Inhibition of Platelet-derived Growth Factor Autocrine Growth Stimulation by a Monoclonal Antibody to the Human α Platelet-derived Growth Factor Receptor

William I. LaRochelle,1 Roy A. Jensen,2 Mohammad A. Heidaran, Mary May-Siroff, Ling-Mei Wang, Stuart A. Aaronson, and Jacalyn H. Pierce

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892

Abstract

A potent neutralizing monoclonal antibody to the human α platelet-derived growth factor (PDGF) receptor (α PDGFR) was raised by immunizing BALB/c mice with 32D cells expressing the human α PDGFR. This monoclonal antibody, designated αR1, immunoprecipitated human, monkey, rabbit, pig, dog, and cat, but not hamster, rat, or mouse α PDGFRs. Comparison with PR292, a monoclonal antibody previously generated against the α PDGFR, showed that both recognized α PDGFR extracellular domains, but neither demonstrated reactivity against the β PDGFR. In vitro binding studies revealed that αR1, but not PR292, detection of the α PDGFR was blocked by either PDGF AA or PDGF BB. These results strongly suggest that the receptor ligand-binding domain spatially overlapped with the αR1 epitope. Monoclonal antibody αR1 also inhibited PDGFR stimulation of [3H]thymidine uptake by 32D cells expressing the α PDGFR (32D αR) as well as autocrine growth stimulation of 32D αR cells transfected with and expressing PDGF AA or PDGF BB. Therefore, monoclonal antibody αR1 may be useful in the detection and growth inhibition of malignancies in which PDGF autocrine stimulation and/or α PDGFR overexpression plays an important role(s).

Introduction

PDGF1 is a major serum mitogen for cells of mesenchymal origin such as glial cells, fibroblasts, and smooth muscle cells (1, 2). Biochemical evidence has established the existence in vivo of three PDGF isoforms: PDGF AA, PDGF BB, and PDGF AB (3). PDGF BB is the human homologue of the v-sis oncogene product (4–6). Cell responsiveness to PDGF depends on signal transduction through cell surface α or β PDGFRs (7–9). The α PDGFR is triggered by all PDGF isoforms, whereas the β PDGFR is activated by PDGF BB and, to a lesser extent, by PDGF AB (10–12).

In a previous study, we introduced expression vectors for the α and β PDGFR complementary DNAs into an IL-3-dependent murine hematopoietic cell line, 32D, which did not normally express these genes (12). Our data revealed that each PDGFR, in the presence of the appropriate ligand, coupled with signal transduction pathways inherently present in these cells. Furthermore, activation of either receptor induced readily detectable mitogenic and chemotactic responses. PDGFR activation also resulted in tyrosine autophosphorylation of each receptor, tyrosine phosphorylation of a variety of endogenous substrates, inositol phospholipid metabolism, and intracellular Ca2+ mobilization.

Although the physiological role of PDGF is associated with wound healing and development, the interactions of PDGF with its cognate PDGFR have been implicated in neoplasia (13–15) and arteriosclerosis (16, 17). PDGF has also been identified in fibrotic diseases (18) such as scleroderma (19) and glomerulonephritis (20). In the present report, we describe an immunocchemical approach for development of a potent antagonist of PDGFR function. We generated a Mab designated αR1, directed against the human α PDGFR. Mab αR1 neutralized PDGFR by inhibiting ligand interaction with the α PDGFR. We also tested the potential of this Mab as a diagnostic tool for identification of α PDGFRs as well as its efficacy in blocking autocrine loops involving PDGF and the α PDGFR.

Results

Isolation of a Human α PDGFR Monoclonal Antibody and Immunocchemical Characterization of α PDGFR Immunocchemical Probes. In order to generate monoclonal antibodies to the human α PDGFR, BALB/c mice were immunized with mouse 32D cells transfected with the human α PDGFR (12). Nine of 12 mice demonstrated an immune response, as measured by ELISA and immunoprecipitation assay using baculovirus-expressed human α PDGFR (21). Mice with the highest titer were selected for fusion. An α PDGFR hybridoma was selected by ELISA utilizing human α PDGFR purified from a baculovirus expression system and confirmed by immunoprecipitation analysis. After cloning of the hybridoma, culture fluids were harvested, and immunoglobulin fractions were prepared by Protein A-Sepharose chromatography.

