An Antibody Reactive with Domain 4 of the Platelet-derived Growth Factor β Receptor Allows BB Binding while Inhibiting Proliferation by Impairing Receptor Dimerization*

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A panel of murine monoclonal antibodies was generated against the extracellular domain of the human platelet-derived growth factor (PDGF) β receptor (PDGFRβ). These antibodies were assayed for both the ability to inhibit binding of PDGF BB to PDGFRβ* cells as well as the capacity to inhibit PDGF BB-mediated mitogenesis. As expected, all antibodies that could prevent PDGF BB binding also inhibited mitogenesis. However one antibody (M4TS.11), with no detectable ability to inhibit PDGF BB binding, was a potent inhibitor of proliferation induced by PDGF BB. Further characterization indicated that M4TS.11 impaired PDGFRβ dimerization, revealing the mechanism by which it prevented PDGF BB-mediated mitogenesis. Using domain deletion mutants of the extracellular portion of PDGFRβ, the determinant recognized by this antibody was localized to the fourth extracellular domain of PDGFRβ, indicating that this domain, which is not involved in ligand binding, actively participates in receptor dimerization and signal transduction. The M4TS.11 antibody could also inhibit PDGF BB-mediated proliferation of responsive cells from both the baboon and the rabbit, indicating the determinant recognized by the antibody is not limited to humans and making it possible to use this antibody to evaluate the therapeutic benefit of interfering with PDGF in animal models of human disease.

Platelet-derived growth factor (PDGF) is a mitogen and chemoattractant for cells of mesenchymal origin, such as fibroblasts, smooth muscle cells, and glial cells (1–3). PDGF is encoded by two genes, the products of which are designated A and B. The active PDGF molecule is a disulfide-linked dimer of these polypeptides and thus can exist in three forms: the homodimers AA or BB or the heterodimer AB (4).

Studies examining the interaction of PDGF with responsive cells have revealed the existence of two specific receptors designated α and β and encoded by separate genes. Each receptor type is composed of five extracellular immunoglobulin-like domains attached to an intracellular tyrosine kinase domain via a transmembrane segment (5). This structural organization is the prototype for the PDGF receptor family of protein-tyrosine kinases, which includes stem cell factor receptor, colony stimulating factor receptor, and Flk-2 (6).

The PDGF dimer stimulates responsive cells by cross-linking two receptor subunits (5, 6). The different forms of PDGF exhibit different affinities for the two forms of the PDGF receptor. The PDGFRα can interact with all three forms of PDGF; PDGFRβ can only interact with PDGF BB and AB. This pattern of reactivity dictates that PDGF AA can signal the cell only through homodimers of PDGFRα; PDGF BB can signal the cell through homodimers of PDGFRα or PDGFRβ or the heterodimer PDGFRαPDGFRβ, and PDGF AB can stimulate cells through either homodimers of PDGFRα or the PDGFRαPDGFRβ heterodimer (7).

As a potent mitogenic and chemotactic agent, PDGF has been implicated as a contributing factor in a number of pathologic conditions that involve the migration and proliferation of PDGF-responsive cells. Such conditions include arteriosclerosis (8), restenosis following coronary bypass surgery or balloon angioplasty (9), nephritis (10), scleroderma (11), and some neoplasias (12). Thus, interfering with the biologic activities of PDGF may be of therapeutic value for one or more of these conditions.

We have generated and characterized a panel of murine monoclonal antibodies against the extracellular portion of the PDGFRβ and examined the ability of these antibodies to inhibit PDGF BB-specific binding and/or induction of mitogenesis. One of these antibodies exhibits no effect on PDGF BB binding to the receptor but does inhibit PDGF-mediated mitogenesis by impairing receptor dimerization. This antibody cross-reacts with PDGFRβ from other species, making it an ideal candidate to study the therapeutic potential of an antibody PDGF antagonist in animal models of human disease.

EXPERIMENTAL PROCEDURES

Cell Lines and Cytokines—The Chinese hamster ovary cell line CHO/dhFr and the rabbit cornea cell line SIRC were obtained from the American Type Culture Collection. Baboon primary aortic smooth muscle cells (SMC) were kindly provided by J. Anderson and S. Hanson at Emory University (Atlanta, GA). Purified PDGF BB was obtained from Boehringer Mannheim.

