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CHC promotes tumor growth and angiogenesis through regulation of HIF-1α and VEGF signaling

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ABSTRACT

Pancreatic adenocarcinoma is an aggressive disease with a high mortality rate. In this study, we have newly generated a monoclonal antibody (mAb), Pa65-2, which specifically binds to pancreatic cancer cells and tumor blood vessels. The target protein of Pa65-2 is identified as human clathrin heavy chain (CHC). *In vitro* and *In vivo* study showed that suppression of CHC either by shRNA or by Pa65-2 inhibited tumor growth and angiogenesis. One of the key functions of CHC was to bind with the hypoxia-inducing factor (HIF)-1α protein, increasing the stability of this protein and facilitating its nuclear translocation, thereby regulating the expression of VEGF. Taken together, our findings indicate that CHC plays a role in the processes of tumorigenesis and angiogenesis. Pa65-2 antibody or CHC shRNA can potentially be used for pancreatic cancer therapy.

1. Introduction

About 95% of pancreatic cancer cases are adenocarcinomas. The overall five-year survival rate of pancreatic adenocarcinoma is about 5%. It is the fourth leading cause of cancer death in the United States [1]. Pancreatic cancer often recurs after initial treatment despite the use of chemotherapy or radiation therapy [2]. At present, there is no effective treatment for pancreatic cancer. The most commonly used medicine to treat pancreatic cancer is gemcitabine (GEM), a pyrimidine nucleoside drug, but it is only moderately effective [3].

Two monoclonal antibody (mAbs) drugs currently in clinical trial for targeted therapy against pancreatic cancers are cetuximab [4] and bevacizumab [5,6], targeting epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), respectively. However, clinical trial data showed that using either cetuximab or bevacizumab, in combination with small molecule drugs had no significant improvement in the overall survival of pancreatic cancer patients [4–7]. Since EGFR and VEGF may not be suitable target for pancreatic cancer treatment, it is important to identify a suitable target for targeting therapy of pancreatic cancer.

We have generated several mAbs recognizing pancreatic cancer cells. One of these mAbs, Pa65-2, can recognize clathrin heavy chain (CHC). Clathrin, encoded by the *CLTC* gene at 17q23.2, is a trimer of heavy chains (~190 kDa each), each paired with a light chain (25–27 kDa). The basic unit of its assembly is the triskelion, which has a flexible, three-armed polyhedral cage-like structure [8]. Clathrin plays a crucial role in clathrin-mediated endocytosis (CME) pathway, a major route for membrane trafficking. It is involved in the ubiquitous uptake of ligand–receptor complexes, membrane transporters, and adhesion molecules [9]. Clathrin also stabilizes the fibers of the spindle apparatus during mitosis [10]. However, the main mechanism of CHC in tumorigenesis remains unknown.

In this study, we found that CHC may regulate the stability of the α subunit of hypoxia inducible factor-1 (HIF-1α). When cells are exposed to hypoxic conditions, HIF-1α is stabilized, which subsequently upregulates several downstream genes to promote cell survival in low-oxygen conditions. HIF-1α accumulation mediates cellular and systemic adaptive responses to maintain oxygen homeostasis. It also upregulates hypoxia-inducible genes, which are involved in angiogenesis, erythropoiesis, energy metabolism as well as cell survival decisions in all metazoan species [11]. In cancerous conditions, cells within rapidly growing solid tumors are exposed to chronic or intermittent hypoxia. Therefore, tumor cells encounter powerful selective pressure from hypoxia during their progression, invasion, and metastasis [12]. An elevated expression of hypoxia-responsive proteins is a poor prognostic sign

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in many types of solid tumors [13–15], and the results from several studies suggest that agents acting directly or indirectly against the expression of HIF-1α have anticancer effects [16,17].

In the present study, we found that CHC associates with the HIF-1α, increases the stability of this protein, and facilitates its nuclear translocation, thereby regulating VEGF gene expression in cancer cells. Our newly generated Pa65-2 inhibited tumor growth and angiogenesis, suggesting that this mAb can potentially be used to inhibit tumor angiogenesis and tumorigenesis in pancreatic cancer.

