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HYPOXIA-DEPENDENT EXPRESSION OF ADAM8 IN HUMAN PANCREATIC CANCER CELL LINES

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Background: One of the most characteristic features of malignant tissue is the high level of intratumoral hypoxia, which is considered as a powerful factor for induction of tumor aggressiveness and malignant progression. Pancreatic cancer (PC) is a near fatal disease with very unfavorable clinical outcome despite the application of different treatment regimes. It was shown that PC is characterized by high level of hypoxia. *Aim:* To clarify the correlation between tumor hypoxia and ADAM8 protein expression. *Materials and Methods:* ASPC-1, Panc-1, BxPC-3, Capan-1, MiaPaCa-2, Colo-357, Su8686 and T3M4 cell lines were used in the study. Expression of mRNA ADAM8 was evaluated by real-time quantitative polymerase chain reaction method. Immunoblot analysis was used to evaluate the expression of ADAM8 protein. *Results:* Hypoxia induced a 2.5−5.9-fold increase of ADAM8 mRNA levels of in the examined pancreatic cancer cell lines except Panc-1 ($p = 0.046$). On the protein level, hypoxia induced a 1.2−5.9-fold increase of the ADAM8 prodomain removal form (90 kDa) in 5/8 pancreatic cancer cells. Moreover, hypoxia induced a 1.3−2.0-fold increase of the remnant form ADAM8 (60 kDa) in 4/8 pancreatic cancer cell lines: Aspc-1, Colo-357, Panc-1, T3M4. *Conclusion:* It was observed the clear tendency in the increase both of ADAM8 mRNA and ADAM8 protein levels in pancreatic cancer cell lines under hypoxia compared to normal conditions of oxygenation. A potential role of ADAM8 as a hypoxia-dependent protein in the pathogenesis and evolution of pancreatic cancer that is characterized by high level of intratumoral hypoxia can be suggested.
Key Words: pancreatic cancer, invasion, ADAM8, hypoxia.

The high level of intratumoral hypoxia is one of the most characteristic features of malignant tissue. This phenomenon is the most important in differences between tumor and normal tissues as well as benign tumors [1–3]. The investigation results both experimental and clinical studies has allowed on considering tumor hypoxia as a powerful factor for angiogenesis induction [4] facilitating tumor progression and metastatic spread [5, 6].

Pancreatic cancer is a near fatal disease with very unfavorable clinical outcome despite the application of different treatment regimes [7]. One of the major causes of therapy failure of pancreatic cancer is its systemic dissemination and extraordinary local tumor progression, in particular invasion into the adjacent tissues. It is remarkable that pancreatic cancer featured by high aggressiveness is characterized by high level of hypoxia. Koong et al. [8] have provided the direct evidence due to pO_2 measurements using polarographic electrode that significant tumor hypoxia exists in human pancreatic cancer. Several studies shown that pancreatic cancer hypoxia is associated with its aggressiveness and positively impact on malignant progression, including metastasis and chemoresistance [9, 10].

It is well known that extracellular proteases have been closely associated with metastatic phenotype of tumor cells. There are rather clear evidences of a direct correlation between elevated level of tumor matrix metalloproteinases (MMPs) activity and ability of tumor to invade and metastasize [11, 12]. In our previous experiments it was shown the direct correlation between

MMPs activity and hypoxia level in primary tumor that was accompanied with active metastasizing in lung of tumor-bearing animals [13]. The results obtained have allowed us to suppose that hypoxia affects positively MMPs activity in tumor tissue resulting in the enhancement of metastasis.

Recently, the attention of scientists has been focused on the relatively new class of proteases, namely ADAMs (A Desintegrin And Metalloprotease domen) that are considered as a new factors associated with the invasiveness of the malignant tumors. They are the members of a family of transmembrane proteins, which implicated in cell-cell interaction, proteolysis of membrane proteins, and various aspects of carcinogenesis [14]. ADAM8 is processed by autocatalysis into two forms: one is derived by removal of a prodomain (processed form) and the other is a remnant protein composed of the extracellular region, with a disintegrin domain at the amino terminus [15].

It was shown that some ADAMs are overexpressed in malignant tumors and may be exploited as prognostic markers [16–19]. The overexpression of ADAM9, ADAM15 and ADAM17 was observed in pancreatic cancer [20–22]. The special interest is concentrated on ADAM8, because it is not inhibited by tissue inhibitors of metalloproteinases (TIMPs) and is different from other proteolytic proteins [23]. Correlation between ADAM8 expression and malignant progression as well as worse prognosis were observed in lung cancer, prostate carcinoma, kidney cancer and glioblastoma [16–19]. ADAM8 overexpression in human pancreatic cancer was shown in our previous study [24].

