Protein Kinase C θ (PKCθ) Expression and Constitutive Activation in Gastrointestinal Stromal Tumors (GISTs)

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ABSTRACT

KIT expression is a key diagnostic feature of gastrointestinal stromal tumors (GISTs), and virtually all of the GISTs express oncogenic forms of the KIT or PDGFRA receptor tyrosine kinase proteins, which serve as therapeutic targets of imatinib mesylate (Gleevec; Novartis, Basel, Switzerland). However, KIT expression can be low in PDGFRA-mutant GISTs, increasing the likelihood of misdiagnosis as other types of sarcoma. We report that the signaling intermediate protein kinase C θ (PKCθ) is a diagnostic marker in GISTs, including those that lack KIT expression and/or contain PDGFRA mutations. PKCθ is strongly activated in most GISTs and hence may serve, along with KIT/PDGFRA, as a novel therapeutic target.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract (1). They are thought to originate from the interstitial cell of Cajal lineage, which forms part of the myenteric plexus in the gastrointestinal tract and regulates peristalsis (2, 3). Most GISTs contain oncogenic gain-of-function KIT mutations and feature strong expression of the constitutively activated KIT (CD117) receptor tyrosine kinase protein (4). Constitutively activated KIT has proven to be the most important therapeutic target in GISTs, with clinical responses and durable control of disease occurring in >80% of patients receiving the selective tyrosine kinase inhibitor imatinib mesylate (Gleevec; Novartis, Basel, Switzerland) for inoperable GIST (5, 6). However, a minority of GISTs lack KIT protein expression, and some KIT-negative GISTs contain gain-of-function PDGFRA mutations as an alternate oncogenic mechanism to KIT mutation (7–9). Notably, GISTs with PDGFRA mutations can respond to imatinib, emphasizing the need for diagnostic markers that distinguish KIT-negative GISTs from histologically similar tumors (9).

Protein kinase C θ (PKCθ) is a serine/threonine kinase that is transcriptionally up-regulated in GISTs compared with other soft tissue tumors (10, 11). PKCθ also is the PKC family member that is selectively expressed in the interstitial cell of Cajal lineage (12–14). The PKC family consists of at least 11 related protein kinases that can be divided into three subgroups based on their structural and biochemical properties. PKCθ, together with PKCδ, PKCe, PKCη, and PKCμ, belongs to the so-called novel PKC subgroup, which is characterized by non-calcium-dependent responsiveness to phorbol ester/diacylglycerol. In T lymphocytes, PKCθ is a key signaling molecule in T-cell receptor activation pathways, serving as a positive regulator of cell survival (15–18). PKCθ inhibition also results in p53-independent cell cycle arrest in various cell types, including mesenchymal (NIH-mouse fibroblast) cells (19). These findings suggest that PKCθ protein expression may be relevant diagnostically and therapeutically in GISTs (20). We report here that PKCθ protein is expressed strongly and is constitutively phosphorylated and enzymatically active in GISTs, irrespective of KIT expression and mutational status. By contrast, PKCθ protein expression is not detectable, or at most weak, in tumors that are histopathologic mimics of GIST. Our findings demonstrate that PKCθ is a diagnostic marker and has therapeutic promise for GIST.

