Effects of PPAR and RXR ligands in semaphorin 6B gene expression of human MCF-7 breast cancer cells

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Abstract. This study tests the hypothesis that the activators of peroxisome proliferator-activated receptors (PPARs) and 9-cis-retinoic acid receptor (RXR) regulate human semaphorin 6B (Sema6B) gene expression. The human MCF-7 breast adenocarcinoma cell line was chosen because it expresses Sema6B at a high level. The Sema6B mRNA level was analyzed by RT-PCR and the semaphorin 6B protein content was determined using a polyclonal antibody that we have produced and characterized. Treatments with fenofibrate (a PPARα activator) and troglitazone (a PPARγ ligand) strongly decreased the Sema6B mRNA. The drop in Sema6B mRNA level and in protein content was more important when the treatment combined the action of fenofibrate or troglitazone and 9-cis-retinoic acid. On the other hand, no significant change was observed in the Sema6B mRNA and protein levels when the cells were exposed to the combined action of GW610742 (a PPARβ activator) and 9-cis-retinoic acid. These data suggest that PPARα/RXR and PPARγ/RXR heterodimers are involved in the regulation of Sema6B gene expression and open new perspectives concerning the participation of these nuclear receptors in cell recognition and migration.

Introduction

Semaphorins are members of a large, highly conserved family of signalling molecules that were identified initially for their role in axon guidance and neural network formation (1,2) and later recognized to control a broad range of functions, from cell migration and immune response to angiogenesis and cancer (3). So far, more than 20 semaphorins have been identified and divided into 8 classes. The first two classes are found in invertebrates, classes 3-7 are vertebrate semaphorins, while class 8 contains viral semaphorins (4). The semaphorins are secreted membrane-anchored or transmembrane proteins characterized by a conserved semaphorin domain which is approximately 500 amino acids long. In contrast to other semaphorins, class-6 semaphorins do not contain additional structural motifs such as immunoglobulin like domains (classes 2-4 and 7), thrombospondin repeats (class 5) and basic domains (class 3) (5). However their cytoplasmic domains contain several proline-rich potential SH3 domain binding sites and interact with several signalling proteins such as Abl (6) and enabled/vasodilator-stimulated phosphoprotein-like protein (6.7). Sema6B was also shown to bind the SH3 domain of the protooncogene, c-src (8).

It has been recently shown that the human Sema6B gene exhibits a responsive element for PPARs (9). The latter are members of the superfamily of nuclear hormone receptors and present three isotypes: PPARα, PPARβ (also called PPARδ, NUC-1, or FAAR), and PPARγ (10). PPARs are activated by their respective ligands, form heterodimers with the retinoid X receptor and bind to responsive elements of target genes (11). It has been reported that Sema6B mRNA level is decreased in human T98G glioblastoma cells by PPARα agonists (9). However, no indication concerning the effect of these activators at the protein level was given and the impact of the other PPAR isotypes was not investigated. The aim of the present study was precisely to complete these previous investigations. The MCF-7 breast adenocarcinoma cell line was chosen because it is a non-neural cell line allowing extension of our previous data; it expresses the Sema6B gene at a high level and exhibits in vitro invasive capacity. Our results demonstrate that the combined treatments of MCF-7 cells with fenofibrate (a potent PPARα activator) or troglitazone (a PPARγ agonist) and 9-cis-retinoic acid (the specific ligand of RXR) strongly decrease Sema6B gene expression at the transcriptional and translational levels. On the other hand, GW610742, an activator of PPARβ does not induce any significant change. The finding...
that PPARα or PPARγ and RXR activators down-regulate Sema6B gene, opens new perspectives for these nuclear receptors concerning their sphere of activity.