At the same time the possible correlation between tumor hypoxia and ADAMs expression was not fully investigated till now. This study was aimed to evaluate the effect of hypoxia on ADAM8 expression in cell lines of human pancreatic cancer.

MATERIALS AND METHODS

Cell lines. ASPC-1, Panc-1, BxPC-3, Capan-1 and MiaPaCa-2 cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). Colo-357, Su8686 and T3M4 cell lines were a gift from R.S. Metzgar (Duke University, Durham, NC, USA). Cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany) and were incubated in a 5% CO_2 humidified atmosphere.

Real-time quantitative polymerase chain reaction (qRT-PCR). All reagents and equipment for mRNA/cDNA preparation were supplied by Roche Applied Science (Mannheim, Germany). mRNA of human pancreatic cell lines was prepared by automated isolation using the MagNA pure LC instrument and isolation kit I (for cells) and kit II (for tissues). cDNA was prepared using the first-strand cDNA synthesis kit for RT-PCR (AMV) according to the manufacturer's instructions. The primer sequences for ADAM8 were obtained from Search-LC (Heidelberg, Germany). Real-time PCR was performed using the LightCycler FastStart DNA SYBR Green kit. The number of specific transcripts was normalized to the average expression of two housekeeping genes (cyclophilin B and HPRT) and presented as adjusted transcripts/µl cDNA, as described previously [25].

Immunoblot analysis. Cells were lysed in a lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2mM EDTA pH 8.0, and the Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics GmbH, Mannheim, Germany) and 1% SDS. 17–25 µg proteins were separated on NuPAGE Novex Bis-Tris 4–12% gels (Invitrogen, Karlsruhe, Germany) and electro-blotted onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% milk in 20 mM Tris HCl, 150 mM NCl, 0.1% Tween-20), followed by overnight incubation with a rabbit polyclonal ADAM8 antibody (dilution 1 : 250 in blocking solution) (Chemicon International, Temecula, CA, USA). The membranes were then washed in TBS-T and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience, Buckinghamshire, UK). Antibody detection was performed with an enhanced chemiluminescence reaction (ECL, Amersham Bioscience). Equal loading and transfer was confirmed using γ-tubulin and ERK-2 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). For semiquantitative analysis of the immunoblots, densitometry was carried out and the signal intensity of ADAM8 expression was normalized to its corresponding signal intensity of γ-tubulin.

Hypoxia treatment. Cells were grown to ~ 70% confluence in 10 cm tissue culture dishes. For hypoxia treatment, cells were incubated in a hypoxic chamber with fresh complete medium (Billups-Rothenberg, Inc., Del Mar, CA, USA) with an 89.25%/10%/0.75% mixture of $N_2/CO_2/O_2$ for 24 h. After the indicated time of hypoxia exposure, RNA and proteins were extracted from hypoxic and normoxic pancreatic cancer cells as described above.

Statistical analysis. Results are expressed as a mean ± SEM. Statistical analysis was performed using non-parametric Mann — Whitney test.

RESULTS

QRT-PCR was performed to evaluate the expression of ADAM8 mRNA in pancreatic cancer cell lines affected by hypoxia compared to normal condition of these cell lines. Therefore, different amounts of ADAM8 mRNA within a range of 16–922 copies/µl cDNA were detected in all examined pancreatic cancer cell lines under normoxia (Table 1). Then, the effects of hypoxia on ADAM8 mRNA levels were examined in pancreatic cancer cells. Hypoxia induced an increase of ADAM8 mRNA levels of 2.5–5.9-fold in all examined pancreatic cancer cell lines except Panc-1 with the lowest basal level of ADAM8 mRNA (Table 1). BxPC-3, Capan-1 and Su8686 have shown the highest relative increase of ADAM8 mRNA expression levels under the effects of hypoxia. The clear tendency to increase levels of ADAM8 mRNA under hypoxia condition as compared to normoxia ones was observed.