MATERIALS AND METHODS

Study Group. Comparative PKCθ immunoblot analyses were performed in total cell lysates from 53 frozen tumor specimens, including GISTs (n = 20) and potential GIST mimics (n = 33). The GISTs included 15 cases that were KIT positive and 5 that were KIT negative by immunohistochemistry (IHC; DAKO, Carpinteria, CA). Each of the KIT-positive GISTs had genomic KIT mutations, which were located in exon 11 (n = 9), exon 9 (n = 4), exon 13 (n = 1), or exon 17 (n = 1). These KIT-positive and KIT-mutant GISTs were of various primary sites, including gastric (n = 9), small bowel (n = 5), and peritoneal (n = 1). Each of the KIT-negative GISTs had PDGFRA mutations, including three with exon 18 mutations encoding D842V substitutions and two with in-frame exon 12 deletions. Each of these KIT-negative and PDGFRA-mutant GISTs was gastric (n = 5). The diagnosis of GIST in the KIT-negative cases was based on tumor location, typical GIST histopathology, and CD34 immunoreactivity. The histologic GIST mimics, for the immunoblot studies, were leiomyosarcomas (n = 14), leiomyomas (n = 3), malignant peripheral nerve sheath tumors (n = 3), benign schwannomas (n = 2), synovial sarcomas (n = 2), desmoid tumors (n = 2), paragangliomas (n = 3), malignant melanomas (n = 2), and undifferentiated carcinomas (n = 2). In all, 28 of the 33 histologic mimics were from the gastrointestinal tract or from the abdominal or pelvic regions, where primary or metastatic GISTs most often are considered in a differential diagnosis. For the smooth muscle tumors, specifically, the primary sites were esophageal (n = 1), gastric (n = 1), retroperitoneal (n = 10), and pelvic (extraperitoneal; n = 5).

PKCθ immunohistochemical analyses were performed in 27 GISTs, which were predominantly KIT-negative cases, and in 40 histologic mimics of GIST, including leiomyosarcomas (n = 20), benign schwannomas (n = 10), and desmoid tumors (n = 10). The KIT-negative GISTs (n = 18) were gastric (n = 11), generalized intra-abdominal (n = 3), omental (n = 2), mesenteric (n = 1), and small bowel (n = 1). These tumors had PDGFRA exon 12 mutations (n = 2), PDGFRA exon 14 mutation (n = 1), PDGFRA exon 18 mutations (n = 10), KIT exon 11 mutation (n = 5), KIT exon 9 mutation (n = 1), or were KIT/PDGFRA-wild-type (n = 3). The KIT-positive GISTs (n = 9) were gastric (n = 3), small bowel (n = 3), omental (n = 1), retroperitoneal (n = 1), and liver metastasis with generalized intra-abdominal involvement (n = 1). The leiomyosarcomas were retroperitoneal (n = 14), uterine (n = 2), and other (n = 4). The desmoid tumors were small bowel, mesenteric, and retroperitoneal (1 of each) and other (n = 7). The schwannomas were retroperitoneal (n = 2) and other (n = 8).
Cell Lines and Reagents. GIST882 is a primary human GIST cell line with an activating homozygous missense mutation in KIT exon 13, encoding a K642E mutant KIT oncoprotein (21). The Jurkat T-cell acute lymphoblastic leukemia cell line was obtained from American Type Culture Collection (Manassas, VA). SARC152 is a primary human cell line established from a high-grade spindle cell sarcoma. Normal thymus protein lysate was from BD Biosciences Clontech (Palo Alto, CA), and normal rat cerebrum protein lysate was from BD Transduction Labs (Lexington, KY).

Antibodies. Antibodies for total PKC were goat polyclonal sc-1875 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal clone 27 (catalogue no. 610089; BD Biosciences PharMingen, San Diego, CA). Antibodies for phosphorylated PKC were polyclonal rabbit to phospho-Thr538 and phospho-Ser676 (Cell Signaling, Beverly, MA). Incubating Western blots done to validate specificity for phospho-PKC and phospho-Ser676 (Cell Signaling, Beverly, MA). Incubating Western blots PDGFRA Y754 (Santa Cruz Biotechnology), and phosphatidylinositol 3'-kinase (Upstate Biotechnology, Lake Placid, NY); and polyclonal goat to myelin basic protein (Santa Cruz Biotechnology).

Western Blot Analysis. Whole cell lysates of frozen tumors were prepared by mincing the specimens in ice-cold lysis buffer [1% NP40, 50 mM Tris HCl, 10 mM sodium citrate buffer (pH 8.0), 100 mM sodium fluoride, 30 mM sodium PPI, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium orthovanadate containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride, and additional sodium orthovanadate 2 mM, followed by homogenization with a Tissue Tearor (BioSpec, Bartlesville, OK) and clearing by centrifugation. Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Electrophoresis and immunoblot analysis were performed as described previously. Blot immunostains were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ), and chemiluminescence signals were captured and quantified using a FUJI LAS1000plus system with Science Lab Image Gauge 4.0 software (FujiFilm Medical Systems, Stamford, CT).