Transfectants Producing Soluble and Membrane-bound PDGFRβ—A gene encoding the PDGFRβ lacking the nucleotides encoding a portion of the 5′ end, was obtained from the American Type Culture Collection. The missing portion of the gene, which included the secretion signal sequence, was constructed by oligonucleotide synthesis and used to assemble the complete PDGFRβ gene with an XbaI site at each end. This fragment was inserted into the XbaI site of the plasmid pVK (13) and co-transfected together with plasmid pVgl (13), which contains a dhfr gene, into CHO/dhFr− cells using the calcium phosphate method essentially as described (14). Methotrexate-resistant transfectants expressing the PDGFRβ were identified by indirect immunofluorescence using a commercially available antibody (Genzyme) and cloned by sin-
gle cell sorting using a FACStarPlus (Becton Dickinson). The resulting cell line (CHO C4) was responsive to PDGF BB as evidenced by increased DNA synthesis and cell proliferation in the presence of the growth factor as demonstrated previously (15).

To produce a soluble form of PDGFRβ (sPDGFRβ), the gene encoding the extracellular portion of the human PDGFRβ was cloned into the pBluescript plasmid and inserted into DH5α competent cells, and the recombinant plasmid was purified. The protein was purified by a combination of heparin-Sepharose and caprylic acid chromatography. The protein was >95% pure based on SDS-polyacrylamide gel electrophoresis analysis.

Monoclonal Antibodies—A panel of monoclonal antibodies was generated against the extracellular portion of the human PDGFRβ by immunizing outbred Swiss Webster mice with 50 μg of purified sPDGFRβ in Ribi adjuvant (Immunol Research Labs). The mice received booster immunizations of 50 μg of PDGFRβ every 1–2 weeks. Mice were bled 2 weeks after each boost, and the sera were tested with sPDGFRβ by ELISA. The mice exhibiting the highest serum titer of anti-PDGFRβ activity were sacrificed 3 days after receiving a final boost of 50 μg of sPDGFRβ, and hybridomas were prepared. Hybridoma supernatants were tested for reactivity with sPDGFRβ by an ELISA (see below). Hybridomas that exhibited reactivity were expanded and cloned. All monoclonal antibodies reactive with soluble PDGFRβ were assayed for reactivity against cell-surface-expressed PDGFRβ on the surface of CHO C4 transfectants by flow cytometry. Only antibodies demonstrating reactivity with cell-surface-expressed PDGFRβ were further characterized.

ELISA—Immunon 196-well plates (Dynatech Laboratories Inc.) were coated with sPDGFRβ by adding 100 μl of a 0.5 μg/ml solution of sPDGFRβ in phosphate-buffered saline to each well. After an overnight incubation at 4°C, 200 μl of phosphate-buffered saline plus 1% bovine serum albumin and 0.5%Tween 20 were added to each well to block unoccupied protein binding sites. After a 1-h incubation at room temperature, the wells were washed three times with phosphate-buffered saline plus 1% Tween 20. Dilutions of purified antibody or antibody-containing supernatants (100 μl) were added to each well, and the plates were incubated for 1 h at room temperature, after which they were washed three times as described above. One hundred μl of a 1 μg/ml solution of horseradish peroxidase-conjugated GAM IgG (Tago, Inc.) was added to each well, and the plates were incubated for 1 additional h at room temperature, after which they were washed three times. One hundred μl of peroxidase substrate (Bio-Rad) was added to each well, the plate was incubated for 15–60 min, and absorbance at 415 nm was determined.

Antibody Inhibition of Radiolabeled PDGF BB Binding to Cells—CHO C4 cells were harvested, washed twice with cold Dulbecco’s modified Eagle’s medium, and resuspended at 10⁶ cells/ml in Dulbecco’s modified Eagle’s medium. The assay was carried out in triplicate by incubating 100 μl of the cell suspension with either no antibody (to determine maximum binding), 5 μg of the indicated antibody, or 100 μg of cold PDGF BB (to saturate specific binding sites and determine nonspecific binding) in 12 × 75 mm polypropylene tubes for 15 min at 4°C. Each sample received 1.0 ng of ¹²⁵I-labeled PDGF BB (Amersham Life Science, Inc.) and was incubated for an additional 60 min at 4°C. Unbound ¹²⁵I-labeled PDGF BB was separated from bound to the cell by layering the sample over a mixture of 80% dibutylphthalate, 20% olive oil in a Beutnergelfix test tube (Sarstedt, Inc.) and microliter pipets to pellet the cells. The tubes were then incubated at room temperature. Varying concentrations of anti-PDGFRβ or control antibodies were added. Following a 2-h incubation with antibody, PDGF BB was added. The cells were incubated overnight, then 1 μCi of (¹⁰⁸)Hthymidine was added to each well. The cells were incubated for an additional 4 h then harvested using a PHD cell harvester to collect cells in a vial containing 5 μl of (¹⁰⁸)Hthymidine incorporation in each well was determined with a scintillation counter. The same assay was used to measure the inhibitory activity of the antibodies on baboon SMC and the rabbit cornea cell line SIRC.