As shown in Fig. 1A, α PDGFR Mab 1 (αR1) readily immunoprecipitated the baculovirus recombinant human α PDGFR, which was recognized to a lesser extent by PR292, a commercially available Mab. The specificity of its immunoreactivity was confirmed using antisera to α PDGFR peptides 959–973 (anti-PR1) or 1074–1089 (anti-PR2). Under the same conditions, neither control Mab MOPC21 nor normal rabbit sera immunoprecipi-
tated the α PDGFR. Moreover, Mab αR1 failed to recognize the human β PDGFR (data not shown), further demonstrating its α PDGFR specificity and the lack of epitope conservation between these two related receptor molecules. Since baculovirus-expressed proteins are not terminally glycosylated (21), Mab αR1 recognition of the α PDGFR appears to be independent of its terminal glycosylation.

In order to more precisely characterize the relative affinity of αR1 and PR292, these Mabs were analyzed by ELISA using purified recombinant human α PDGFR (Fig. 1B). Mab αR1 demonstrated saturable binding to the α PDGFR and possessed a half-maximal binding affinity of 50 pm. Mab PR292 bound with a half-maximal binding affinity of greater than 200 pm. Neither Mab bound the β PDGFR in the solid phase ELISA, confirming the results of immunoprecipitation studies (data not shown).

**Immunoochemical Interspecies Recognition and Human Tumor Expression of the α PDGFR.** We next tested whether the Mab αR1 epitope was conserved across mammalian species. As shown in Fig. 2, Mab αR1 recognized human, monkey, pig, dog, cat, and rabbit α PDGFRs to a similar extent as anti-PR2. However, Mab αR1 failed to immunoprecipitate hamster, rat, or mouse α PDGFRs, which were readily detectable by anti-PR2. Anti-PR1 also immunoprecipitated α PDGFRs in the entire panel of cell lines (data not shown). These results indicated that Mab αR1 recognized an epitope of the α PDGFR at least partially distinct from that detected by anti-PR1 or anti-PR2, and one that is not conserved between human and rodent species.

We also tested whether Mab αR1 recognized α PDGFRs expressed by selected human tumor cell lines previously shown to possess these receptors (13, 14). As demonstrated in Fig. 3, Mab αR1 immunoprecipitated the α PDGFR from whole cell lysates of synovial cell sarcoma-derived A3243 cells. The α PDGFR was also immunoprecipitated from astrocytoma-derived A204 or A1235 cells. Mab αR1 also recognized α PDGFRs expressed by human embryonic lung fibroblasts, but not by mouse NIH 3T3 fibroblasts, as previously observed. As an additional control, MOPC21 immunoprecipitated no α PDGFRs.

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**Fig. 1.** Characterization of immunoochemical probes for the human α PDGFR. Murine monoclonal antibodies αR1 (Lane 1), PR292 (Lane 2), MOPC21 (Lane 3), rabbit anti-PR1 peptide serum (Lane 4), anti-PR2 peptide serum (Lane 5), and normal rabbit serum (Lane 6) were used to immunoprecipitate purified tyrosine-phosphorylated human α PDGFR expressed in baculovirus. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine Mab (A). In B, Mabs αR1 (A), PR292 (B), and MOPC21 (C) were titrated using a solid phase ELISA against purified human α PDGFR expressed in baculovirus.

**Fig. 2.** Interspecies immunoreactivity of human α PDGFR immunoochemical probes. Monoclonal antibody αR1 (Lane 1), MOPC21 (Lane 2), and anti-PR2 peptide serum (Lane 3) were utilized to immunoprecipitate cell lysates from PDGF BB-stimulated human, monkey, pig, dog, cat, rabbit, hamster, rat, and mouse fibroblasts, as described “Materials and Methods.” Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine Mab.
Mapping of Monoclonal Antibody Epitopes to the α PDGFR Extracellular Domain. In order to test whether Mab αR1 recognized an extracellular or intracellular epitope, we performed flow cytometry on intact 32D cell transfectants expressing the α PDGFR. These cells have been shown to orient PDGFRs in a normal fashion such that their ligand-binding domains are accessible at the cell surface (8, 12). As shown in Fig. 4, Mab αR1 specifically recognized α PDGFR-expressing 32D cells (32D αR) as indicated by a 10- to 100-fold increase in fluorescence intensity compared to an isotype-specific control monoclonal antibody. Although PR292 also specifically recognized 32D αR, neither Mab αR1 nor PR292 recognized untransfected 32D cells or 32D transfectants containing the β PDGFR (data not shown). Recognition of the α PDGFR extracellular domain was consistent with the possibility that Mab αR1 recognized an epitope residing in the ligand-binding domain of the receptor.