Immunohistochemistry. PKC IHC was performed using the mouse monoclonal antibody, clone 27 (catalogue no. 610089; BD Biosciences PharMingen), at a 1:100 dilution with 30 min microwave antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). The EnVision Plus system (2001 Image Gauge 4.0 software (FujiFilm Medical Systems, Stamford, CT)).

In Vitro Kinase Assay. Endogenous PKC was immunoprecipitated from lysates of serum-starved GIST882 (1 mg of protein) using a goat polyclonal anti-PKC (Santa Cruz Biotechnology) and protein A-Sepharose beads. After overnight incubation and washing in lysis buffer, immunoprecipitates were resuspended in kinase buffer containing 10 μM [γ-32P]-ATP (5 μCi per reaction) and myelin basic protein (1 μg per reaction) as substrate. Reactions were incubated for 30 min at 30°C with gentle shaking, then subjected to SDS-PAGE, transferred to nitrocellulose, and developed by autoradiography. The membrane subsequently was stained with polyclonal antibodies to PKC and myelin basic protein.

RESULTS AND DISCUSSION

PKC Is Strongly Expressed and Phosphorylated in GISTs, Regardless of KIT or PDGFRα Mutational Status. Immunoblot studies revealed PKC expression in each of 20 GISTs, irrespective of KIT (n = 15) or PDGFRα (n = 5) mutation type (Fig. 1). PKC expression was ∼50% lower in the PDGFRα-mutant GISTs than in most KIT-mutant GISTs (Fig. 1). The relative levels of PKC expression were identical when detected with a goat polyclonal antibody (Fig. 1) or with a monoclonal mouse antibody (data not shown). Phosphorylation of PKC T538 and S676, which is requisite for PKC activation, was demonstrated in each of the 20 primary GISTs (Fig. 1). PKC phosphorylation levels in KIT and PDGFRα mutant GISTs generally paralleled the expression of total PKC. Therefore, per molecule, phosphorylation of PKC was similar in all of the GISTs and was independent of the KIT and PDGFRα mutational mechanisms. PKC expression and phosphorylation were higher in the GISTs than in human thymus and Jurkat T-cell acute lymphoblastic leukemia cells (Fig. 2A). This finding is notable because high levels of PKC expression are restricted to few cell lineages, among which particularly high levels have been reported in thymus and Jurkat cells (22, 23). Similarly, PKC phosphorylation was substantially higher in GISTs than in normal thymus or Jurkat cells (Fig. 2A). PKC and PKC expression were evaluated in GISTs and cerebrum (positive control), given that these PKC isoforms could cross-react with the phospho-PKC antibodies. However, neither PKC nor PKC expression was assessed in GISTs (Fig. 2B), indicating that the phospho-PKC immunoblot stains were highly specific.

PKC activity was evaluated further by in vitro kinase assay in lysates from serum-starved GIST882 cells (Fig. 3). Substantial PKC activity as manifested by myelin basic protein phosphorylation was demonstrated in PKC immunoprecipitates from GIST882 but not in the non-GIST spindle cell sarcoma cell line (SAR152). These findings corroborate constitutive PKC activation in GIST.