Table 1. ADAM8 mRNA expression in pancreatic cancer cells exposed to hypoxia

Cell lines	Culture conditions	Copies/µl cDNA (mean ± SEM)	Hypoxia/normoxia ratio of mRNA expression
Aspc-1	Normoxia	847.0 ± 427.9	
	Hypoxia	2246.0 ± 954.2	2.6
BxPC-3	Normoxia	140.5 ± 7.5	
	Hypoxia	470.0 ± 275.0	3.3
Capan-1	Normoxia	439.7 ± 180.3	
	Hypoxia	1612.0 ± 215.4	3.7
Colo-357	Normoxia	42.0 ± 11.0	
	Hypoxia	104.5 ± 20.5	2.5
MiaPaCa-2	Normoxia	52.5 ± 15.5	
	Hypoxia	136.0 ± 8.0	2.6
Panc-1	Normoxia	15.5 ± 7.5	
	Hypoxia	19.0 ± 4.0	1.2
Su-8686	Normoxia	740.5 ± 287.5	
	Hypoxia	4356.0 ± 1301.0	5.9
T3M4	Normoxia	929.0 ± 215.0	
	Hypoxia	2419.0 ± 834.5	2.6

Immunoblot analysis was performed to compare the mRNA data with the corresponding protein expression level. It has been shown that ADAM8 was present in two forms (60 kDa and 90 kDa) in the all investigated cell lines although the basal level of expression was different being in parallel with the expression of mRNA (Figure). The 90 kDa processed form contains the metalloprotease (MP) domain responsible for the proteolytic activity of ADAM8, and the 60 kDa remnant form mediates cell adhesion [15]. On the protein level, hypoxia induced a 1.2–5.9-fold increase of the ADAM8 prodomain removal form (90 kDa) in 5/8 pancreatic cancer cells. In Panc-1 cells, there was a 0.4-fold decrease of ADAM8 in response to hypoxia, and in BxPC-3 and Su8686 there were no significant changes observed (Table 2). Additionally, hypoxia induced a 1.3–2.0-fold increase of the remnant form ADAM8 (60 kDa) in 4/8 pancreatic cancer cell lines: Aspc-1, Colo-357, Panc-1, T3M4 (see Table 2). From these results we might conclude, that in some pancreatic cancer cell lines tendency to increase levels of ADAM8 protein under effect of hypoxia was observed.
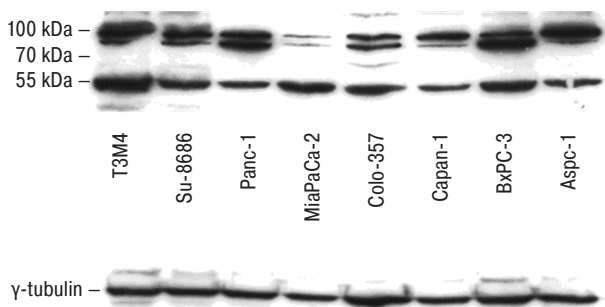
Figure. ADAM8 protein expression in pancreatic cancer cell lines was determined by immunoblotting. Equal loading of the protein samples was confirmed using γ-tubulin antibody. Size markers are indicated on the left (in kDa)

Table 2. ADAM8 protein expression in pancreatic cancer cells exposed to hypoxia

Cell lines	Hypoxia/normoxia ratio of ADAM8 forms protein expression	
	90 kDa	60 kDa
Aspc-1	1.9	1.96
BxPC-3	1.04	0.94 ↓
Capan-1	1.22	1.07
Colo-357	5.91	1.46
MiaPaCa-2	1.73	0.78 ↓
Panc-1	0.41 ↓	1.33
Su-8686	1.09	1.02
T3M4	1.48	1.35

DISCUSSION

ADAMs are a large family of transmembrane proteins that are involved in proteolysis, making them candidates for mediating the remodeling of the extracellular matrix (ECM). ADAMs affects cell adhesion and cell migration, which is important under physiological conditions. During tumor development these effects might influence the ability of cancer cells to metastasize, making these proteins possible targets for anti-tumor therapy [26]. ADAM8, one of the members of the ADAM family, is overexpressed in various human tumors [16–19]. It has been shown previously that hypoxia influences the expression of some ADAM proteins [27, 28]. Namely, oxidative stress induced ADAM9 protein expression in human prostate cancer cells and chronic hypoxia reduce expression ADAM10 and TACE proteins in the human neuroblastoma but without altering their mRNA levels [27, 28].

In our study ADAM8 mRNA expression has been shown to increase in all pancreatic cancer cell lines under hypoxic conditions although ADAM8 protein expression increased in 5 of 8 pancreatic cancer cell lines under hypoxic condition. Interesting, BxPc-3 and Su8686 cell lines have not shown changes of ADAM8 protein expression (one of the two presented active forms) under hypoxia. It was shown the decrease of ADAM8 protein expression (90 kDa form only) under hypoxia in Panc-1 cell line. 90 kDa processed form contains metalloprotease domain responsible for proteolytic activity of ADAM8 and is a potential shedding protein. 60 kDa form of ADAM8 protein mediates cell adhesion. It was also shown increase of ADAM8 90 kDa form expression in MiaPaCa-2 cell line that might indicate the increase of shedding ability of ADAM8 protein under hypoxia in this cell line. It was observed the increase of both forms of ADAM8 protein expression under hypoxia in T3M4 cell line at the same level.