Domain Deletion Mutants of PDGFRβ—Domain deletion mutants of PDGFRβ were prepared by cloning the PDGFRβ gene fragment (with various extracellular domains deleted) into the XhoI site of a version of pVig that had the IgG1 constant region replaced with the human lambda constant region cDNA. This allowed production of fusion proteins that contained varying numbers of the PDGFRβ extracellular domains with the human lambda light chain constant domain at the carboxyl terminus. The mutants included only the first, the first and second, the first through third, and the first through fourth extracellular domains of PDGFRβ fused to the human lambda light chain constant domain. The lambda constant domain served as a tag and allowed the deletion mutants to be captured onto the surface of an ELISA plate. The five-domain extracellular portion of the human PDGFRβ with no lambda constant domain (sPDGFRβ) served as the positive control. The domain deletion mutants were captured onto an ELISA plate using a goat anti-human lambda chain antiserum (Tago). Deletion mutant binding was confirmed using a horseradish peroxidase-conjugated anti-human lambda antiserum. The sPDGFRβ (domains 1–5) was directly coated onto the plate as described above. Reactivity of the antibodies with the domain deletion mutants was determined by incubating antibody with the plate-bound deletion mutants for 1 h, washing away unbound antibody, and developing the assay with a horseradish peroxidase-conjugated GAM IgG.

Analysis of PDGFRβ Dimerization Status—The dimerization status of PDGFRβ on the surface of CHO C4 cells exposed to PDGF BB was carried out in a manner similar to that described previously (17).

RESULTS

Monoclonal Antibodies against PDGFRβ Differ in the Ability to Prevent PDGF BB Binding and Proliferation—We generated a panel of monoclonal antibodies against the extracellular portion of the human PDGFRβ and assayed the antibodies both for the ability to inhibit radiolabeled PDGF BB binding to the receptor as well as the capacity to inhibit PDGF BB-mediated cell proliferation. The antibodies examined fell into three categories. Fig. 1 presents the results for a representative example of each category. Several antibodies, such as M4TS.15, had little or no effect on PDGF BB binding and likewise did not inhibit PDGF BB-mediated proliferation. Another class of antibodies, represented by M4TS.22, inhibited both PDGF BB binding to PDGFRβ and PDGF BB-mediated cell proliferation. A single antibody (M4TS.11) exhibited no inhibitory activity in the PDGF BB binding assay; however, it did display inhibition of PDGF BB-mediated proliferation. Despite the difference in the ability to inhibit binding of PDGF BB to cells expressing PDGFRβ, M4TS.11 and M4TS.22 were indistinguishable in their ability to inhibit PDGF BB-mediated proliferation (Fig. 1B).

M4TS.11 Prevents PDGF BB-mediated Proliferation by Impairing PDGFβ Dimerization—The easily detectable antibody of M4TS.11 to inhibit PDGF BB-mediated proliferation in the absence of the ability to block ligand interaction with receptor implied that the antibody was influencing an event post-ligand binding that was a prerequisite for the induction of the mitogenic signal. PDGFRβ is a receptor tyrosine kinase whose activation is dependent on ligand-mediated dimerization (5). To determine if M4TS.11 impaired the ability of PDGF BB to induce receptor dimerization, we examined the status of PDGFRβ on cells exposed to PDGF BB in the presence and absence of this antibody. CHO C4 cells were exposed to ¹²⁵I-labeled PDGF BB after a preincubation with no antibody or with M4TS.11 or M4TS.15. The cells were then treated with bis(sulfosuccinimidyl) suberate (Pierce) to covalently cross-link the ¹²⁵I-PDGF BB-PDGFRβ complex (17). These cells were...
lysed with Nonidet P-40, and lysate aliquots were subjected to SDS-polyacrylamide gel electrophoresis on a 6% gel. A 6% gel was selected to allow migration of all cross-linked complexes into the gel, and this was confirmed by the lack of radioactivity detected at the top of the gel as well as the ability to recover between 86 and 94% of the counts loaded from the gel lanes (data not shown). Density scans of autoradiographs of the gels indicated that radioactive PDGF BB predominantly migrated in the two areas of the gel that corresponded to the molecular weight of the 125I-labeled PDGF BB cross-linked to one (monomer) or two (dimer) PDGFRβ molecules (unbound 125I-labeled PDGF BB runs off the gel). Cells preincubated with M4TS.11 before exposure to 125I-labeled PDGF BB and cross-linking had approximately 50% that of the level of receptor dimer (with a corresponding 100% increase in receptor monomer) as compared with cells exposed to PDGF BB in the presence M4TS.15 or no antibody (Fig. 2).