PKC Expression in Histopathologic Mimics of GIST Is Weak to Non detectable. Immunoblot assays showed that none of 33 comparison tumors expressed PKC at levels comparable with those in GISTs (Fig. 4A). There was no demonstrable PKC expression in any of 14 leiomyosarcomas, 2 benign schwannomas, 3 paragangliomas, 2 monophasic synovial sarcomas, 2 desmoid tumors, or 2 undifferentiated carcinomas (Fig. 4A). There was weak PKC expression (at levels <5% of those in GISTs) in one of three leiomyomas, two of three malignant peripheral nerve
sheath tumors, and each of two malignant melanomas (Fig. 4, B and C). It is unclear whether the PKCθ expression in the five non-GIST primary tumors reflects weak, or focal, expression in the neoplastic cells, or perhaps expression in admixed reactive T lymphocytes. Identical results were obtained by immunoblot analysis with a mouse monoclonal antibody to PKCθ (data not shown). These studies demonstrate that PKCθ expression generally is at least 20-fold higher in GISTs than in histologic mimics. Importantly, PKCθ expression was not detected in any leiomyosarcomas, which are the most common differential diagnosis with malignant GIST.

The potential diagnostic relevance of PKCθ expression was evaluated further by IHC in conventional paraffin sections. IHC with the monoclonal PKCθ antibody demonstrated cytoplasmic staining in 20 of 27 GISTs (72%; Fig. 5A). PKCθ expression was shown in 13 of 18 (72%) KIT-negative GISTs, including 11 of 13 cases with PDGFRA mutations, and in 7 of 9 (77%) KIT-positive GISTs. IHC staining intensity varied markedly among the GISTs, in keeping with the...
PKCθ expression seen by immunoblot analysis, but PKCθ staining was diffuse in most of the positive cases. By contrast, there was IHC demonstration of PKCθ expression in only 2 of 20 leiomyosarcomas (one with moderate and diffuse staining and the other with weak and focal staining involving <1% of cells; Fig. 5B). One of 10 benign schwannomas also was positive for PKCθ IHC (weak cytoplasmic staining in <5% of cells). All of the 10 desmoid tumors were negative. Correlates with immunoblot analysis (not shown) revealed that IHC with the monoclonal PKCθ antibody was highly specific but less sensitive than immunoblot analysis. By contrast, IHC with the goat polyclonal PKCθ antibody (Santa Cruz Biotechnology; data not shown) was highly sensitive but not specific, being positive in several of the non-GIST tumors with no PKCθ expression by immunoblot analysis. These studies show that a strong PKCθ IHC result with the BD Biosciences Pharmingen monoclonal antibody may be useful to confirm a GIST diagnosis, whereas a negative result is uninformative. Because PKCθ expression can be demonstrated readily in most GISTs by immunoblot analysis, it is likely that more suitable PKCθ antibodies, with greater sensitivity and specificity in paraffin section IHC, can be developed.

In summary, we have shown that PCKθ is expressed strongly in GISTs but not in leiomyosarcoma or in other tumors that are histopathologically similar to GIST. Therefore, PKCθ joins the newly described protein DOG1 as a diagnostic marker of particular relevance in KIT-negative GISTs (24). PKCθ is expressed and phosphorylated in all of the GISTs, irrespective of their KIT or PDGFRα mutational status, demonstrating that PKCθ is a diagnostic marker and potential therapeutic target for GISTs. It is yet unclear whether PKCθ activity in GISTs depends on the constitutively activated KIT and PDGFRα signaling pathways in these tumors or whether PKCθ is activated by mechanisms independent of the KIT and PDGFRα oncogenic signals. However, there is reason to hypothesize that KIT and PDGFRα signaling may be relevant for PKCθ activation. In particular, KIT- and PDGFRα-mediated activation of phosphatidylinositol 3′-kinase and phospholipase C γ may generate the PKCθ cofactors phosphatidylycerine and diacylglycerol, respectively (18), and thereby modulate PKCθ function. Although selective inhibition of KIT and/or PDGFRα with agents such as imatinib mesylate has dramatically improved the outcomes of patients with GIST (6), resistance to single agent imatinib has been noted to develop over time (25). Other kinase inhibitors, such as the multitarget kinase inhibitor SU11248, have shown activity in patients with GIST resistant to imatinib (6). However, new therapeutic targets are needed to optimize the care of patients with this life-threatening disease. The data presented here suggest that PKCθ has many of the attributes that would characterize a GIST-specific target; therefore, it would be worthwhile to test drugs targeting this kinase in patients with imatinib-resistant GIST.

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