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Low-dose Chemotherapeutic Agents Regulate Small Rho GTPase Activity in Dendritic Cells

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Summary

Conventional chemotherapy targets dividing tumor cells and might support antitumor immunity by providing tumor antigens from dying tumor cells to antigen-presenting dendritic cells (DCs). Despite emerging evidence to suggest that phagocytosis of dying tumor cells by DCs requires membrane targeting of specific small Rho guanosine triphosphatases (GTPases), nothing is known with regard to the direct effect of chemotherapeutic agents on low molecular weight Rho GTPases in DCs. Prompted by a recent observation that low-dose chemotherapeutic drug paclitaxel could up-regulate DC maturation and function, here we studied putative regulatory roles for various chemotherapeutic agents in modulating small Rho GTPases in DC. Our results demonstrate that different classes of chemotherapeutic drugs at low nontoxic concentrations regulate activity of Rac, RhoA, and RhoE in murine DC, suggesting that small Rho GTPases might serve as new targets for modulating functional activity of DC vaccines or endogenous DCs in various immunotherapeutic or chemoimmunotherapeutic strategies.

Keywords

chemotherapeutic agents; dendritic cells; Rho GTPases

Chemotherapeutic drugs target dividing cells and induce growth arrest and apoptosis not only in tumor cells but also in nonmalignant cells, including the cells of the immune system. This usually results in acute and cumulative toxicities to normal tissues and limits the dose and duration of chemotherapy. The suppression of the immune system by conventional cytotoxic chemotherapy might support tumor escape mechanisms and promote the propagation of chemoresistant clones by maintaining a cytokine milieu that favors proliferation of tumor cells. Thus, chemotherapy has usually been regarded as unrelated or, more commonly, antagonistic to immunotherapy. However, recent findings suggest that several chemotherapeutic agents may improve the outcome of immunotherapy.[1,2] Cell death induced by chemotherapeutic drugs is often accompanied by tissue destruction and the release of a number of danger signals that lead to the activation of immune cells and may

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facilitate tumor antigen engulfing and processing by the professional antigen-presenting cells (APCs).[3] For instance, chemotherapy with taxol, an antimicrotubule mitotic inhibitor, in advanced ovarian cancer was shown to result in up-regulation of antitumor immunity that has been attributed to taxol-induced tumor apoptosis and release of tumor antigens.[4,5] Epirubicin and doxorubicin, anthracyclines inhibiting DNA and RNA synthesis, have been reported to induce necrosis in tumor cells and increase uptake of tumor cell components by dendritic cells (DCs).[6] Apoptotic death induced by antimetabolic drug 5-fluorouracil can induce class I major histocompatibility complex (MHC) cross-presentation of tumor-associated antigens to cytotoxic T lymphocytes (CTLs).[7] These and other results support the contention that tumor cell death induced by many cytotoxic drugs can be a priming event for antitumor immunity by making large amounts of tumor antigens accessible for APCs.

DCs are professional APCs specializing on the processing and presentation of antigens to T lymphocytes and, thus, playing a key role in inducing and maintaining antitumor immunity. Beyond their unique APC features, DCs display a multiplicity of effector functions that play critical roles in regulating both cell-mediated and humoral immune responses. In the line of the antitumor immune response, DCs are distinctive in engulfing tumor cells killed by immune cells or therapeutic intervention (eg, radiation or chemotherapy) and cross-presenting tumor antigens to CTLs. This makes DCs a perfect candidate for therapeutic cancer vaccines, which currently are widely tested in preclinical and clinical trials.[8,9]

Tumor cells undergoing cell death were established to be useful as a source of tumor antigens in immunizations, particularly in approaches based on DCs.[10,11] However, it is still unclear whether necrotic or apoptotic tumor cells represent the best supply of tumor antigens for loading into DCs for vaccination purposes and both cell deaths were claimed to be superior to each other or equal.[12-14] Furthermore, although DCs mediate antitumor immune responses by stimulating tumor-specific CTLs, the mechanisms of recognition of dying tumor cells and intracellular pathways responsible for endocytic activity of DCs in the tumor environment are still unclear. For instance, exposure of phosphatidylserine (PS) represents one of the key signals for triggering phagocytosis of both apoptotic and necrotic cells,[15,16] including chemotherapy-killed tumor cells, and serves as a powerful “eat-me” signal for engulfing dying tumor cells by DCs. Recently, anthracyclins, the chemotherapeutic agents that inhibit DNA and RNA synthesis and cause tumor cell death, have been reported to stimulate the translocation of calreticulin to the cell surface, which then acts as an “eat-me” signal for DCs, allowing them to phagocytose tumor cells and to prime tumor antigen-specific CTLs.[17] Furthermore, it was just revealed that the release of one of the recently defined damage-associated molecular pattern molecules, the high mobility group box 1 protein (HMGB1), by dying tumor cells and its interaction with Toll-like receptor (TLR) 4 on DCs are required for the efficient activation of tumor antigen-specific T-cell immunity.[18]

Although much attention has been focused on the participating ligands, receptors, and mechanisms of uptake, little is known of the disposition of signaling pathways in DCs that may direct DC behavior after engulfing dead or dying tumor cells.