2. Materials and methods

2.1. Cell lines and culture

Human pancreatic adenocarcinoma cell lines (MIA PaCa-2 and AsPC-1), and a human skin fibroblast cell line (CCD-1112Sk), were purchased from American Type Culture Collection (ATCC). These cells were cultured in accordance with cell bank protocols and had been passaged for less than 6 months after resuscitation. Normal nasal mucosal (NNM) epithelia were a primary culture derived from a nasal polyp [18]. Human umbilical vein endothelial cells (HUVECs) were purchased (Lonza) and grown in EBM-2 medium (Lonza).

2.2. Generation of monoclonal antibodies

Monoclonal antibodies against MIA PaCa-2 were generated following a standard procedure [19] with slight modifications [20]. Briefly, female BALB/cJ mice were immunized intraperitoneally with MIA PaCa-2 four times at 3-week intervals. On day 4 after the final boost, splenocytes were harvested from the immunized mouse spleen and fused with NSI/1-Ag4-1 myeloma cells by 50% polyethylene glycol (GIBCO). Those hybridomas, positive for MIA PaCa-2 but negative for NNM, were then subcloned by limited dilution and preserved in liquid nitrogen. Ascites were produced in pristane-primed BALB/cJ mice and mAbs purified with protein G Sepharose 4G gel (GE).

2.3. Identification of the target protein of Pa65-2

MIA PaCa-2 cell lysates were purified by protein G Sepharose (GE), coupled with Pa65-2 and eluted with elution buffer [21]. The eluates were separated by SDS–PAGE. The band of interest was cut from the gel, reduced with dithioerythreitol (DTE) alkylated with iodoacetamide (IAA) and digested with trypsin for 16 h at 37 °C [21]. The digested peptides were analyzed by LC–MS/MS sequencing in the Core Facility for Proteomics and Structural Biology Research at Academia Sinica (Taipei).

2.4. Immunoprecipitation and immunoblotting assay

Cells were extracted with RIPA buffer and the supernatants were immunoprecipitated using either anti-CHC antibody, Pa65-2, mAb X-22 (Affinity), or anti-HIF-1α antibody (BD), then analyzed by immunoblotting [22]. The signals were developed using enhanced chemiluminescence reagents (ECL) (Thermo).

2.5. Cell proliferation analysis and invasion assays

RT-CES (ACEA), a microelectronic cell sensor system, was used to count the number of living cells. Cells (5×10^3) were seeded into each sensor-containing well in microtiter plates. The electronic sensors provided a continuous (every 6 h), quantitative measurement of the cell index in each well. Cell growth was measured for 72 h, and cell indices for each well were recorded at all time points. Cell invasion was assayed in 24-well Biocoat Matrigel invasion chambers (8 μm; Millipore) according to the manufacturer's directions. Cells were counted under a microscope in five predetermined fields. Assays were performed in triplicate.

2.6. shRNA transfection and luciferase reporter gene assays

Lentiviruses (pLKO.1) containing the CHC shRNA ID TRCN0000007984 (Academia Sinica, Taipei) and pLKO.1 empty vector controls were generated and used to infect MIA PaCa-2 cells. For plasmid transfection, 293T cells were seeded at a density of 60% in a six-well plate and incubated for 24 h. The cells were co-transfected with 1.5 μg of CHC shRNA or pLKO.1 empty vector controls with pCMVΔR8.91 and pMD.G, using Lipofectamine2000 (Life Technologies), according to the recommended protocol. Conditioned medium was exchanged the following day and lentivirus-containing supernatant was harvested 48 h later. For lentiviral infection, cells were seeded at a density of 70% confluence and incubated for 24 h. Lentiviral infection was added 10% (v/v) of lentivirus-containing medium to the cell culture and incubated for 48 h, transduced cells were selected using puromycin (2 μg/ml, Sigma) for 4 days [23,24]. Transfection efficacy achieved 80%. The VEGF reporter plasmids contains nucleotides −2274 to +379 of the VEGF gene inserted into luciferase reporter pGL2-Basic (Promega) as previously described [25]. VEGF promoter primer sequences are presented in Supplementary Table 1. Luciferase reporter gene assays were conducted using the Renilla Luciferase Assay System (Promega) according to the manufacturer's directions. The Renilla luciferase was constructed for normalization of transfection efficiency. Relative light units were calculated as the ratio of Firefly luciferase to Renilla luciferase activity (normalized luciferase activity).