Colo-357 cell line was characterised by the highest increase of level of ADAM8 90 kDa form expression under hypoxia compared to normoxia condition of this cell line cultivation. As we known, BxPc-3 Panc-1 and MiaPaCa-2 cell lines originated from primary tumor of pancreatic carcinoma (G3), Aspc-1-ascites (G2), Su8686, Capan-1-liver metastasis (Su8686-G2/3, Capan-1-G1) and T3M4-lymph metastasis (G2). It is needed to remark that observed changes in expression of ADAM8mRNA and ADAM8 protein were registered in cells exposed *in vitro* to hypoxia during 24 h. At the same time, tumor tissue *in vivo* is characterized by the continuous exposure of tumor cells to chronic hypoxia resulting in the rearrangement of molecular profile of tumor. This fact may partly explain why increase of both mRNA and protein ADAM8 expression was not substantial under relatively short-term exposition to hypoxia.

In the previous work it has been shown that ADAM8 protein presents in the normal pancreatic tissues in one active form only (60 kDa form remnant protein which is a potential adhesion molecule). In comparison to this fact, ADAM8 protein found to be expressed in two active forms: potential shedding protein and remnant protein in the pancreatic tissues under chronic pancreatitis and pancreatic cancer [24].

Thus, in the present study we observed the tendency of increasing both ADAM8 mRNA and ADAM8 protein levels in pancreatic cancer cell lines under hypoxia compared to normal conditions of oxygenation. A potential role of ADAM8 as a hypoxia-dependent protein in the pathogenesis and evolution of pancreatic cancer that is characterized by high level of intratumoral hypoxia may be suggested. Pancreatic cancer cell lines with different levels of ADAM8 expression under hypoxia compared to normoxia may be exploited for screening of new anticancer substances, in particular hypoxia-dependent.

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ЗАВИСИМАЯ ОТ ГИПОКСИИ ЭКСПРЕССИЯ БЕЛКА ADAM8 В КЛЕТОЧНЫХ ЛИНИЯХ РАКА ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ ЧЕЛОВЕКА

Введение: известно, что характерной чертой злокачественных опухолей является внутриопухолевая гипоксия, положительно влияющая на злокачественную прогрессию. Рак поджелудочной железы (РПЖ) является очень агрессивной опухолью, обладающей высокой способностью к инвазии в близлежащие ткани и органы, что обусловливает неудовлетворительные результаты лечения. Показано при этом, что РПЖ относится к новообразованиям с высокой степенью гипоксии. *Цель:* установление возможной зависимости экспрессии белка АДАМ8 и его мРНК от уровня оксигенации клеток. *Материалы и методы:* использовали клеточные линии РПЖ человека. Экспрессию мРНК и белка ADAM8 определяли методами qRT-PCR и вестерн-блот-анализа соответственно. *Результаты:* установлено, что гипоксия индуцировала повышение уровня мРНК ADAM8 в 2,5–5,9 раза в исследованных клеточных линиях РПЖ, за исключением клеток линии Panc-1. В клетках линий BxPC-3, Capan-1 и Su8686 выявлено наиболее значительное возрастание уровня экспрессии мРНК ADAM8 при гипоксии ($p = 0,046$). С помощью вестерн-блот-анализа показано, что экспрессия удаляемой формы продомена ADAM8 (90 kDa) усиливалась в несколько раз при гипоксии в 5 из 8 изученных клеточных линий РПЖ. Гипоксия индуцировала повышение экспрессии остаточной формы ADAM8 (60 kDa) в 4 из 8 клеточных линий РПЖ: Aspc-1, Colo-357, Panc-1, T3M4, хотя изменения оказались умеренно выраженными. *Выводы:* в клеточных линиях РПЖ человека отмечается четкая тенденция к повышению при гипоксии уровня экспрессии, как мРНК, так и белка ADAM8, что позволяет предположить зависимость экспрессии от уровня оксигенации и потенциальную роль ADAM8 в развитии и прогрессии РПЖ, который отличается высокой степенью внутриопухолевой гипоксии.
Ключевые слова: рак поджелудочной железы, инвазия, ADAM8, гипоксия.