Regulation of a wide variety of cellular activities, from endocytosis and cell motility to cytokinesis and intracellular trafficking, falls largely to the Rho family of guanosine

triphosphatases (GTPases). The low molecular weight Rho GTPases control DC adherence, antigen processing and presentation, migration, chemotaxis, and endocytosis.[19-22] Differential role of the small Rho GTPase family members, including RhoA and RhoB, Rac1/2/3, and Cdc42, in the capacity of DCs to phagocytose apoptotic cells (efferocytosis) and prime T cells via cross-presentation has been also reported.[23-26] Furthermore, cell stimulation with PS induces translocation and activation of Rac1 and promotes Cdc42 activation and phosphorylation of mitogen-activated protein kinase through Rac1/PS signaling,[27] confirming the earlier conclusion that PS is capable of signaling changes in the actin cytoskeleton and these changes require both Cdc42 and Rac1.[28]

Despite emerging evidence to suggest that efferocytosis of dying tumor cells by DCs requires membrane targeting of specific small Rho GTPases, nothing is known with regard to the direct effect of chemotherapeutic agents on low molecular weight Rho GTPases in DCs. We previously reported regulation of small G proteins in DCs in the tumor environment,[22] and modulation of DC maturation and function by low-dose chemotherapeutic drug paclitaxel.[29] Herein, we studied putative regulatory roles for various chemotherapeutic agents in modulating small Rho GTPases in DCs. Our results demonstrate that different classes of chemotherapeutic drugs at low nontoxic concentrations regulate activity of Rac, RhoA, and RhoE in murine DCs, suggesting that small Rho GTPases might serve as new targets for modulating functional activity of DC vaccines or endogenous DCs in various immunotherapeutic or chemoimmunotherapeutic strategies.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice, 6 to 8 weeks old, were obtained from Taconic and housed in transparent plastic cages under pathogen-free conditions, controlled temperature and humidity, and a 12-hour light-dark cycle, with sterile food and water ad libitum.

Generation of DCs

The generation of murine bone marrow-derived DCs was carried out as described.[21] Briefly, mouse hematopoietic progenitors were isolated from the bone marrow of femur and tibia and depleted of erythrocytes, T and B lymphocytes, and adherent cells. Nonadherent cells were resuspended (0.5×10^6/mL) in culture medium (Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum, 100 units/100 μg/mL penicillin/streptomycin, 1-mM sodium pyruvate, 2-mM L-glutamine, and 0.1-mM nonessential amino acids) containing granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) 4 and cultured for 6 to 7 days.

MTT Assay

Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is based on the ability of a mitochondrial dehydrogenase from viable cells to cleave yellow MTT and form dark blue impermeable formazan crystals. Murine 3LL lung cancer and RM1 prostate cancer tumor cells were plated in flat-bottom 96-well plates at 2×10^3 and 5×10^3/well and treated with a range of

concentrations of cytotoxic agents (0 to 100,000 nM) for 48 h. Then the cells were washed, resuspended in 100-μL medium, and treated with MTT reagent (5-mg/mL MTT in phosphate-buffered saline, 20 μL) for 3 hours before lysing with a detergent (200 μL DMSO, dimethyl sulfoxide). Absorbance was recorded at 570 nm. The results were expressed as effective dose 10% (ED_{10}) reflecting the highest concentrations of the cytotoxic agents that causes <10% inhibition of optical density 570 values, that is, cell activity.

Annexin V Binding Assay

To evaluate apoptosis in control and drug-treated DCs, the Annexin V binding assay was used. After coincubation with different concentrations of chemotherapeutic agents for 48 hours, DCs were washed and double stained with fluorescein isothiocyanate-conjugated Annexin V (PharMingen) and propidium iodide (PI, 10 μg/mL, Sigma). Cells undergoing early apoptosis were determined as the percentage of Annexin V-positive/PI-negative cells by FACScan with Cell Quest 1.0 Software package (Becton Dickinson). The results were expressed as ED_{10}, showing the highest concentrations of the chemotherapeutic agents that cause apoptosis in <10% of DCs.

Rac and RhoA Activity Assay

Activity of Rac1/2/3 and RhoA in control and cytotoxic drug-treated DCs was determined by G-LISA Rac and G-LISA RhoA Activation Assays (Cytoskeleton), respectively, according to the manufacturer's protocols. DC cultures were treated by indicated concentrations of chemotherapeutic agents for the first 72 hours, washed, resuspended in complete medium supplemented with GM-CSF and IL-4, and harvested on day 6. DC lysates were prepared, followed by assessing and equalizing protein concentrations. Active GTP-bound Rac or RhoA in cell lysates were detected with Rac-specific or RhoA-specific antibody, respectively, and the levels of Rac or RhoA activation in each sample were determined by comparing readings from activated cell lysates versus nonactivated cell lysates. The results were expressed as optical density at 490 nm.