Some Mabs which bind extracellular receptor domains have been shown to aggregate or cap receptors and subsequently induce their internalization (22). Thus, we investigated whether Mab αR1 might cause down-regulation of α PDGFR receptors. Following Mab αR1 incubation with the 32D αR cells for 1 h at 4 or 37°C, there was no decrease in surface immunofluorescence (data not shown). These results support the concept that Mab αR1 recognizes an external domain epitope whose binding does not induce α PDGFR internalization.

Mab αR1 Potently Neutralizes PDGF and Its Epitope Overlaps with PDGF Binding Determinants. The effects of Mab αR1 on PDGF ligand binding and mitogenic stimulation were next investigated. To measure PDGF competition for Mab αR1 recognition of the receptor, a solid phase ELISA-type binding assay was utilized. Briefly, wells were coated with α PDGFRs, and the ability of PDGF AA or PDGF BB to block Mab αR1 detection of the α PDGFR was quantitated. As shown in Fig. 5, either PDGF AA or BB blocked α PDGFR detection by Mab αR1 in the range of 25 to 250 ng/ml but failed to block PR292, anti-PR1, or anti-PR2 recognition over the same concentration range. The similar efficiencies of PDGF AA or PDGF BB inhibition of Mab αR1 binding suggested that both α PDGFR ligand-binding domains spatially overlapped with the Mab αR1 epitope. Since both PDGFs blocked Mab αR1 binding to the α PDGFR, we next tested whether Mab αR1 inhibited PDGF AA or PDGF BB binding to the α PDGFR in 32D cells expressing the α PDGFR. As shown in Fig. 6, Mab αR1, but not control MOPC21, inhibited 125I-PDGF AA or 125I-PDGF BB binding half-maximally in the range of 500 to 800 pM.

We further tested whether Mab αR1 inhibited PDGF AA- or PDGF BB-induced DNA synthesis in 32D αR cells triggered with either ligand. As shown in Fig. 7, 100 ng/ml PDGF AA or PDGF BB were inhibited half-maximally at Mab αR1 concentrations of around 700 and 400 pM, respectively. At concentrations between 5 and 50 nM, Mab αR1 totally abolished [3H]thymidine uptake induced by either PDGF, but not by IL-3, further demonstrating the specificity of Mab αR1. Under the same conditions, a control monoclonal antibody showed no inhibition of PDGF AA- or PDGF BB-induced [3H]thymidine uptake, and Mab PR292 inhibited only weakly. Mab αR1 further failed to inhibit PDGF BB-induced DNA synthesis in 32D βR cells triggered with PDGF BB (data not shown). At concentrations 25- to 100-fold greater than those used here for Mab αR1, we observed that Mab PR292 neutralized PDGF AA- and PDGF BB-induced DNA synthesis 40 to 60% (data not shown). All of these results indicated that Mab αR1 is a potent and specific inhibitor of α PDGFR-mediated responses.

Mab αR1 Abrogates Autocrine Proliferation of 32D Cell Transfectants Coexpressing PDGF AA or PDGF BB and the α PDGFR. Supernatransfection of 32D αR with
expression vectors for PDGF AA or PDGF BB results in the creation of an autocrine loop. Such cell lines exhibit IL-3-independent proliferation in vitro and are malignant in vivo (23). Because these cell lines provide a model for the study of PDGF autocrine growth stimulation of the α PDGFR, we tested whether Mab αR1 was a specific antagonist of their proliferation. As shown in Table 1, Mab αR1 specifically inhibited the proliferation of 32D cell transfectants coexpressing the α PDGFR and either PDGF AA or PDGF BB by approximately 50%. The same antibody showed no inhibition of 32D cell transfectants coexpressing the β PDGFR and PDGF BB or 32D cells grown in the presence of exogenous IL-3 (Table 1). A control monoclonal antibody also showed no inhibition of PDGF AA or PDGF BB autocrine growth stimulation of 32D αR (data not shown). Thus, Mab αR1 was able to at least partially abrogate α PDGFR autocrine proliferation.

