (1/100,000). The membrane was processed and treated with chemiluminescent reagents, and the bands were visualized on Kodak film.

Values are the results of one representative of three independent experiments.

dhCdc42 and dnRac1 transduction reduced endocytic activity of DC

To study the regulation of DC endocytosis by small Rho GTPases in the tumor microenvironment, VV-based technique of DC transfection was used as described previously (27, 35). Endocytic activity of DC transduced with VV-based constructs encoding dnCdc42, dnRac1, and dnRhoA was assessed by the analysis of dextran 40 uptake and fluorescent latex bead uptake. Flow cytometry analysis showed that DC transduction with dnCdc42 and dnRac1 significantly decreased DC endocytic activity (Fig. 3, A, C, and D). Dextran 40 uptake by dnCdc42- and dnRac1-transduced DCs was reduced by up to 1.5 times when assessed as the percentage of positive cells or MFI (Fig. 3C). Quantification of fluorescent latex bead uptake by DC indicated that dnCdc42 and dnRac1 caused down-regulation of phagocytosis in transduced DC by up to two times (p < 0.05; Fig. 3D). At the same time, transduction of DCs with dnRhoA did not significantly decrease DC endocytosis (p > 0.05; data not shown). Thus, these results suggest that transduction with dnCdc42 and dnRac1 caused changes in DCs similar to those induced by tumor cell lines: both treatments reduced levels of active Cdc42 and Rac1 in DCs and inhibited DC endocytosis.

Transduction of DCs with caCdc42 and caRac1 restored endocytic activity of DCs inhibited by tumor cells

To confirm that Cdc42 and Rac1 are directly involved in tumor-induced suppression of DC endocytosis, we examined whether the up-regulation of small Rho GTPase activity may reverse the inhibitory effect of tumors on DC function. To increase the Rho GTPase activity in DC, RM1-treated and nontreated DCs were transduced with VV-based constructs encoding caCdc42 and caRac1. Transfection of DC with caRhoA was not tested given that transduction with caCdc42 and caRac1 caused changes in DCs similar to those induced by tumor cell lines: both treatments reduced levels of active Cdc42 and Rac1 in DCs and inhibited DC endocytosis. To confirm that Cdc42 and Rac1 are directly involved in tumor-induced suppression of DC endocytosis, we examined whether the up-regulation of small Rho GTPase activity may reverse the inhibitory effect of tumors on DC function. To increase the Rho GTPase activity in DC, RM1-treated and nontreated DCs were transduced with VV-based constructs encoding caCdc42 and caRac1. Transfection of DC with caRhoA was not tested given that our data showed no alteration of RhoA activity in DC in the tumor microenvironment. DCs transduced with the VV/CD56 construct were used as a control. Coincubation with RM1 cells decreased endocytosis. However, flow cytometry analysis of dextran 40 uptake and quantification of fluorescent latex bead uptake by DCs have shown that it was restored by Cdc42 and Rac1 activation (Fig. 3, B–D). Whereas activation of Rac1 completely reversed the inhibitory effect of tumor cells on DC endocytosis, activation of Cdc42 only partly (50–55%) restored DC endocytosis in RM1-pre-treated DCs. Furthermore, caRac1 transduction of control DC increased dextran 40 uptake by up to 30% (p < 0.05), whereas caCdc42 transduction of control DC did not significantly change dextran 40 uptake when compared with CD56-control-transduced DCs (Fig. 3C). To further evaluate this phenomenon, we count the number of DCs with internalized beads using confocal microscopy. The results demonstrated that activation of Cdc42 and Rac1 in DC did not significantly change the phagocytic activity of control DC, but it raised phagocytosis from 60% to 90% (p < 0.05) in those DCs that were inhibited by tumor cells (Fig. 3D). Therefore, these results directly demonstrate that Rac1 and Cdc42 play a crucial role in the down-regulation of DC endocytosis in the tumor microenvironment.

Tumor cells decreased the levels of active Cdc42 and Rac1 in DC

Because Rho GTPases are involved in endocytosis regulation (33, 34), we next tested whether Cdc42, Rac1, and RhoA were involved in the tumor-induced inhibition of DC function. To determine whether tumor cells alter the activity of small Rho GTPases in DCs, the levels of active Cdc42, Rac1, and RhoA in control and RM1-treated DCs were evaluated by a pull down assay. Active Cdc42 and Rac1 were isolated from the cell lysates by incubation with PAK-1 PBD and detected by Western blotting (Fig. 2). The bands corresponding to active small Rho GTPases on the Western blots were quantified by densitometry as relative intensity units (RIU) and presented as a ratio of RIU of active (PAK-1 PBD-agarose-bound) Cdc42 or Rac1 to the RIU of equal volume of total Cdc42 or Rac1 in DC lysates, respectively. Coincubation of DCs with tumor cells decreased the ratio of active to total Cdc42 from 0.72 ± 0.09 in nontreated DCs to 0.39 ± 0.05 in RM1 cell-treated DCs (p < 0.05). The ratio of active to total Rac1 was decreased from 0.85 ± 0.12 to 0.48 ± 0.06 (p < 0.05). Active RhoA was isolated from DC lysates by incubation with Rhotekin RBD, detected by Western blot, and quantified by densitometry as well. The level of RIU of active RhoA in DC was not significantly changed by coincubation with tumor cells (p > 0.05, data not shown). Therefore, these data indicate that Cdc42 and Rac1 may be primarily involved in the tumor-induced dysregulation of endocytic function of DC.
Discussion