**Mapping Antibody Reactivity Using PDGFRβ Domain Deletion Mutants**—To identify the portion of the PDGFRβ recognized by the M4TS antibodies, domain deletion mutants of the extracellular portion of PDGFRβ were constructed and expressed in a soluble form. The deletion mutants included the first, first and second, first through third, and first through fourth extracellular domains of PDGFRβ fused to the human lambda immunoglobulin constant domain (see "Experimental Procedures"). Each deletion mutant was tested for reactivity with M4TS.11, M4TS.15, or M4TS.22. This analysis revealed that M4TS.11 exhibited reactivity only when extracellular domain 4 of the PDGFRβ was present (Fig. 3). M4TS.15 required the presence of extracellular domain 2, whereas reactivity with M4TS.22 was dependent on the presence of extracellular domain 3 (Fig. 3).

**Reactivity of M4TS Antibodies with PDGFRβ from Various Species**—It was of interest to determine if the M4TS.11 and M4TS.22 antibodies could react with the PDGFRβ from species other than human and if they would function as a PDGF BB antagonist for these species. PDGFRβ expression has been reported for a variety of cell types derived from a number of mammalian species, and human PDGF BB can induce proliferation in these cells. Baboon SMC (18) and the rabbit SIRC line (19) express PDGFRβ and are responsive to human PDGF BB. Preliminary indirect immunofluorescence experiments revealed that both M4TS.11 and M4TS.22 could bind baboon SMC, but only M4TS.11 reacted with SIRC cells (data not shown). Fig. 4 demonstrates that preincubation with either M4TS.11 or M4TS.22 significantly decreased PDGF BB-mediated proliferation of the baboon SMC. M4TS.11, but not M4TS.22, exhibited a similar effect on the rabbit SIRC cell line

![Fig. 1. A, effect of anti-PDGFRβ antibodies on 125I-labeled PDGF (125I-PDGF) BB binding to PDGFRβ+ cells. CHO C4 cells expressing the human PDGFRβ were preincubated with 5 μg of the indicated antibody. Addition of 100 ng of unlabeled PDGF BB (Excess PDGF) served to indicate the level of nonspecific 125I-labeled PDGF BB binding. B, effect of anti-PDGFRβ antibodies on PDGF BB-mediated proliferation. CHO C4 cells were preincubated with various concentrations of M4TS.11 (●), M4TS.15 (▲), or M4TS.22 (■) for 2 h, after which PDGF BB was added. [3H]Thymidine incorporation was determined 20 h later. Values given in both panels are the average of triplicates with the standard deviation indicated.](image1)

![Fig. 2. The M4TS.11 antibody impairs PDGF BB-mediated receptor dimerization. 125I-Labeled PDGF BB was covalently cross-linked to PDGFRβ on CHO C4 cells in the presence of M4TS.11 (B) or M4TS.15 (C) or in the absence of antibody (A). Cell lysates were electrophoresed to separate complexes of 125I-PDGF BB bound to PDGFRβ monomers and PDGFRβ dimers, and the resulting gels were subjected to autoradiography. Each lane has been scaled individually to account for minor differences in the number of counts loaded. The area of migration of the dimer and monomer are labeled D and M on the plots, respectively, and the area integrated on each scan is indicated by the brackets. The 200,000-dalton marker migrated 67 mm into the gel.](image2)
those that had little or no effect on ligand binding or PDGF BB-induced proliferation, 2) those that prevented interaction of the ligand with the receptor and thus prevented ligand-induced proliferation, and 3) a single antibody, M4TS.11, that had no detectable effect on ligand binding but was a potent inhibitor of ligand-induced proliferation.