Materials and methods

Chemicals. Fenofibrate and 9-cis-retinoic acid were from Sigma Chemical Co. (St. Louis, MO, USA). Troglitazone was from Calbiochem-Novabiochem Corporation (San Diego, CA). GW610742 was a generous gift from GlaxoSmithKline (Stevenage, UK). Eagle minimum essential medium and Iscove's modified Dulbecco's medium, fetal bovine serum (FBS), primers for RT-PCR, dNTP, reverse transcriptase, Taq polymerase and RNase were purchased from Invitrogen (Cergy Pontoise, France). TNT-coupled wheat germ extract system for in vitro translation was provided by Promega Corp. (Madison, WI, USA). The cocktail of protease inhibitors (Complete) and the BM chemiluminescence kit were from Roche (Mannheim, Germany). The polyclonal antibody directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon International (Temecula, CA, USA) and goat anti-rabbit biotinylated IgG and streptavidin/ peroxidase mix were from Dako S.A. (Trappes, France). The Vector NovaRed substrate kit for peroxidase was obtained from Intercihim (Montluçon, France). All other products were of reagent grade.

Cell culture and treatments. T98-G (12) and U-87 MG (13) glioblastoma cells, SH-SY5Y (14) neuroblastoma cells, HepG2 hepatoblastoma cells (15) and THLE-5B liver cells (16), HT-29 (17) and HCT 116 (18) colorectal carcinoma cells, MRC-5 (19) and A-549 (20) lung cells, L-293 kidney cells (21), and MCF-7 breast adenocarcinoma cells (22) were grown in Eagle's minimum essential medium with 10% (v/v) FBS concentration was applied for the culture of Caco-2 colorectal adeno-carcinoma cells (17). Chronic myelogenous leukemia K-562 cells (23) were grown in Iscove's modified Dulbecco's medium, fetal bovine serum (FBS), primers for RT-PCR, dNTP, reverse transcriptase, Taq polymerase and RNase were purchased from Invitrogen (Cergy Pontoise, France). TNT-coupled wheat germ extract system for in vitro translation was provided by Promega Corp. (Madison, WI, USA). The cocktail of protease inhibitors (Complete) and the BM chemiluminescence kit were from Roche (Mannheim, Germany). The polyclonal antibody directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon International (Temecula, CA, USA) and goat anti-rabbit biotinylated IgG and streptavidin/ peroxidase mix were from Dako S.A. (Trappes, France). The Vector NovaRed substrate kit for peroxidase was obtained from Intercihim (Montluçon, France). All other products were of reagent grade.

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Real-time RT-PCR. Real-time PCR analyses were performed in a fluorescent iQ Cycler (Bio-Rad, Marnes-la-Coquette, France) with specific primers (Table I) yielding short PCR products suitable for SYBR Green detection. Samples in duplicate were processed in 96-well plates with the following conditions. Amplification reactions contained cDNA equivalent to 100 ng RNA, 300 nM of the sense and antisense primers in a volume of 20 μl using the iQ SYBR Green supermix (Bio-Rad). The thermal cycling conditions used were 95°C for 3 min to activate Taq polymerase, followed by 50 cycles of 10 sec 94°C, 60°C, 72°C and 95°C for 5 sec. At the end of each run, melting-curve profiles were produced (cooling the samples to 70°C and heating to 90°C at a ramp rate of 0.1°C/3 s with continuous measurement of fluorescence) to confirm amplification of specific transcripts.

Production and characterization of anti-semaphorin 6B antibodies. The polyclonal anti-semaphorin 6B antibodies were raised against the amino-acid sequence, NH2-FLEEFETYRPDRC-COOH, present in the extracellular part of the human transmembrane semaphorin 6B (25). The peptide sequence is conserved in rats (29) and humans but is specific to semaphorin 6B among semaphorins as determined from a preliminary experiment in which a peptide sequence was produced by Sigma Chemical Corp.

A chimeric protein containing the full length sequence of rat Sema6B (formerly R-SemaZ) C-termally tagged with a c-myc sequence was produced by in vitro transcription and translation. The latter were performed using a rat Sema6B-c-myc pcDNA1.1 plasmid (29), the TNT-coupled wheat germ extract system and L-[35S]-methionine. Translated products were either directly submitted to SDS-PAGE on 8% gel
followed by autoradiography or incubated with anti-Sema6B antibody (diluted 1:100) and the complexes were immunoprecipitated using protein A-Sepharose and then analyzed by SDS-PAGE and autoradiography.