Evaluation of RhoE

For Western blot studies, cells were lysed in lysis buffer (1% Triton ×100, 0.1% sodium dodecyl sulfate, 50-mM Tris-HCl, 150-mM NaCl, 10-μg/mL aprotinin, 40-μM leupeptin, 1-mM dithiothreitol, and 0.6-mM phenylmethylsulfonyl fluoride, pH 8) by incubating on ice for 30 minutes. Then, equal amounts of proteins (50 μg) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidine diflouride membranes, and blocked with 5% nonfat milk in TBS-Tween. After blocking, blots were incubated overnight with anti-RhoE antibody (1:500, Upstate). After washing in TBS-Tween, blots were incubated with horseradish peroxidase-conjugated antimouse immunoglobulin G antibodies (1:100,000; Pierce). β-actin served as a housekeeping control protein. All blots were developed using the enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech). Finally, membranes were scanned and each band was analyzed by densitometry using UN-SCAN-IT software package (Silk Scientific Inc). Expression of RhoE protein in each sample was evaluated as a ratio between the RhoE protein and corresponding β-actin densities.

Reagents

Murine recombinant GM-CSF and IL-4 were from PeproTech and used at 1000 units/mL to direct DC differentiation from bone marrow hematopoietic precursor cells. The cytotoxic agents paclitaxel, cisplatin, and taxotere were from Mayne Pharma and vinblastine, vincristine, 5-azacytidine, methotrexate, cyclophosphamide, mitomycin C, doxorubicin, carboplatin, flutamide, tamoxifen, and bleomycin were from Calbiochem. All other reagents were from Sigma.

Statistical Analysis

For a single comparison of 2 groups, the Student *t*-test was used after evaluation for normality. If data distribution was not normal, a Mann-Whitney rank sum test was performed. For the comparison of multiple groups, analysis of variance was applied. For all statistical analyses, $P<0.05$ was considered to be significant. Data are presented as mean ± SEM.

RESULTS

Establishing Low Nontoxic Concentrations of Chemotherapeutic Agents

We have recently reported that paclitaxel at low concentrations does not induce apoptosis of lung cancer cells, but abolishes the ability of tumor cells to inhibit DC function.[29] To extend these findings and reveal the mechanisms of this intriguing effect, here we first established the nontoxic concentrations, that is, those that cause <10% inhibition of metabolic activity of tumor cells in the MTT assay, of 13 common cytotoxic drugs by titrating down their inhibitory effect on 2 tumor cell lines (0 to 100,000 nM). Different classes of cytotoxic agents, including antimicrotubule agents, topoisomerase inhibitors, alkylating agents, antimetabolites, platinum agents, and other groups, were screened (Table 1). As can be seen, 5 out of 13 tested substances, including cyclophosphamide, cisplatin, carboplatin, flutamide, and tamoxifen, demonstrated no significant inhibitory effect on 3LL and RM1 tumor cells at the concentrations lower than 1000 nM. Other therapeutic agents displayed suitable dose-dependent inhibition of tumor cell proliferation and were titrated down to the concentrations that did not significantly affect tumor cell line viability in the MTT assay. For instance, vinblastine and vincristine showed the lowest ED_{10} values for the lung cancer cell line 3LL (<1 nM), whereas the ED_{10} values for other agents were ≤10 nM.

Next, the same agents were tested for their ability to induce apoptosis in DCs. Murine DCs were treated with a range of concentrations of chemotherapeutic drugs (0 to 5000 nM) and the levels of apoptosis was assessed by the Annexin V/PI binding assay (Table 1). The results revealed that the ED_{10} values of tested cytotoxic drugs for the tumor cell lines were similar or lower than ED_{10} values for DCs. Together, these data allowed establishing the concentrations of chemotherapeutic agents that are toxic for neither tumor cell lines nor DCs. These low concentrations of drugs were used to determine their effect on small Rho GTPases in DCs.

Regulation of Rac Activity in DCs by Chemotherapeutic Agents

Typical Rho GTPases act as molecular switches and alternate between their active GTP-bound form and the inactive guanosine diphosphate (GDP)-bound form, suggesting that assessing their levels in cells by Western blot does not reflect the state of their activity for many family members. To evaluate small Rho GTPases in DCs, we assessed membrane-bound and cytoplasmic levels of RhoG proteins, reflecting active and inactive pools, respectively, or used the pool-down assay earlier.[21,22] Here, to test whether chemotherapeutic agents might affect small Rho GTPases in DCs, we used novel Activation Assay kits, which allow screening of multiple effector molecules. The low nontoxic concentrations of cytotoxic agents were estimated in Table 1 and were used in some pilot studies. Furthermore, here we focused on the suboptimal concentrations, that is, the concentrations that were 5 times lower than those obtained above (Table 1) to ensure no, even minimal, toxicity for DCs. The results of the experiments characterizing Rac1/2/3 activity in DCs are summarized in Figure 1. As can be seen, 6 out of 13 tested drugs exhibited significant effects on Rac activation in DCs. This includes vincristine (200 pM), methotrexate (1 nM), cyclophosphamide (100 nM), paclitaxel (1 nM), mitomycin (10 nM), and doxorubicin (2 nM), which up-regulated Rac activity in DCs ($P<0.05$), and cisplatin (100 nM), which caused its down-regulation ($P<0.05$). The highest level of inhibition of Rac activity in DCs was shown for cisplatin (3.2-fold), whereas the strongest activator of Rac in DCs was cyclophosphamide, which increased the level of GTP-bound Rac in DCs up to 170% ($P<0.01$).

