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The Adhesion GPCR CD97/ADGRE5 inhibits apoptosis

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ABSTRACT

The Adhesion G protein-coupled receptor (GPCR) CD97/ADGRE5 is induced, upregulated, and/or biochemically modified in various malignancies, compared to the corresponding normal tissues. As tumor cells are generally more resistant to apoptosis, we here studied the ability of CD97 to regulate tumor cell survival under apoptotic conditions. Stable overexpression of wild-type CD97 reduced serum starvation- and staurosporine-induced intrinsic and tumor necrosis factor (TNF)/cycloheximide-induced extrinsic apoptosis, indicated by an increase in cell viability, a lower percentage of cells within the subG0/G1 phase, expressing annexin V, or having condensed nuclei, and a reduction of DNA laddering. Protection from cell death by CD97 was accompanied by an inhibition of caspase activation and modulation of anti- and pro-apoptotic members of the BCL-2 superfamily. shRNA-mediated knockdown of CD97 and, in part, truncation of the seven-span transmembrane (TM7) region of CD97 increased caspase-mediated apoptosis. Protection from apoptosis required not only the TM7 region but also cleavage of the receptor at its GPCR proteolysis site (GPS), whereas alternative splicing of its extracellular domain had no effect. Together, our data indicate a role of CD97 in tumor cell survival.

1. Introduction

CD97/ADGRE5 is a prototypic member of the Adhesion class of G protein-coupled receptors (Adhesion GPCRs), which plays essential roles in various developmental processes as well as in tumorigenesis (Yona et al., 2008; Langenhan et al., 2013; Hamann et al., 2015). Adhesion GPCRs are large molecules composed of an extended extracellular domain, containing numerous protein folds and a juxtamembranous GPCR autoproteolysis-inducing (GAIN) domain (Langenhan et al., 2013), a seven-span transmembrane (TM7) domain, and an intracellular domain. The GAIN domain facilitates autocatalytic cleavage at the GPCR proteolysis site (GPS) into an extracellular N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remain non-covalently associated, giving rise to the remarkable bipartite structure of Adhesion GPCRs (Lin et al., 2004; Araç et al., 2012). Cooperation and interaction of the NTF and the CTF are essential for receptor-mediated cellular functions (Prömel et al., 2012; Langenhan et al., 2013; Hsiao et al., 2014).

CD97 is a member of the epidermal growth factor (EGF)-TM7 subfamily of Adhesion GPCRs (ADGREs), which possess variable numbers of EGF-like domains at the end of the NTF. Due to alternative RNA splicing, isoforms with three (125), four (1235), and five (1–5) EGF-like domains are expressed, which facilitate interactions with the binding partners CD55 and chondroitin sulfate B (Hamann et al., 1996; Stacey et al., 2003). Two other binding partners, integrin α5β1 and CD90 (Thy1), bind to the GAIN domain within the NTF (Wang et al., 2005; Wandel et al., 2012).

CD97 is widely expressed in normal human tissues with high abundance in hematopoietic, epithelial, and muscle cells. In the corresponding malignant tumors, CD97 is found to be induced or

Abbreviations: Ab, antibody; AV, annexin V; CHX, cycloheximide; EGF, epidermal growth factor; GAIN, GPCR autoproteolysis-inducing; GPCR, G protein-coupled receptor; GPS, GPCR proteolysis site; mAb, monoclonal antibody; pAb, polyclonal antibody; PI, propidium iodide; SEM, standard error of the mean; shRNA, short hairpin RNA; TM2, two-span transmembrane; TM7, seven-span transmembrane; TNF, tumor necrosis factor.

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upregulated (Aust et al., 1997, 2002, 2006; Steinert et al., 2002; Wobus et al., 2004; Ward et al., 2013). In leio- and rhabdomyosarcoma, CD97 is in part N-glycosylated, whereas normal muscle cells express the "naked" CD97 protein core (Aust et al., 2006; Zyryanova et al., 2014), indicating tumor-specific post-translational modification of the receptor. Expression levels of CD97 correlate with dedifferentiation in thyroid carcinoma (Aust et al., 1997; Ward et al., 2013). In colorectal carcinoma, CD97 is overexpressed at the invasion front, and the presence of CD97-postive scattered tumor cells correlates with tumor stage (Steinert et al., 2002; Galle et al., 2006). In gall bladder carcinoma and glioblastoma, the level of CD97 expression is inversely related with overall survival of the patients (Wu et al., 2012; Safaee et al., 2013). Beside conventional clinical studies that specifically addressed the expression of CD97 during tumorigenesis and its prognostic relevance, the involvement of CD97 in cancer was confirmed in hypothesis-free clinical screenings. Gene expression studies and characterization of the leukemia cell surface proteome identified CD97 as a marker for minimal residual disease in acute lymphoblastic leukemia (ALL) (Coustan-Smith et al., 2011), accounted CD97 for the most informative differences between normal and malignant cells in this leukemia (Mirkowska et al., 2013), and identified CD97 as a leukemic stem cell marker in acute myeloid leukemia (AML) (Bonardi et al., 2013). Finally, CD97 is a direct target of tumor suppressor microRNA-126 in MDA-MB-231 cells (Lu et al., 2014).