Discussion

In the present report, we describe a potent neutralizing monoclonal antibody directed toward the extracellular domain of the α PDGFR. Mab αR1 was demonstrated to exhibit broad species immunoreactivity for α PDGFRs in species as diverse as human and rabbit. Further characterization of the Mab αR1 epitope revealed that it was unique to the α PDGFR and not conserved in the β PDGFR. Biochemical analysis indicated that its binding was independent of terminal glycosylation. Other studies have revealed that Mab αR1 recognizes the α PDGFR by immunoblotting after nondenaturing but not denaturing PAGE.* Thus, the epitope appears to be conformational as opposed to linear in nature.

Efforts to elicit immune responses and generate monoclonal antibodies without time-consuming protein purification steps have taken advantage of rodent fibroblast transfectants expressing such proteins as v-fms or the colony-stimulating factor 1 receptor (22). We demonstrated here that 32D, a cell line capable of growth in suspension culture, is applicable to this approach. 32D cells expressing the α PDGFR developed high serum titers to the α PDGFR in the majority of immunized mice. We have also observed high serum titers to the β PDGFR after immunization with 32D β PDGFR transfectants.4 The fact that these cells grow in suspension allows their harvesting without the use of agents that might destroy a surface-exposed protein to which antibodies are to be elicited. Thus, this cell system may be generally applicable to the generation of monoclonal antibodies to foreign cell surface proteins introduced into 32D cells by transfection.

Characterization of Mab αR1 revealed that PDGF AA or BB effectively inhibited its in vitro interactions with the α PDGFR. Conversely, Mab αR1 inhibited exogenous PDGF AA- or BB-induced DNA synthesis by 32D αR transfectants. These findings are consistent with the conclusion that αR1 recognizes an epitope that spatially overlaps with the PDGFR-binding domain rather than indirectly interfering with PDGFR binding. Further support derives from another Mab, PR292, which also interacts with the α PDGFR extracellular domain but fails, over the concentration range used here, to inhibit PDGFR ligand binding. However, it is not possible to exclude that αR1 induces allosteric alterations that indirectly affect ligand binding. More detailed mapping of the Mab αR1 epitope combined with analysis of specific external domain deletion mutants and/or chimeras with the β PDGFR may help to resolve these possibilities.

Previous studies have demonstrated functional autocrine PDGFR stimulation in certain human tumor cell lines (13, 14). Moreover, amplification and/or overexpression of the α PDGFR gene has been documented in human malignancies including glioblastomas and astrocytomas (13–15). Mab αR1 was capable of significantly inhibiting the in vitro proliferation of 32D cells exhibiting an experimentally designed PDGF/α PDGFR autocrine transform-

* R. A. Jensen, W. J. LaRochelle, and J. H. Pierce, unpublished observations.
Fig. 7. Mab αR1 inhibition of PDGF-induced mitogenesis of 32D αR transfectants. Varying concentrations of Mab αR1 were tested for their ability to inhibit [3H]thymidine incorporation in 32D αR stimulated with PDGF AA (Δ), PDGF BB (A), or IL-3 (O). 32D αR transfectants were simultaneously exposed to varying doses of Mab αR1 in the presence of fixed amounts of each growth factor, as described in "Materials and Methods." Results are the mean of duplicate samples and are representative of three independent experiments.

Materials and Methods

Immunization and Monoclonal Antibody Preparation. The 32D cell line is an IL-3-dependent murine hematopoietic cell line. These cells are propagated in RPMI 1640 with 10% fetal calf serum and 5% WEHI-3B conditioned medium (as a source of IL-3). 32D cells do not normally express α PDGFR. We have previously expressed this receptor in 32D cells by transfection (12). Monoclonal antibodies were prepared by immunizing BALB/c mice i.p. with 106 32D cell transfectants expressing the human α PDGFR at 10-day intervals. Mouse sera were titered against baculovirus-expressed human α PDGFR by solid phase ELISA, and immunorecognition was confirmed by immunoprecipitation analysis. Spleens from mice producing the highest antibody titer against the α PDGFR were fused with Sp2/0 myeloma cells. Hybridomas secreting immunoglobulins directed against the α PDGFR were selected by ELISA and cloned by limiting dilution. Ascites fluid or hybridoma supernatant was purified by Protein A-Sepharose chromatography. Purified immunoglobulin was quantitated by absorbance at 280 nm assuming an extinction coefficient of 1.35. Isotype analysis demonstrated that Mab αR1 was an IgG2a. Mab PR292 was obtained from a commercial source (Genzyme).