Alteration of RhoA Activation in DCs by Chemotherapeutic Agents

The effects of cytotoxic drugs at low concentrations on RhoA activity in DCs differed from the effects of the same drugs on Rac activation. As shown in Figure 2, vincristine (200 nM), 5-azacytidine (2 nM), doxorubicin (2 nM), flutamide (100 nM), and bleomycin (1 nM) up-regulated RhoA activity in DCs ($P<0.05$), with bleomycin having the highest potential (up to 2-fold). Vinblastine (0.2 nM), methotrexate (1 nM), mitomycin C (10 nM), and tamoxifen (100 nM) demonstrated statistically significant inhibition of active RhoA in DCs ($P<0.05$), with mitomycin C showing decrease to 35% of the control values ($P<0.01$). No statistically significant correlation between the effect of cytotoxic drugs on Rac and RhoA activity in DCs was seen, suggesting that tested agents might pursue different intracellular pathways for regulating small Rho GTPases in murine DCs.

Changing the Levels of RhoE Expression in DCs by Chemotherapeutic Agents

RhoE activity in the cell correlates with the level of its expression and, thus, could be assessed by Western blot.[30] Treatment of murine DCs with cytotoxic drugs at low concentrations resulted in marked alteration of RhoE expression and was drug dependent. Figure 3 demonstrates the results of 1 out of 3 experiments showing similar changes in RhoE expression in DC. As can be seen, paclitaxel (1 nM), 5-azacytidine (2 nM), cisplatin (100 nM), flutamide (100 nM), and bleomycin (1 nM) markedly increased the expression of RhoE, with 5-azacytidine displaying the highest level of RhoE activation up to 140% ($P<0.05$). Two cytotoxic drugs—mitomycin C (10 nM) and carboplatin (100 nM)—significantly down-regulated expression of RhoE protein in DCs ($P<0.5$). Carboplatin

decreased RhoE level by 2-folds ($P<0.01$). Interestingly, with the only exception, the effects of tested chemotherapeutic agents on RhoE activity were similar to their effects on RhoA activity, but did not correlate with the alterations of Rac activation (Table 2).

DISCUSSION

Small Rho GTPases are well characterized in eukaryotic cells where they regulate many aspects of cellular behavior through changes of the actin cytoskeleton. The majority of the Rho family proteins function as molecular switches cycling between the active, GTP-bound and the inactive, GDP-bound forms. When active, these proteins function to remodel the actin cytoskeleton by interacting with a number of downstream effector molecules necessary for cell adhesion, spreading, cell-cell contact, endocytosis, and motility. Early studies showed that Rho proteins regulate cell morphology and the actin cytoskeleton[31,32]; however, it is now clear that they also affect gene expression, cell proliferation, cell survival, and oncogenesis.[33] Unlike typical Rho family proteins, the Rnd subfamily members, including Rnd1, Rnd2, RhoE (also known as Rnd3), and RhoH, are GTPase deficient and are thus expected to be constitutively active. Rnd1 and RhoE expression in fibroblasts or epithelial cells induces loss of actin stress fibers and, therefore, they seem to function antagonistically to RhoA in actin cytoskeleton regulation. Recent data indicate that RhoE has 2 functions, first to regulate the actin cytoskeleton and second to influence cell cycle progression.[34] RhoE expression also increases cell migration speed and might induce signaling associated with inflammation.[35-37]

The key functions of DCs are associated with their motility, endocytosis and exocytosis, attachment to extracellular matrix, and cell-cell contacts. These functions are directly related to the actin cytoskeletal reorganization, and the important role of small Rho GTPases in DC functioning has been reported earlier.[20,21,38,39] Recently, the involvement of Rho GTPases in DC dysfunction in the tumor microenvironment has also been proven.[22] This suggests that small Rho GTPases in DCs might serve as novel targets for improving function and longevity of DC vaccines in cancer. However, there are no data on pharmacologic regulation of Rho proteins in DCs for therapeutic purposes. Here, we reported for the first time that different chemotherapeutic agents were able to modulate activity of small Rho GTPases in DCs at low concentrations, that is, at the concentrations that did not interfere with DC viability and longevity.