Altogether our results demonstrate that 1) tumor cells are able to down-regulate DC endocytosis and 2) this is mediated by dysregulating activation of small Rho GTPases in DCs. These data have supported the theory that small Rho GTPases have functional significance in the regulation of DC activity in cancer. Direct evidence of the involvement of small Rho GTPases in the modulation of DC activity in the tumor microenvironment has been obtained here with a genetic modification of control and tumor-treated DC using VV-based constructs encoding dn or ca forms of Cdc42 and Rac1. Transduction of DC with the dnCdc42 and dnRac1 genes led to reduced DC endocytic activity (Fig. 3A), which was similar to the tumor-induced inhibition of DC endocytosis (Figs. 1 and 3B), and was also associated with tumor-induced down-regulation of the levels of active Cdc42 and Rac1 in DCs (Fig. 2).

Several scenarios, such as altered differentiation and maturation, increased apoptosis, and inhibited Ag processing and presentation have been proposed for the disbalance of the DC system by tumor cells or specific tumor-derived factors (6–8, 36). We have shown here a new molecular pathway of DC inhibition by tumor cells: the down-regulation of endocytic activity through modulating small Rho GTPase activation. The endocytic activity of DCs was assessed by uptake of dextran 40, reflecting mostly receptor-mediated endocytosis and polystyrene beads reflecting phagocytosis. We revealed significant decrease in uptake of dextran 40 and polystyrene beads by DCs generated from tumor-bearing mice ex vivo, as well as by DCs generated in the presence of four different tumor cell lines, including RM1 prostate, MC38 colon, 3LL lung, and B7E3 oral squamous cell carcinomas in vitro. Interestingly, tumor-treated DCs remained at the immature stage, as assessed by the
expression of MHC and costimulatory molecules (32). Because the endocytic activity of DCs was suppressed despite their immaturity, it is reasonable to speculate that the immaturity of DCs in cancer may not be associated with the preservation of their functional activity. Therefore, our results suggest that tumor cells do not simply delay or prevent DC maturation but actively suppress DC function, e.g., endocytosis.

Although there are clear indications that small Rho GTPases are critical for various functions of DC, there is conflicting evidence regarding the specific roles of Rac, Cdc42, and Rho. Several lines of evidence indicate that the Rho family of small GTP-binding proteins, including RhoA, Rac1, and Cdc42, regulate the cytoskeleton architecture and coordinate various cellular functions in DC such as endocytosis, motility, adhesion, and chemotaxis (19–21, 27, 37). For instance, the treatment of immature spleen-derived DC (21) or bone marrow-derived DC (19) with toxin B, an inhibitor of all members of the Rho GTPase family, completely blocks the macropinocytotic uptake of fluorescent fluid-phase markers, which is a form of high-volume, nonspecific endocytosis involving the extension of membrane ruffles. The involvement of the specific Rho GTPase family members was investigated by expression of dominant inhibitory versions of the different Rho GTPases. For instance, administration of dnRac1 or dnCdc42 blocks macropinocytosis in immature DC (19, 21). Significantly, in mature DCs, which have down-regulated macropinocytosis, microinjection of activated Cdc42 recovers endocytosis indicating that the machinery for macropinocytosis controlled by Cdc42 remains intact after maturation. Importantly, Garret et al. (19) showed that the levels of active GTP-bound Cdc42 were markedly reduced in lysates from mature DCs compared with immature DCs. These results implicate both Cdc42 and Rac in regulating endocytosis in DCs and suggest that the shutdown in endocytosis seen during maturation is caused by inactivation of Cdc42 or Rac1 (20). However, the functional significance of these pathways in DCs that have been exposed to the tumor microenvironment has not been yet investigated. Our data demonstrate that crucial DC functions are not only regulated by RhoA, Rac1, and Cdc42 (27) but also are inhibited in tumor-treated DCs (6, 8, 32).

Using affinity-based isolation of active GTP-bound protein, we have revealed that tumor cells decreased the activity of Cdc42 and Rac1 by >40% in RM1-treated DC (Fig. 2). Furthermore, when active Rho GTPases in DC were blocked with VV-based constructs encoding dnCdc42 and dnRac1, uptake of fluorescent latex beads and dextran 40 by DCs was also reduced by 40% (Fig. 3A). Transduction of DC with the constitutively active form of Rac1 completely recovered dextran 40 uptake by DCs altered by tumor cell-conditioned media. These data also support our hypothesis that the Rho GTPase-mediated pathway of the regulation of functional activity of DCs in cancer and suggest that Rac1 and Cdc42 are involved in tumor-suppressed endocytosis of active DCs. Regulation of small Rho GTPases might serve as a new pharmacological tool for protection of DC vaccines from tumor-induced inhibition and functional failure.

Disclosures

The authors have no financial conflict of interest.

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