The association of CD97 expression in human malignant tumors with important clinical parameters, such as tumor stage and disease outcome, raises questions about the molecular and cellular functions of CD97 in tumorigenesis. Data indicate that CD97 regulates tumor cell migration and invasion, although the exact molecular pathway is not known. CD97 expression levels correlated strongly with migration and invasion of colorectal carcinoma cell lines (Steinert et al., 2002). Moreover, CD97 overexpression enhanced random single tumor cell migration in vitro and supported local tumor growth in immunodeficient mice in vivo (Galle et al., 2006). In a mouse model of gastric cancer, CD97 promoted metastatic spread (Liu et al., 2012). In thyroid and prostate cancer cells, CD97 heterodomerized and amplified the lysophosphatidic acid receptor 1 (LPAR1) signaling to promote cell invasion and tumor progression in a Rho-dependent fashion (Ward et al., 2011, 2013). On the other hand, ectopic CD97 in fibrosarcoma cells enhanced TIMP-2 secretion, leading to reduced matrix metalloproteinase activity and impaired cell migration/invasion in vitro and lung macrometastasis in vivo (Hsiao et al., 2014).

Although inhibition of apoptosis is essential in tumorigenesis (Cotter, 2009), the effect of CD97 on this basic cellular process has not been studied yet. Here, we examined whether CD97 can regulate intrinsic apoptosis, triggered by the withdrawal of survival factors or cellular stress, and/or extrinsic apoptosis, triggered by tumor necrosis factor (TNF) receptor stimulation (Taylor et al., 2008). Applying CD97 overexpression and knockdown, we show that CD97 inhibits programmed cell death.

2. Materials and methods

2.1. Stable cell lines

Cell lines, which stably overexpress various CD97 forms or in which CD97 is downregulated by short hairpin RNA (shRNA), were generated by either plasmid transfection using lipofectamine (Life Technologies, Darmstadt, Germany) (Galle et al., 2006) or by viral transduction (Hsiao et al., 2014). HT1080 cells (human fibrosacoma, ATCC code: CCL-121) expressing full-length CD97 containing either three (125) or five (1–5) EGF-like domains, cleavage-deficient CD97 with a mutated GPS, or truncated CD97 with a TM2 only domain were generated as described (Galle et al., 2006; Hsiao et al., 2011).

Table 1
HT1080 cell lines used in this study.

Designation	Description
Plasmid overexpression	
Mock	Inverse CD97 cDNA
CD97(125)	Shortest CD97 isoform expressing EGF-like domains 1, 2, and 5
CD97(125TM2)	Shortest CD97 isoform with truncated TM region
Retroviral overexpression	
Mock	Empty vector
CD97(125)	Shortest CD97 isoform expressing EGF-like domains 1, 2, and 5
CD97(1–5)	Longest CD97 isoform expressing EGF-like domains 1–5
CD97(125GPS)	Shortest CD97 isoform with mutated, cleavage-resistant GPS
CD97(1–5GPS)	Longest CD97 isoform with mutated, cleavage-resistant GPS
Lentiviral shRNA knockdown	
scr shRNA	Scrambled CD97 shRNA
CD97 shRNA1	CD97 shRNA cell line 1
CD97 shRNA2	CD97 shRNA cell line 2

GPS, GPCR proteolysis site; TM, transmembrane; shRNA, short hairpin RNA.

Table 1 and Fig. 1A provide details on the various cell lines used in this study. CD97 surface expression was quantified using flow cytometry. Cells were blocked with phosphate-buffered saline (PBS)/5% goat serum/2% bovine serum albumin for 30 min, stained with the monoclonal antibody (mAb) CLB-CD97/3 (Kwakkenbos et al., 2002) and a secondary fluorochrome-labeled goat anti-mouse Ab in blocking buffer for 30 min at 4 °C, and analyzed on a BD FACSCanto II (BD Biosciences, San Jose, CA, USA).

2.2. Induction and inhibition of apoptosis

Intrinsic apoptosis was induced either by serum withdrawal or by the addition of 0.1 μM staurosporine (Enzo Life Sciences, Lörrach, Germany). Extrinsic apoptosis was induced by adding 5 ng/ml TNF (PeproTech, Rocky Hill, NJ, USA) and 10 μg/ml cycloheximide (CHX; Sigma–Aldrich, St. Louis, MO, USA). Proteolysis of caspases was inhibited with 50 μM carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone (*z*-VAD-fmk; Enzo Life Sciences) or with 10 μg/ml pan-caspase Q-VD-OPH inhibitor (Q-VD; R&D Systems, Minneapolis, MN, USA). Dose and time responses were optimized for staurosporine and the inhibitors (data not shown).

2.3. Cellular assays

Assays were performed according to the manufacturer's protocols and, if applicable, adapted to 384-well or 96-well formats. In multi-well plate assays, one data point in one experiment consisted of four measurements. Each experiment was repeated 3–5 times. To analyze the cell-cycle phase distribution, ethanol-fixed cells were stained with 50 μg/ml propidium iodide (PI; Sigma–Aldrich) in the presence of 25 ng/ml RNaseA (Qiagen, Hilden, Germany) and analyzed on a BD LSR II flow cytometer (Becton Dickinson, Heidelberg, Germany). Cell viability was determined either with the Cell Counting Kit-8 Assay (CCK-8; Sigma–Aldrich) or the CellTiter-Blue™ Cell Viability Assay (Promega, Mannheim, Germany). The number of attached cells was quantified using crystal violet as described (Kueng et al., 1989). The GFP-Certified™ Apoptosis/Necrosis Detection Kit (Enzo Life Sciences) and the TACS Annexin V-FITC Apoptosis Detection Kit (R&D Systems) containing annexin V (AV) and PI were used to discriminate early (AV^+/PI^-) and late (AV^+/PI^+) apoptotic cells. The apoptosis rate was defined as the percentage of AV^+ apoptotic cells. DNA fragmentation was determined as described (Herrmann et al., 1994). Changes in nuclear morphology were examined in cells labeled with 0.001% 4′-6-diamidine-2-phenylindole (DAPI) for 30 min. The percentage of cells with condensed