Traditionally, activity of the “classic” members of the Rho family of small GTPases, such as Rac1/2/3, RhoA/B, and Cdc42, has been assessed using a pull-down method, wherein the Rho–GTP-binding domain, Rac–GTP-binding domain, or Cdc42–GTP-binding domain of their effector protein is coupled to agarose beads, allowing affinity-based detection of the active form in biologic samples. We have used a pull-down method for characterization of Rho, Rac, and Cdc42 in DCs and their role in regulation of DC function in the tumor microenvironment.[21,22] However, this method suffers from several drawbacks, including being time consuming, requiring large amount of cellular protein, being limited in the number of samples, and yielding only semiquantitative results. Unfortunately, it cannot be used for screening purposes, such as analyzing the effect of different pharmacologic agents on small Rho GTPases in DC. We, thus, used a recently introduced G-LISA assay with a 96-

well plate format, which are available for RhoA and Rac, but not for Cdc42 analyses. RhoE activity was determined by Western blot method as its level in a cell directly correlates with its activity.[35]

We have reported here that different chemotherapeutic agents, used at low concentrations, were able to up-regulate or down-regulate the activity of Rac, RhoA, and RhoE in murine bone marrow-derived DCs in vitro (Table 2). Interestingly, from 13 tested drugs, only methotrexate and mitomycin C altered activity of all 3 Rho GTPases, whereas the rest of tested agents only affected either 1 or 2 of them. In general, no correlations between the effects of chemotherapeutic agents on different Rho proteins were seen with the only exception: in 4 out of 5 cases, the effect of cytotoxic drugs on RhoA and RhoE activity in DCs was similar. This is an unexpected observation as RhoE expression was shown to induce a decrease in both RhoA and Rac in other cell types[40] and increased expression of each Rnd protein induced loss of stress fibers acting antagonistically to RhoA.[35] In part, this is because of RhoE interaction with the RhoA effector ROCK I, a serine/threonine kinase that regulates the formation and contractility of stress fibers: RhoE binding inhibits ROCK I from phosphorylating its downstream target myosin light chain phosphatase, thus increasing the activity of the phosphatase to dephosphorylate myosin II, which results in reduced actomyosin contractility.[35,37] However, the function of RhoE in DCs and its interaction with the other members of the small Rho GTPase family in DCs is unknown. Interestingly, as has been reported for other cell types, RhoE might participate in the stimulation of the inflammatory response: RhoE expression was shown to induce a decrease in both RhoA and Rac, actin cytoskeleton disorganization, and also stimulate both the Interleukin-1 receptor associated kinase/extracellular signal regulated kinase/nuclear factor-κB pathway and the cyclooxygenase-2 expression associated with the inflammatory response.[40] In keratinocytes, RhoE serves as a prosurvival factor acting upstream of p38, Jun-N-terminal kinase, p21, and cyclin D1.[41] Interestingly, modulation of Rho GTPases in DCs by low concentrations of chemotherapeutics was accompanied by phenotypical changes of DCs including expression of MHC class II, CD40, and costimulatory molecules CD80 and CD86. Moreover, chemotherapeutic agents in low concentrations were able to alter the DC ability to present antigen to antigen-specific T cells. However, contribution of small Rho GTPases in modulating DC phenotype and function induced by chemotherapeutics is unknown and under investigation in our laboratory. Our preliminary results revealed that toxin B, an inhibitor of classic small Rho GTPases, blocked the ability of several cytotoxic drugs to regulate function of DCs. More studies and tools are needed to reveal the role of non-classic family member RhoE in DCs and its regulation by different pharmacologic agents for therapeutic purposes.

Furthermore, modulation of small Rho proteins by chemotherapeutic agents in immune cells has also not yet been investigated, and there are only a few reports demonstrating the effect of cytotoxic drugs on the Rho family members in other cell types. For instance, cisplatin, which induces DNA damage and pocket protein-dependent G_1 checkpoint arrest accompanied by a decrease in cyclin D1 expression, was shown to induce RhoE expression in fibroblasts.[34] Our results also demonstrated the up-regulation of RhoE in DCs by low concentrations of cisplatin (Fig. 3 and Table 2). In lymphoma cells, resistance to etoposide and doxorubicin-induced apoptosis might be mediated by RhoG nucleotide dissociation

inhibitor expression, which forms a complex with the GDP-bound form of the Rho family of monomeric G proteins and thus, may serve as a nodal point regulating the activation state of Rho GTPases. The mechanism for the antiapoptotic activity of RhoG nucleotide dissociation inhibitor may derive from its ability to inhibit caspase-mediated cleavage of Rac1 GTPase, which is required for maximal apoptosis to occur in response to cytotoxic drugs.[42] Recently, Rho GTPases have also been implicated in genotoxic stress induced by doxorubicin treatment in some tumor cell lines.[43] However, the precise role of Rho GTPases in cell sensitivity to cytotoxic agents is still poorly understood and more studies are required to understand the role of this pathway in regulating DC function and longevity in the tumor environment when chemotherapy is in use.