The polyclonal antibodies produced were characterized by immunocytochemistry analysis performed as described by Plénat et al. (30). Frozen sections (5 μm) were incubated overnight at 4˚C with the rabbit anti-semaphorin 6B antibodies (diluted 1:1600), incubated with goat anti-rabbit biotinylated IgG solution (diluted 1:200) for 1 h at room temperature, and washed again. Sections were washed with 0.1 M potassium phosphate buffer containing 0.1% (v/v) Tween-20, incubated with streptavidin/peroxidase mix (diluted 1:200) for 1 h at room temperature. Sections were washed and peroxidase substrate (Vector NovaRed) was added for 5 min. Enzymic reaction was stopped by immersing the slides in bidistilled water. Negative controls were achieved with preimmune serum.

The characterization of the antibodies was also performed by immunoblotting with membrane-enriched fractions from MCF-7 cells. The latter were collected in TES buffer (20 mM Tris-base, pH 7.4, 1mM EDTA, pH 8.0, 150 mM NaCl, 0.25 M sucrose and a cocktail of protease inhibitors) and homogenized on ice with 30-40 cycles in Dounce homogenizer. The homogenate was centrifuged for 10 min at 500 x g at 4˚C. The resulting supernatant (crude cell extract) was spun for 90 min at 100000 x g at 4˚C. The supernatant following this spin represents the S100 cytoplasmic fraction. The membrane-enriched pellet after the foregoing centrifugation was solubilized for 1 h in solubilization buffer (10 mM Tris-base, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM DTT and 0.1% NP-40) and centrifuged at 13000 x g for 3 min at 4˚C to remove debris. Proteins from crude cell extracts, S100 and membrane fractions were submitted to Western blotting.

Western blot analysis. Concentrations of proteins from crude cell extracts, S100 or membrane fractions were determined (31). Protein samples were fractionated on 8% SDS-PAGE, transferred onto membrane filters. Immunoreactivity was detected using the produced anti-Sema6B antibody (diluted 1:500). GAPDH was used as an internal standard and detected by a specific antibody (diluted: 1/5000). Enhanced chemiluminescence was carried out according to the manufacturer's protocol.

Statistical analysis. Densitometric analyses of RT-PCR and Western blotting assays were carried out using a Gel Doc 2000 system. The data represent the mean ± SD of three independent experiments. The statistical significance of differences between data from RT-PCR and Western blotting analyses were assessed by Student's-t-test. The acceptable level of significance was P<0.05.

Results

Semaphorin 6B gene expression in different human cell lines. Thirteen well-characterized human cell lines were examined for Sema6B expression. Three independent cell cultures were carried out for total RNA extraction and semi-quantitative RT-PCR analysis with the specific primers for Sema6B and GAPDH genes. The ratios between the intensities of the Sema6B and GAPDH (used as internal standard) signals were determined. The bar graphs represent the mean ± SD from 3 independent experiments.

