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TFPI or uPA–PAI-1 complex affect cell function through expression variation of type II very low density lipoprotein receptor

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ABSTRACT

Very low density lipoprotein receptors (VLDLR) including type I and type II are known to affect cell functions by binding to its extracellular ligands. However, the effect of these ligands on VLDLR expression remains elusive. Tissue factor pathway inhibitor (TFPI) and urokinase plasminogen activator and plasminogen activator inhibitor 1 (uPA–PAI-1) complex, two ligands of VLDLR, were used to examine their effects on VLDLR expression. TFPI treatment decreased type II VLDLR expression, inhibited cell proliferation and migration, and degradated β-catenin in SGC7901 cells. However, uPA–PAI-1 complex, increased type II VLDLR expression with promoted cell proliferation and migration and stabilization of β-catenin. These results indicated that extracellular ligands can change the expression of type II VLDLR to affect cell proliferation and migration.

1. Introduction

The very low density lipoprotein receptor (VLDLR) is a member of the low density lipoprotein receptor (LDLR) superfamily. Two forms of VLDLR, with (type I) and without (type II) the *O*-linked sugar domain, are generated through alternative splicing [1]. Type I VLDLR is mainly distributed in heart and skeletal muscles relating to fatty acid metabolism, whereas type II VLDLR is predominant in non-muscle tissue, including kidney, spleen, adrenal gland, lung, etc [1–3]. There are also reports demonstrating that VLDLR isoforms are expressed in various cancer tissues [4,5], suggesting that VLDLR is likely to be involved in the pathogenesis of cancers. A previous study has shown that transient overexpression of the type I VLDLR in COS-7 cells strongly inhibited cell growth in a ligand-independent manner, which has been demonstrated to be mediated by the *O*-linked sugar domain of type I VLDLR [6]. Our recent study has also shown that the stable transfection of SGC7901 cells with type II VLDLR recombinant DNA induced cell proliferation and migration, while type I VLDLR transfection decreased cell proliferation and migration [7]. Both the previous report and our recent study have demonstrated a ligand-independent effect of VLDLR.

Besides the receptor mediated ligand-independent effects, VLDLR can also exert various functions by binding to their ligands [8]. By using VLDLR ligands, the tissue factor pathway inhibitor (TFPI) [9,10] and the complex of urokinase-type plasminogen activator and plasminogen activator inhibitor 1 (uPA–PAI-1) [11,12], it has been reported that uPA–PAI-1 complex can promote cell proliferation and migration through VLDLR by activating extracellular signal-regulates kinase (ERK) pathway [11,12] and TFPI can inhibit cell proliferation through VLDLR by inducing *JADD45B* and p38 signal pathway [9,10]. These studies showed a ligand-dependent effect of VLDLR. Nevertheless, it is unclear whether these ligands exert their roles through altering the expression levels of these two subtypes of VLDLR.

In this study, the two VLDLR ligands (TFPI or uPA–PAI-1) and the human gastric adenocarcinoma cell line SGC7901 were employed to investigate the effect of these ligands on VLDLR subtypes expression and cell proliferation and migration. Our results showed that TFPI treatment decreased the expression of type II VLDLR without affecting type I VLDLR. However, uPA–PAI-1

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complex increased type II VLDLR and decreased type I VLDLR expression. Cell proliferation and migration were further found to be decreased by TFPI and increased by uPA–PAI-1 complex, respectively. Furthermore, a decrease of ERK1/2 phosphorylation and degradation of β-catenin was observed in SGC7901 cells upon TFPI treatment. The treatment of uPA–PAI-1 complex, however, increased the phosphorylation of ERK1/2 and stabilized β-catenin by reducing its phosphorylation. These results suggested that extracellular ligands can change the expression of type II VLDLR to affect cell proliferation and migration.

2. Materials and methods

2.1. Reagents

The uPA was from Chemicon International (Temecula, CA, USA). TFPI and PAI-1 were obtained from Calbiochem (San Diego, CA, USA). The uPA–PAI-1 complex (10 nM) was pre-formed by mixing uPA with PAI-1 at a 1:1 molar ratio at 37 °C for 20 min. (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) was purchased from Sigma (St. Louis, MO). IgG6A6 hybridoma cells were from ATCC.

2.2. Preparation of monoclonal antibodies against the C-terminal of VLDLR

IgG6A6 hybridoma cells, which were prepared as previously described [13], were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37 °C. BALB/c female mice were injected with 0.5 ml Freund's incomplete adjuvant. After a week, the mice were injected with 1×10^6 IgG6A6 cells suspended in 0.5 ml phosphate-buffered saline (PBS). The ascites were collected two weeks later and the antibodies were purified by protein A affinity chromatography.