This is a particularly important issue because a combinatorial use of chemotherapeutic agents and DC vaccine for cancer treatment began to attract a lot of attention recently. Cyclophosphamide before DCs pulsed with tumor cell lysates or intralesional DCs augmented the antitumor effects of DC vaccine in melanoma and colon carcinoma models.[44,45] Combination of DCs pulsed with irradiated tumor cells and gemcitabine, a nucleoside antimetabolite, significantly increased survival of tumor-bearing mice in a murine pancreatic carcinoma model.[46] Similar increase in animal survival was reported for a combination of tumor antigen-transfected DCs and an alkylatinglike agent temozolomide in a murine glioma model.[47] Unfortunately, there are only a few reports on successful combination of chemotherapy and DC vaccines and the mechanisms of their synergy are mostly unknown. Doxorubicin, 5-fluorouracil, gemcitabine, and paclitaxel, but not mitomycin C, were shown to induce apoptosis of tumor cells resulting in increased immunogenicity of tumor cells.[48,49] It is also possible that potentiation of antitumor activity of DC vaccine by chemotherapy might be because of epitope spreading against tumor antigens[50] induced by the modulation of both tumor cells and DCs after chemotherapy.

Another immunologic mechanisms of chemotherapy may be linked to a recently described release of the nuclear chromatin protein HMGB1 from dying tumor cells treated with chemotherapy. Extracellular HMGB1 mediates a number of important functions including endothelial cell activation, stromagenesis, recruitment and activation of innate immune cells, and also DC maturation that, in the setting of cancer, leads to a chronic inflammatory response.[51,52] In its role as a damage-associated molecular pattern molecule, HMGB1 induces the release of proinflammatory cytokines from monocytes and DCs, such as IL-12, IL-6, IL-1α, and IL-8, and recruits and matures DCs, up-regulating CD40, CD54, CD80, CD83, and MHC class II molecules.[53] It seems that the release of HMGB1 by dying tumor cells is mandatory to license host DCs to process and present tumor antigens.[54] Several receptors have been identified for HMGBI including Receptor for Advanced Glycation Endoproducts, TLR 2, TLR 4, syndecan, and thrombomodulin.[53] On DCs, HMGB1 as an alarmin protein interacts with TLR4; DCs require signaling through TLR4 and its adaptor MyD88 for efficient processing of tumor antigen and cross-priming of antitumor T cells in vivo.[18]

Finally, up-regulation of function of DCs in a tumor-bearing host induced by low-dose chemotherapy may play an important role in elevating efficacy of other immunotherapeutic approaches, for instance associated with injection of tumor antigen(s) or immunostimulatory

cytokines. Such strategy could exploit the mutual effects of therapy to treat cancers owing to the different mechanisms of action of chemotherapy and immunotherapy.

A major limitation for combination chemoimmunotherapy is that the chemotherapeutic drugs induce apoptotic death of immune cells, including APCs, reducing the efficacy of a combination treatment significantly. Strategies that limit negative influence of chemotherapeutics on DCs would, therefore, enhance the efficacy of combined chemoimmunotherapy. One strategy to reach this goal has recently been reported, where pretreatment of DC vaccine with nitric oxide, before the intratumoral administration, protects them from chemotherapy-induced apoptosis.[55] Although protecting administered DCs, this approach, similar to other approaches with a conventional chemotherapy, does not limit the toxic effect of chemotherapy in vivo. We have recently introduced an alternative strategy and demonstrated that nontoxic low-dose chemotherapy before intralesion injection of DCs beneficially alters the tumor microenvironment, protects DCs from tumor-induced inhibition, up-regulates DC function, and significantly augments the antitumor potential of DC vaccine.[29] Our new data on modulation of Rho GTPase signaling in DCs by low-dose chemotherapeutics support this new approach and opens new opportunities in modulating DC function in the tumor environment.

Altogether, our findings suggest that small Rho GTPases may be involved in the regulation of DC activity by low concentrations of chemotherapeutic agents. These findings provide new insights into the molecular mechanism involved in recently described antitumor potential of low-dose chemotherapy combined with DC vaccines in the in vivo tumor models. Further evaluation of small molecule inhibitors that interfere with Rho GTPase activation in DCs can serve as useful tools for affecting Rho-mediated signal transduction cascades and may lead to the development of novel cancer therapeutics.

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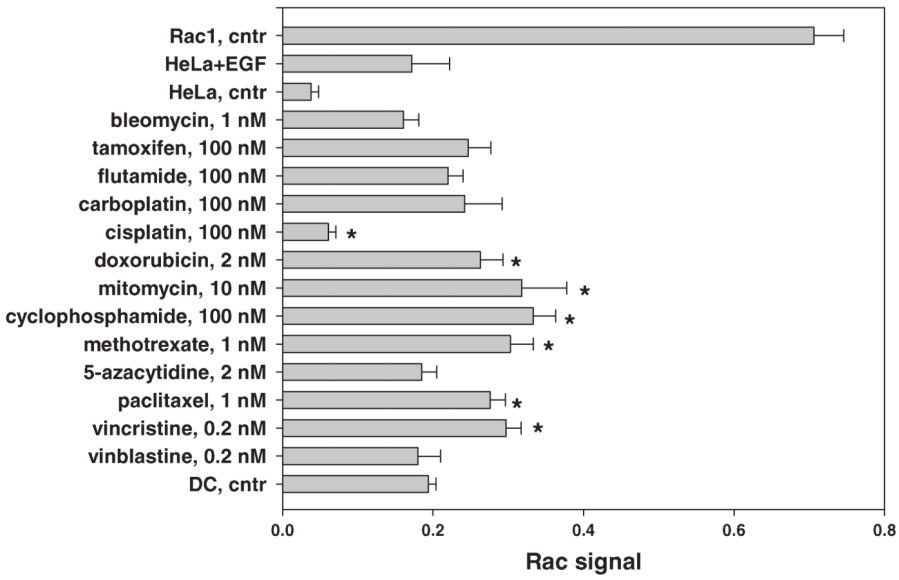

FIGURE 1.