Table I. Sequences of primers used in RT-PCR analyses.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Techniques</th>
<th>Sequences (5'-3') sense</th>
<th>Sequences (5'-3') antisense</th>
<th>Refs.</th>
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<tr>
<td>Sema6B</td>
<td>Semi-quantitative RT-PCR</td>
<td>GGGATGCTCTTCCACAGCTACT</td>
<td>AGGTAGTTAACTCCATCGCAATC</td>
<td>(33)</td>
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<tr>
<td>GAPDH</td>
<td>Semi-quantitative RT-PCR</td>
<td>GTGTAAGGTCGGGCTCAACG</td>
<td>GGGATCAGGCTCCTGGGAAGG</td>
<td>(34)</td>
</tr>
<tr>
<td>CPT1α</td>
<td>Semi-quantitative RT-PCR</td>
<td>TGCTTTACAGGGCATAACTG</td>
<td>TGGAAATCGGGATCCCCAA</td>
<td>(35)</td>
</tr>
<tr>
<td>Sema6B</td>
<td>Real-time RT-PCR</td>
<td>TCAGGACACCCACTACCCCGTGAT</td>
<td>AATGGCAGAGCTCGGTAAACGTA</td>
<td>(33)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Real-time RT-PCR</td>
<td>ACCCACTCCTCCACCTTTGAC</td>
<td>TCCACACCCCTTTGCGCAGTAG</td>
<td>(34)</td>
</tr>
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Figure 1. Sema6B mRNA expression in different human cell lines. Total RNA was extracted from cells after 5 days of culture and submitted to semi-quantitative RT-PCR using specific primers for Sema6B and GAPDH genes. The ratios between the intensities of the Sema6B and GAPDH (used as internal standard) signals were determined. The bar graphs represent the mean ± SD from 3 independent experiments.

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The highest levels were expressed by MCF-7 breast adenocarcinoma cells and HepG2 hepatoblastoma cells. The Sema6B gene displayed average expression in HCT-116 colorectal carcinoma cells, A-549 lung cells and T98G glioblastoma cells. The lowest levels of Sema6B mRNA were found in L-293 kidney cells, chronic myelogenous leukemic K-562 cells, HT-29 and Caco-2 colorectal adenocarcinoma cells, SH-SY5Y neuroblastoma cells, MRC-5 lung cells, U-87 MG glioblastoma cells and THLE-5B liver cells. On the basis of these data, the MCF-7 cell line was selected for the following studies as it expresses the Sema6B gene at a high level, exhibits in vitro invasive capacity, and is frequently used as a model in studies dealing with semaphorins (32,33).

Effects of PPAR activators on Sema6B mRNA level. The effects of 500 μM fenofibrate, 10 μM troglitazone and 1 μM GW610742 on the Sema6B mRNA level were determined in MCF-7 cells by semi-quantitative RT-PCR. As shown in Fig. 2, a significant decrease in the Sema6B mRNA content of MCF-7 cells occurred within 6 h of fenofibrate treatment. A 4-fold decrease was noted after 24-h treatment. On the other hand, the CPT1α mRNA level was increased in fenofibrate-treated MCF-7 cells (Fig. 3), indicating that the fenofibrate treatment was efficient. A significant decrease was observed in Sema6B mRNA level when MCF-7 cells were treated with troglitazone for 24 h (Fig. 4). No significant change was noted in Sema6 mRNA content when cells were cultured with GW610742 for 24 h (data not shown).

Combined actions of PPAR and RXR agonists on Sema6B mRNA level. As PPARs heterodimerize with RXRs before binding to response elements of target genes, we investigated by semi-quantitative RT-PCR the combined effects of their activators on the Sema6B mRNA level of MCF-7 cells treated for 24 h. No significant change was noted in the mRNA level
when the cells were treated with 1 μM 9-cis-retinoic acid alone (Fig. 5). On the other hand, as expected, a marked decrease of Sema6B mRNA expression was observed upon treatment with 500 μM fenofibrate alone. The decrease was more important when MCF-7 cells were simultaneously treated with fenofibrate and 9-cis-retinoic acid (Fig. 5). The latter data were confirmed by real-time RT-PCR analyses (Fig. 6).

The combined action for 24 h of troglitazone and 9-cis-retinoic acid decreased the Sema6B mRNA level (Fig. 4) whilst that of GW610742 and 9-cis-retinoic acid did not induce any significant changes (data not shown).
Antibody specificity. Until now, the expression of the Sema6B protein has not been studied. As a first step in the expression analyses of this protein we produced a polyclonal antibody raised against a peptide located in the extracellular domain of the human semaphorin 6B. We verified that the signals obtained were specific for the Sema6B protein and not a consequence of a cross-reaction.