PDGFR Immunoprecipitation and ELISA. Human α PDGFR, expressed in baculovirus and constitutively phosphorylated on tyrosine (21), was purified by immunoaffinity chromatography using an anti-phosphotyrosine agarose immunoaffinity column (Upstate Biotechnology). Briefly, cells were harvested by centrifugation from a 500-ml spinner culture. A crude membrane fraction was prepared and divided into four aliquots. Each aliquot was processed by solubilization in 10 mM Tris(pH 7.4)-50 mM NaCl-0.5 mM EDTA-0.2% Triton X-100, and the insoluble material was removed by low speed centrifugation. The supernatant was batch loaded onto an anti-phosphotyrosine agarose column overnight, washed extensively, and then eluted with 10 mM phenyl phosphosphate (Sigma). α PDGFR was purified to over 95% homogeneity, as assayed by silver staining and confirmed by immunoblotting.

For immunoprecipitation analysis, 50 ng of affinity-purified tyrosine-phosphorylated PDGF receptor were incubated for 4 h with 33 mM Mab αR1 in PBS-1% BSA-0.1% Triton X-100. One hundred μl of a 1:1 slurry of Protein A-Sepharose CL4B were added for 1 h and vigorously shaken. The immunocomplex was washed three times with PBS-0.1% Triton X-100. The pellet complex was solubilized in SDS running buffer-100 mM dithiothreitol and repelleted, and the supernatant was loaded on a 7% SDS-polyacrylamide gel. Phosphorylated α PDGFR was detected by Western blot analysis with an anti-phosphotyrosine Mab. For ELISA analysis, 20 ng of purified α PDGFR were added to each well of a Falcon 3912 flexible assay plate. The ELISA was performed essentially as described (24). Polyclonal antibodies to α PDGFR residues 959-973 (anti-PR1) or 1074-1089 (anti-PR2) were described previously (12, 21).

Species Recognition by PDGFR Immunochimical Probes. Cell lines used for immunoprecipitation analysis of the α PDGFR were D17 (dog), BHK (hamster), rab9 (rabbit), AG1523 (human), HFSSV (monkey), CV1 (rat), NIH 3T3 (mouse), cat (National Institute of General Medical Sciences Cell Repository no. GM06207), and pig fibroblasts (a kind gift of Dr. Timothy Fleming). Cells were starved 18 h in serum-free media (DMEM) and then triggered with 250 ng/ml PDGF BB. After harvesting by centrifugation, the cell pellet was solubilized in radioimmunoprecipitation assay buffer (20 mM Tris(pH 7.4)-1% Triton X-100-5 mM EDTA-50 mM NaCl-10 mM sodium pyrophosphate-1 mM sodium orthovanadate-1 mM phenylmethylsulfonyl fluoride-10 μg/ml each of leupeptin, pepstatin, and aprotinin), and the lysate was cleared by centrifugation. The supernatant was immunoprecipitated with 5 μg of Mab or 5 μl of peptide antisera as described above. After immunoprecipitation, proteins

<p>| Table 1: Monoclonal antibody αR1 inhibition of 32D PDGFR transfectants expressing PDGF AA or PDGF BB |</p>
<table>
<thead>
<tr>
<th>Cell line</th>
<th>[3H]Thymidine incorporation (cpm x 10^4)</th>
<th>Percentage inhibition</th>
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</thead>
<tbody>
<tr>
<td>32D αR + IL-3</td>
<td>244</td>
<td>245</td>
</tr>
<tr>
<td>32D αR + AA</td>
<td>315</td>
<td>146</td>
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<tr>
<td>32D αR + BB</td>
<td>126</td>
<td>64</td>
</tr>
<tr>
<td>32D βR + BB</td>
<td>431</td>
<td>435</td>
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</table>