Chemotherapeutic agents differentially regulate Rac activity in bone marrow-derived DCs. DCs were differentiated from the bone marrow hematopoietic precursors in cultures supplemented with GM-CSF and IL-4 and treated with indicated concentrations of different cytotoxic drugs for the first 72 hours. DCs were collected at day 6, washed, and lysed. Lysates were clarified by centrifugation and, after determination of protein concentration, tested in G-LISA Rac Activation Assay. DC, cntr indicates DCs treated with medium alone; HeLa cntr, HeLa cells used as a baseline control; HeLa+EGF, activated HeLa cells used as a positive control; Rac1, cntr, recombinant Rac1 protein. The results are represented as the mean ± SEM (N = 3). *P<0.05 (1-way ANOVA). ANOVA indicates analysis of variance; DCs, dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin.

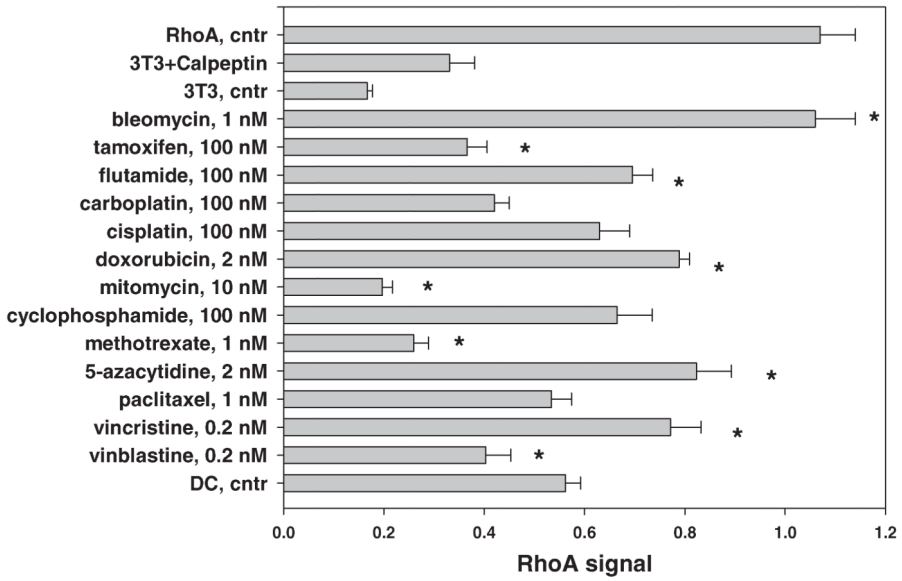

FIGURE 2.

Chemotherapeutic agents differentially regulate RhoA activity in DCs. DCs were differentiated from the bone marrow hematopoietic precursors in cultures supplemented with GM-CSF and IL-4 and treated with indicated concentrations of different cytotoxic drugs for the first 72 hours. DCs were collected at day 6, washed, and lysed. Lysates were clarified by centrifugation and, after determination of protein concentration, tested in G-LISA RhoA Activation Assay. DC, cntr indicates DCs treated with medium alone; RhoA, cntr, recombinant RhoA protein; 3T3 cntr, Swiss 3T3 mouse fibroblast cell line used as a baseline control; 3T3+Calpeptin, 3T3 cells treated with calpeptin (0.1 mg/mL, 30 min) served as a positive control.. The results are represented as the mean ± SEM (N = 3). *P<0.05 (1-way ANOVA). ANOVA indicates analysis of variance; DCs, dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin.