In vitro-translated rat Sema6B was used for immunoprecipitation assays, taking advantage of the human peptide sequence chosen for immunization being conserved in the corresponding rat sequence. Fig. 7 shows that the antibody recognized a protein band (lane 3) exhibiting the same apparent molecular weight as that obtained for in vitro-translated rat Sema6B (lane 1).

In immunocytochemical assays, a reactivity was detected in most MCF-7 cells (Fig. 8B). Higher magnifications reveal a strong membrane staining as expected for a transmembrane protein (Fig. 8C). Using the preimmune serum as a control, no signal was obtained (Fig. 8A).

Immunoblotting with crude extracts of MCF-7 cells, showed a protein band with an apparent molecular weight of approximately 110 kDa (Fig. 9). The latter is very close to the calculated value, 95 kDa. The difference is probably due to the fact that semaphorin 6B is a glycosylated protein. In addition, the antibody detected protein in the membrane fraction and not in the supernatant fraction (cytosolic fraction) confirming the immunocytochemical data.

Combined actions of PPAR and RXR agonists on Sema6B protein content. MCF-7 cells were simultaneously treated with 1 μM 9-cis-retinoic acid and 500 μM fenofibrate or 10 μM troglitazone or 1 μM GW610742 for 72 h. Our Western blot analyses revealed that the semaphorin 6B protein content was substantially decreased in MCF-7 cells treated with 9-cis-retinoic acid and fenofibrate (Fig. 10) or with 9-cis-retinoic acid and troglitazone (Fig. 11). On the other hand, the semaphorin 6B protein content was not significantly changed by the simultaneous treatment of MCF-7 cells with 9-cis-retinoic acid and GW610742 (data not shown).
Discussion

The involvement of semaphorins in cancer is suggested by several reports (34). The class-3 semaphorins, in particular semaphorin 3B and semaphorin 3F, function as potent inhibitors of tumor development in small cell lung carcinomas (32,33,35,36). These semaphorins modulate the adhesive and migratory properties of responsive malignant cells and function as inhibitors of angiogenesis. Their anti-angiogenic properties contribute to their anti-tumorigenic properties (37-39). In contrast with these inhibitory semaphorins, there is now some evidence indicating that semaphorins, such as semaphorin 3C (40-42), semaphorin 3E (43), semaphorin 4D (44), semaphorin 5A (45) and semaphorin 6D (6), may contribute to tumorogenesis or tumor progression.

To evaluate a possible role of Sema6B in the metastatic process, we analyzed its expression in different human cancer cell lines, some of which display in vitro invasive capacity. In a first step, semi-quantitative RT-PCR was used for a rough comparison of Sema6B gene expression levels. Despite the fact that the method used does not tell to what extent the gene is expressed in absolute terms, it does allow comparison of the levels of the Sema6B gene expression between the different cell lines examined. Our analysis showed that the highest levels of Sema6B mRNA levels were found in MCF-7 breast cells and HepG2 hepatoblastoma cells. The Sema6B mRNA levels noted in HCT 116 colorectal carcinoma cells, A-549 lung cells and T98-G glioblastoma cells were in an average range whereas the lowest levels were observed for the other cell lines examined.

Until now, the mechanisms involved in the regulation of the genes encoding the various semaphorins have not been investigated. We found that the Sema6B gene harbours a functional PPAR binding site in its promoter (9). Using a specific anti-Sema6B antibody that we have produced and characterized, the present study shows that fenofibrate induces a significant decrease in the semaphorin 6B protein content of MCF-7 breast adenocarcinoma cells. It extends at the functional level, our data show that the semaphorin 6B gene is expressed at a high level in human MCF-7 cells that exhibit in vitro invasive capacity. Its level is down-regulated by fenofibrate or troglitazone and 9-cis-retinoic acid, the latter being an anti-proliferative, differentiation-promoting agent. As semaphorin 6B is now emerging as a key regulator of tumor progression, our data suggest that PPARα/RXR and PPARγ/RXR heterodimers could play a role in this process by regulation of the Sema6B gene expression.

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