2.7. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

RNA extractions were performed using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. First strand cDNA was synthesized from 1.0 μg of total RNA by SuperScript III reverse transcriptase (Life Technologies). CLTC, VEGF, HIF-1α, erythropoietin (EPO), platelet-derived growth factor-β (PDGF-β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers sequences are presented in Supplementary Table 1. Real-time PCR was performed using a LightCycler 480 System (Roche). Cycling temperatures were as follows: denaturing 94 °C, annealing 60 °C, and extension 70 °C. Data were normalized by the expression level of GAPDH in each sample.

2.8. Hypoxia assay

For hypoxia experiments, MIA PaCa-2 cells were grown in a Ruskinn Hypoxic Chamber (APM-50D, 18 Astec, Japan) and treated with either 1% O_2; 5% CO_2 for 18 h, or given DFX, deferoxamine (100 μM, Sigma) treatment for 5 or 16 h. For proteasome inhibitor treatment, MG132 (10 μM, Sigma) was added to culture medium then incubated 5–17 h.

2.9. Chromatin immunoprecipitation (ChIP)

The protocol for chromatin immunoprecipitation (ChIP) has been described previously [26]. Briefly, control and shCHC-expressing MIA PaCa-2 cells were fixed with 1% formaldehyde, lysed in lysis buffer, sonicated, and clarified by centrifugation. The supernatant was immunoprecipitated with anti-CHC, anti-HIF-1α (Abcam) or NM-IgG (Sigma) antibodies. The precipitates were then amplified by the LightCycler 480 System. The relative abundance of specific sequences in immunoprecipitated DNA was determined using the $\Delta\Delta C_t$ method with C_t obtained for total extracted DNA (Input DNA) as a reference value. The amount of immunoprecipitated target was quantified by real-time PCR, and the value of immunoprecipitated target was calculated as the ratio of IP DNA to the total amount of input DNA used for the immunoprecipitation (IP/input) to obtain relative-fold enrichment value. ChIP primers sequences are presented in Supplementary Table 1.

2.10. Immuno-electron microscopy

Cells were gently scraped out of the flasks using a cell scraper (Costar) and fixed in paraformaldehyde and glutaraldehyde. Following fixation, cell pellets were washed with buffer and 30% glycerol and gently agitated overnight at room temperature. Cells were subjected to freeze substitution in an AFS (Leica), in which they were dehydrated by methanol at −91 °C for 4–5 days. Cells were later warmed to −50 °C, embedded in Lowicryl HM20, and polymerized at −50 °C by UV. Ultrathin sections of 90 nm thickness were obtained using an Ultracut UC7 (Leica). The sections were incubated with the Pa65-2 mouse IgG or anti-HIF-1α rabbit IgG. The secondary antibodies (Jackson), goat anti-mouse IgG ($F(ab')_2$ fragment) conjugated with 18 nm gold particles, or goat anti-rabbit IgG ($F(ab')_2$ fragment) conjugated with 12 nm gold particles, were then applied to their respective sections. Finally, the sections were stained with uranyl acetate and lead citrate, and examined by TEM (Hitachi).

2.11. Inhibition of cell internalization by Pa65-2

EGF uptake assays were carried out using a fluorescence-based approach, as previously described [27]. Cells were washed with serum-free medium, incubated in 1% BSA in serum-free medium at 37 °C, and pretreated with Pa65-2 or NM-IgG (50 μg/ml) at 37 °C. They were then incubated with or without Alexa 555-EGF (1 μg/ml, Life Technologies) at 37 °C or 4 °C. The cells were imaged using a Leica TCS SP confocal microscope (Leica).

2.12. Immunofluorescent staining

Cells were incubated with Pa65-2 and anti-HIF-1α antibodies, and then with FITC- or Rhodamine-conjugated secondary antibodies (Jackson). Images were captured by confocal microscopy (Leica). For cell surface staining, MIA PaCa-2 cells were seeded on coverslips and grown to 80% confluence. The live cells were incubated with Pa65-2 for 30 min at 4 °C. After being washed twice with PBS, the cells were fixed by 4% paraformaldehyde and blocked by adding 3% BSA. The cells were