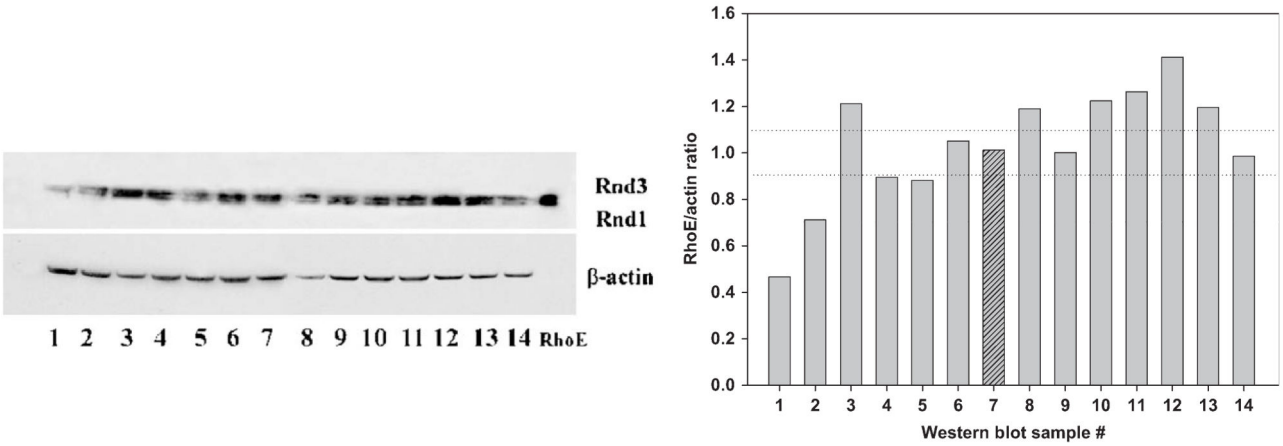

FIGURE 3.

Alteration of RhoE protein expression in DCs by chemotherapeutic agents. DCs were prepared and treated with cytotoxic drugs as described in Figure 1 and 2 legends. Western blot analysis of RhoE in DC lysates was carried out with specific antibodies recognizing both Rnd1 and Rnd3 (RhoE) proteins (left panel). β-actin served as a housekeeper control. RhoE lane corresponds to the control RhoE protein. Relative expression of RhoE was determine by densitometry and calculated as the ratios between the density of RhoE bands and the density of corresponding β-actin bands (right panel). The results of 1 representative experiment are shown; 3 independent experiments showed similar data. Lanes: 1, carboplatin; 2, mitomycin C; 3, methotrexate; 4, vinblastine; 5, vincristine; 6, doxorubicin; 7, control DCs (no treatments); 8, paclitaxel; 9, cyclophosphamide; 10, bleomycin; 11, 5-azacytidine; 12, flutamide; 13, cisplatin; and 14, tamoxifen. DCs indicates dendritic cells.

TABLE 1

Determination of the Highest Concentrations of Chemotherapeutic Agents That Does not Inhibit the Metabolic Activity of Tumor Cells and do not Induce APO of DCs

Chemotherapeutic Agents	ED_{10MTT}* 3LL	ED_{10MTT}* RM1	ED_{10APO}† DC (nM)
Antimicrotubule			
Vinblastine (velban)	≤1nM	≤5nM	≤5
Vincristine (oncovin)	≤1nM	≤1nM	≤5
Paclitaxel (taxol)	≤10nM	≤10nM	<50
Antimetabolites			
5-azacytidine (vidaza)	≤10nM	<50 nM	<50
Methotrexate (rheumatrex, trexall)	≤10nM	≤5nM	≤5
Alkylating agents			
Cyclophosphamide (cytoxan)	Resistant‡	Resistant	<500
Mitomycin C (mutamycin)	<50 nM	<50nM	<500
Topoisomerase inhibitors			
Doxorubicin (adriamycin)	≤10nM	≤10nM	≤10
Platinum agents			
Cisplatin (platinol)	Resistant	Resistant	<500
Carboplatin (paraplatin)	Resistant	Resistant	<500
Hormonal agents			
Flutamide (drogenil, eulexin)	Resistant	Resistant	<500
Tamoxifen (nolvadex)	Resistant	Resistant	<500
Others			
Bleomycin (blenoxane)	≤10nM	≤5nM	<50

APO indicates apoptosis; DC, dendritic cell; MTT, (3-(4,5-Dimmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay; PI, propidium iodide; RM1, murine prostate cancer cell line; 3LL indicates murine lung cancer cell line.

*ED_{10MTT}, Effective dose10; the concentration of a chemotherapeutic agent that causes <10% inhibition of tumor cell activity in the MTT assay.

†ED_{10APO}, The concentration of a chemotherapeutic agent causing <10% of apoptosis in dendritic cells by the Annexin/PI binding assay.

‡Cells are resistant to the treatment if corresponding ED_{10}* is more than 1000 nM.

TABLE 2

Summarized Data on the Effects of Low Concentrations of Chemotherapeutic Agents on Small Rho GTPase Activity in DCs In Vitro

Chemotherapeutic Agents		Rac	RhoA	RhoE
Antimicrotubule				
Vinblastine	0.2nM		↓	
Vincristine	0.2nM	↑	↑	
Paclitaxel	1 nM	↑		↑
Antimetabolites				
5-azacytidine	2 nM		↑	↑
Methotrexate	1 nM	↑	↓	↑
Alkylating agents				
Cyclophosphamide	100nM	↑ ↑		
Mitomycin C	10 nM	↑	↓ ↓	↓
Topoisomerase inhibitors				
Doxorubicin	2 nM	↑	↑	
Platinum agents				
Cisplatin	100nM	↓ ↓		↑
Carboplatin	100nM			↓ ↓
Hormonal agents				
Flutamide	100nM		↑	↑ ↑
Tamoxifen	100nM		↓	
Others				
Bleomycin	1 nM		↑ ↑	↑

DC indicates dendritic cell.