2.3. Cell culture

SGC7901 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were incubated in serum-free medium for 12 h before the treatment with 1 μM TFPI or 10 nM uPA–PAI-1 complex for indicated times.

2.4. Western blotting analysis

The cell pellet was lysed in buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenylmethysulfonyl fluoride, 1 mg/L aprotinin and 0.02 mg/L leupeptin. Following cell protein quantitation, 60 μg of protein per sample were subjected to 7.5% SDS–PAGE. The proteins separated on the PAGE gel were transferred to a nitrocellulose membrane, which was blocked for 2 h at room temperature. The membrane was then incubated overnight at 4 °C with monoclonal antibodies against VLDLR, against β-actin (Santa Cruz Biotechnology, CA, USA) or polyclonal rabbit anti-human phosphop44/42 MAP kinase (Cell Signaling Technology, Beverly, MA, USA) and rabbit polyclonal anti-rat ERK2 (C-14) (Santa Cruz Biotechnology) or mouse monoclonal anti-human β-catenin and Rabbit anti-phospho-β-catenin (S33/S37) (R&D Systems, Minneapolis, MN, USA), followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) system and the level of protein expression determined using Image Quant TL software (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.5. Cell proliferation assays

The cells were seeded at a density of 5×10^3/well and cultured in RPMI 1640 containing 10% FBS for 24 h and then in serum-free RPMI 1640 for another 24 h. The cells were treated with 1 μM TFPI or 10 nM uPA–PAI-1 complex for 12, 24, 36 and 48 h and then incubated for 4 h with 5 mg/ml MTT. Each treatment was done with 10 wells in all assays. Dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals. The absorbance was measured with an ELISA-plate reader (Bio-Tek Instruments, Inc) at 490 nm.

2.6. Cell migration assays

The cells were preincubated with 1 μM TFPI in serum-free RPMI 1640 for 12 h at 37 °C or with 10 nM uPA–PAI-1 complex for 15 min and then transferred to the top chamber at a density of 10^6 cells/ml (100 μl). The bottom chamber contained RPMI 1640 supplemented with 10% FBS. The cells were allowed to migrate for 6 h at 37 °C. Non-migrating cells were removed from the top surfaces with a cotton swab. The membranes were fixed in 4% paraform and stained with hematoxylin. The cells penetrated to the bottom surface of each membrane were counted with random 10 fields on each microscope slide.

2.7. RT-PCR

Four micrograms of total RNA, isolated using Trizol solution, were applied to PCR reaction and cDNA was synthesized by reverse-transcription under the conditions as follows. The primers used were 5′-GGA TGA CAT CAA GGG CAT TCA G-3′ and 5′-GTC ACA GTC CGC CAA ATG AAC C-3′ for MMP-2, 5′-CAA GTG GGC TAC GTG ACC TAT GAC-3′ and 5′-CCC TTT CCT CCA GAA CAG AAT ACC-3′ for MMP-9, and 5′-TGA GAC CTT CAA CAC CCC AG-3′ and 5′-GCC ATC TCT TGC TCG AAG TC-3′ for β-actin. The thermal cycling conditions were 94 °C for 4 min and 94 °C, 30 s; 57 °C, 45 s and 72 °C, 45 s, for 35 cycles. The final cycle was extended at 72 °C for 10 min. PCR products identified by 2% agarose gel electrophoresis were analyzed using Image Quant TL software.

2.8. Statistical analysis

Experiments were repeated a minimum of three times unless otherwise noted. Data are expressed as the mean ± S.D. The significance was determined by one-way ANOVA for comparisons among multiple groups and student *t* test for comparisons between two groups with SPSS12.0. The statistical significance of $P < 0.05$ or 0.01 or 0.001 was indicated in the figure legends.

3. Results

3.1. Cell proliferation and migration

When treated with TFPI for 36 h, the cell proliferation was gradually decreased to 51 ± 6% of that of the control values (Fig. 1A). The cell migration was inhibited to 58 ± 7% of that of the control cells after 12 h of TFPI treatment (Fig. 1B). In comparison, the cell growth was increased to 173 ± 7% of that of the control after 36 h with uPA–PAI-1 complex incubation (Fig. 1A). The migration for the cells preincubated with uPA–PAI-1 complex for 15 min was increased to 301 ± 26% of that of the non-stimulating cells after 6 h (Fig. 1B).