The ability of M4TS.11 to inhibit mitogenesis in the absence of any detectable effect on PDGF BB binding to the receptor implied that the antibody was interfering with an event required for triggering mitogenesis but distinct from ligand binding. Ligand-induced dimerization of transmembrane tyrosine kinases such as PDGFRβ is requisite for transmission of the mitogenic signal (6). Examination of the status of PDGFRβ on cells preincubated with M4TS.11 and then exposed to PDGF BB revealed a decrease in the level of receptor dimer and a corresponding increase in receptor monomer as compared with the levels observed in cells preincubated with a control antibody (Fig. 2). This indicates that M4TS.11 impairs the ability of PDGF BB to induce receptor dimerization, a characteristic that makes it a PDGF BB antagonist that is equivalent in potency to an antibody such as M4TS.22 that directly inhibits PDGF BB binding.

Deletion mutants allowed us to map the determinant recognized by M4TS.11, M4TS.15, and M4TS.22 to the fourth, second, and third extracellular Ig-like domains, respectively. Domain 3 is required for M4TS.22 binding, indicating that the antibody recognizes a determinant that either resides in domain 3 or is composed of portions of domain 3 and domain(s) 1 and/or 2. The first three domains of PDGFRβ are required to form the ligand binding site (20), thus M4TS.22 reacts with the receptor at a site near to, or possibly identical with, that part of PDGFRβ that binds PDGF BB. This is consistent with the observation that M4TS.22 can inhibit PDGF BB-induced proliferation by preventing the interaction of the ligand with the receptor. M4TS.15, an antibody that has no effect on PDGF BB binding or proliferation, reacts with each of the deletion mutants that contains domain 2. Thus, despite the involvement of domain 2 in forming the ligand binding site, the portion recognized by M4TS.15 is spatially distinct from that which interacts with PDGF BB. The requirement of domain 4 for reactivity with M4TS.11 indicates that the determinant recognized by this antibody is distinct from the ligand binding portion of the PDGFRβ formed by domains 1–3 and suggests that domain 4, despite being uninvolved in PDGF BB binding (20), does participate in transmitting the mitogenic signal to the cell. Each of these antibodies appears very different from the anti-PDGFRβ monoclonal antibody 2A1E2, which inhibits ligand binding and mitogenesis but reacts with the fifth extracellular domain of PDGFRβ (21).

The mechanism by which M4TS.11 inhibits PDGF BB-induced mitogenesis appears very similar to that of antibodies that have been described by Blechman et al. (17, 22) against the stem cell factor receptor (SCFR). The SCFR, which is the product of the c-kit proto-oncogene, is a receptor protein kinase that, like PDGFRβ, transmits a signal when dimerized by the divalent ligand, SCF (23). Antibodies that bind to the fourth Ig-like domain of SCFR inhibit SCF-induced dimerization but have no effect on SCF binding (17, 22). These findings, coupled with the inability of a domain-four deletion mutant of SCFR to dimerize in the presence of SCF, have led to the hypothesis that divalent ligand binding per se is insufficient for receptor dimerization but rather exposes an intrinsic dimerization site on the receptor that mediates subsequent dimerization and thus, signal transmission (17). The behavior of M4TS.11, which binds to the analogous domain four of the PDGFRβ, is consistent with the above hypothesis and supports the contention that the proposed mechanism may be a general feature of receptor tyrosine kinase signaling (17). In addition, the reactivity of M4TS.11 with PDGFRβ from a wide variety of species indicates that the determinant recognized by the antibody is evolutionarily conserved. This is a characteristic expected of a portion of the molecule essential for proper function.

The unique ability of M4TS.11 to inhibit PDGF BB-mediated proliferation while allowing the interaction of this ligand with PDGFRβ may make it an especially efficient PDGF BB antagonist in vivo, as local fluctuations in the concentration of PDGF BB should have no effect on the binding of antibody to the receptor. An antibody that inhibits PDGF BB-induced proliferation by blocking ligand binding could theoretically be displaced by high local concentrations of the ligand, possibly negating the inhibitory effect and making it a less effective antagonist than M4TS.11.

The involvement of PDGF in a variety of human disease conditions makes it an attractive target for therapy, and neutralizing the activity of PDGF with an antibody is a viable strategy. Advances over the past decade have made human treatment with monoclonal antibodies a feasible therapeutic approach. The major complication of the human anti-murine antibody response has largely been eliminated by “humanization” techniques, which transform a murine monoclonal antibody into a molecule that is indistinguishable from a human antibody.
immunoglobulin (24). The excellent safety profile and extended half-life of such antibodies (25) make them ideal candidates for applications such as interfering with the PDGF system. We have successfully humanized both the M4TS.11 and M4TS.22 antibodies and retained all the characteristics of the original murine versions, thereby making these antibodies excellent candidates for potential development as therapeutic agents.

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