Exponentially growing cell lines were incubated with 330 mM Mab αR1, and [3H]thymidine incorporation was measured after 2 days, as described in "Materials and Methods." Results represent the mean of duplicate samples and are representative of three independent experiments.
were separated using a 7% SDS-polyacrylamide gel, transferred to Immobilon-P, and probed with anti-phosphotyrosine Mab. Bound primary antibody was detected with 125I-Protein A. Tumor cell lysates were prepared and analyzed in the same manner, except that immunoprecipitated α PDGFR was immunoblotted with anti-PR2 followed by 125I-Protein A.

In Vitro and 32D αR Binding Assays. Affinity-purified α PDGFR (50 ng/well) was plated in a 96-well format as described for the solid phase ELISA. Plates were blocked with PBS-4% BSA-0.02% azide for 30 min. PDGF AA or PDGF BB was added at the indicated concentration in PBS-1% BSA-0.05% Tween 20–0.02% azide. Next, Mab αR1, PR292, or MOPC21 was used at 300 pm concentration for 18 h. Peptide antisera were added at a dilution of 1:100. After washing four times with PBS-0.05% Tween 20–0.02% azide, either rabbit anti-mouse or goat anti-rabbit conjugated to alkaline phosphatase was used to detect bound primary antibody. After a 2-h incubation, excess secondary antibody was removed by washing, and 1 mg/ml alkaline phosphatase substrate 304 (Sigma) was added to 100 nm bicarbonate buffer-1 mM MgCl2 (pH 9.8). Optical density was read at 405 nm. Maximal inhibition was defined as the difference between the optical density obtained with each antibody in the absence of PDGF competitor and that measured in the presence of excess PDGF (100 ng).

For inhibition of 125I-PDGFR AA or PDGF BB binding to 32DαR, transfectants were harvested by centrifugation, washed in DMEM, gently resuspended in HB binding buffer (DMEM-25 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (pH 7.4)-1 mg/ml BSA), and maintained at 37°C. Next, 1.2 × 103 32D αR cells, Mab αR1 at the indicated concentration, and 2 ng of iodinated PDGF AA or PDGF BB were incubated in HB buffer for 2 h at 16°C. After 1 h, the cell suspension was layered onto 300 μl of a chilled oil mix (n-butyl phthalate (Fischer)-bis(2-ethylhexyl)phthalate (Kodak), 1:5:1). Cells were centrifuged in an Eppendorf microfuge at 10,000 × g for 10 min at 4°C. The cell pellet was removed and counted in a Beckman 5500 gamma counter. Specific PDGF binding was calculated by subtraction of background binding, defined as counts remaining after incubation with 100-fold excess of unlabeled PDGF.

Mab αR1 Inhibition of 32D αR Growth Proliferation. 32D transfectants (2 × 105 cells/ml) expressing the α PDGFR (12) were washed in PBS and triggered overnight in RPMI 1640 medium with 10% FBS containing recombinant IL-3 (100 ng/ml), PDGF AA (100 ng/ml), or PDGF BB (100 ng/ml) and varying concentrations of Mab αR1 or control Mab. The next day, cells were incubated with [3H]thymidine (0.5 μCi) for 5 h, washed, and harvested onto glass filters with an automatic harvester (Skatron). Incorporated [3H]thymidine was counted in a Beckman 5500 scintillation counter. 32D αR cells treated with FBS alone incorporated less than 0.1% of the counts incorporated in the presence of IL-3.

We have previously generated 32D transfectants expressing the α PDGFR and either PDGF AA or PDGF BB, or the β PDGFR and PDGF BB (23). These dual transfectants did not require IL-3 for growth due to autocrine stimulation of their PDGF receptors by PDGF, and they were tumorigenic in nude mice. In order to determine whether Mab αR1 blocked autocrine stimulation, the different 32D transfectants were seeded at 5 × 104 cells/ml and RPMI 1640 medium containing 10% FBS with or without 330 nm Mab αR1 was added for 48 h. Next, the cells were treated with [3H]thymidine for 5 h and processed as described. A control IgG2a Mab (Caltag) showed no inhibition of PDGF autocrine growth stimulation.

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References


