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Hypoxia enhances the interaction between pancreatic stellate cells and cancer cells via increased secretion of connective tissue growth factor

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ABSTRACT

Background: Pancreatic cancer (PC), a hypovascular tumor, thrives under hypoxic conditions. Pancreatic stellate cells (PSCs) promote PC progression by secreting soluble factors, but their functions in hypoxia are poorly understood. This study aimed to clarify the effects of hypoxic conditions on the interaction between PC cells and PSCs.

Methods: We isolated human PSCs from fresh pancreatic ductal adenocarcinomas and analyzed functional differences in PSCs between normoxia (21% O_2) and hypoxia (1% O_2), including expression of various factors related to tumor–stromal interactions. We particularly analyzed effects on PC invasiveness of an overexpressed molecule—connective tissue growth factor (CTGF)—in PSCs under hypoxic conditions, using RNA interference techniques.

Results: Conditioned media from hypoxic PSCs enhanced PC cell invasiveness more intensely than that from normoxic PSCs ($P < 0.01$). When co-cultured with PSCs, PC cell invasion was more enhanced under hypoxia than under normoxia ($P < 0.05$). Among various soluble factors, which were related to invasiveness, CTGF was one of the overexpressed molecules in hypoxic PSCs. A higher level of CTGF expression was also found in supernatant of hypoxic PSCs than in supernatant of normoxic PSCs. PC cell invasiveness was reduced by *CTGF* knockdown in hypoxic PSCs co-cultured with PC cells ($P < 0.05$).

Conclusion: Hypoxia induces PSCs' secretion of CTGF, leading to enhancement of PC invasiveness. CTGF derived from hypoxia-stimulated PSCs may be a new therapeutic target for pancreatic cancer.

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1. Introduction

Pancreatic cancer (PC) is one of the most lethal cancers and the fourth-leading cause of tumor-related deaths in the industrialized world [1]. The vast majority of patients with PC have poor outcomes, because early diagnosis is uncommon and conventional therapeutics such as surgical resection, chemotherapy, and radiotherapy have limited efficacy [1,2].

Severe hypoxia is a physiologic characteristic unique to solid tumors [3]. Hypoxic regions of solid tumors often surround necrotic zones and harbor viable cells, and also contribute to the invasive and metastatic characteristics of aggressive cancers [4]. Many reports have shown hypoxia to exist in a large variety of solid tumors using *in vivo* imaging or clinical samples [5,6]. PC also exists under hypoxic conditions, as proven by the needle measurement of intratumoral O_2 [7]. Hypoxia is a driving force in PC progression [4,8]. Excessive desmoplasia in PC maintains the hypoxic condition and contributes to poor prognosis, resistance to chemotherapy and radiation therapy, and increased metastatic potential [9,10].

PC is characterized by profuse desmoplasia, characterized as a proliferation of fibrotic tissue with an aberrant extracellular matrix (ECM) [11,12]. This desmoplastic reaction is associated with PC progressiveness, invasiveness, and proliferation. Thus, tumor–stromal interaction is a major factor in promoting PC aggressiveness [12]. Pancreatic stellate cells (PSCs) are the principal source of the aberrant ECM observed in chronic pancreatitis and PC [13,14]. Like hepatic stellate cells, which are the most important cell type for matrix production in hepatic fibrosis, PSCs store fat droplets containing vitamin A in their cytoplasm [15]. Pancreatic stellate cells are transformed into their activated phenotype upon stimulation by various autocrine or paracrine factors. They express α-smooth muscle actin (α-SMA) and produce various ECM proteins, such as collagen type I and fibronectin [16]. Soluble factors secreted by activated PSCs promote proliferation, migration, invasion, and survival against conventional PC therapies [14].

Recent studies have shown that hypoxia-inducible factor 1α (HIF-1α) and carbonic anhydrase 9 (CA9), known as hypoxia-regulated markers, are expressed in fibroblasts within pancreatic tumors [17,18]. These findings suggest that hypoxic conditions concomitantly exist both in PC cells and in surrounding stroma, including PSCs. Although the roles of PC cells in hypoxia have been extensively studied, those of PSCs in hypoxia are little known.

The aim of this study was to investigate hypoxia-induced or hypoxia-promoted effects in PSCs that affect cancer progression. We examined behavioral differences in tumor–stromal interaction between PSCs in hypoxia and in normoxia, using *in vitro* experiments. In particular, we focused on some soluble molecules that PSCs secrete in hypoxia, and investigated the biologic mechanism by which hypoxia-stimulated PSCs promote PC progression. We then focused on connective tissue growth factor (CTGF), which is involved in desmoplasia [19] and is reported to be expressed in the tumor–stromal border area of pancreatic ductal adenocarcinoma in the mouse model with *Kras* activation [20].

2. Materials and methods

2.1. Cells and culture condition

Human PSCs were isolated from fresh PC surgical specimens using the outgrowth method described previously [13]. Passage numbers 2–4 were used for the assays. In addition, two PC cell lines, SUIT-2 and PANC-1 (Dr H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), were used. Pancreatic cancer cell lines and human PSCs were maintained until subconfluent in Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 g/mL), and penicillin (100 U/mL) at 37°C. The study was approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Declaration of Helsinki.

2.2. Hypoxic conditions and preparation of the conditioned media

Subconfluent PSCs were incubated in a hypoxic incubator with 1% O_2, 5% CO_2, and 94% N_2. Oxygen levels in the culture chambers were continuously monitored. Control experiments included parallel cultures in which cells were exposed to normoxia (21% O_2, 5% CO_2, and 74% N_2). Culture medium was preconditioned to the correct O_2 level in hypoxic experiments. Conditioned DMEM with 1% FBS, following 24-h incubation of PSCs under normoxic or hypoxic conditions, was harvested and used for the next assay as normoxia-conditioned media (NCM) or hypoxia-conditioned media (HCM).

2.3. Immunohistochemical procedures and evaluation

Sections were cut at 4-μm thickness from paraffin-embedded material, deparaffinized in xylene, and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was achieved by microwaving sections in citrate buffer at pH 6.0. A Histofine SAB-PO kit (Nichirei, Tokyo, Japan) was used for immunohistochemical labeling. Each section was exposed to 10% nonimmunized rabbit serum for 10 min to block nonspecific binding of the antibodies, followed by incubation with a mouse monoclonal anti-CTGF antibody (sc-101586; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100) or CA9 (NB100-417; Novus Biologicals, Littleton, IL; 1:500) at 4°C overnight. Sections were then sequentially incubated with a biotinylated anti-mouse immunoglobulin solution for 20 min, followed by peroxidase-labeled streptavidin for 20 min. The reaction products were visualized using 3,3′-diaminobenzidine as a chromogen, followed by nuclear counterstaining with hematoxylin. Cytoplasmic immunoreactivity was detected in the stromal cells and carcinoma cells.