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Title	Hypoxia enhances the expression of autocrine motility factor and the motility of human pancreatic cancer cells
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Citation	British Journal of Cancer, 86(12), 1914-1919 https://doi.org/10.1038/sj.bjc.6600331
Issue Date	2002-06-17
Doc URL	https://hdl.handle.net/2115/11317
Type	journal article
File Information	bjc.pdf

HYPOXIA ENHANCES THE EXPRESSION OF AUTOCRINE MOTILITY FACTOR AND THE MOTILITY OF HUMAN PANCREATIC CANCER CELLS

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A running title: AMF expression promoted by hypoxia

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Summary

    The incidence of distant metastases is higher in the tumors

with low oxygen pressure than in those with high oxygen pressure.

It is well known that hypoxia induces the transcription of various

genes involved in angiogenesis and anaerobic metabolism

necessary for the growth of tumor cells *in vivo*, suggesting that

hypoxia   may   also   induce   the   transcription   of

metastasis-associated genes.   We sought to identify the

metastasis-associated genes differentially expressed in tumor

cells under hypoxic conditions with the use of a DNA microarray

system.   We found that hypoxia enhanced the expression of

autocrine motility factor (AMF) mRNA in various cancer cells

and also enhanced the random motility of pancreatic cancer cells.

AMF inhibitors abrogated the increase of motility under hypoxic

conditions.   In order to explore the roles of hypoxia-inducible

factor-1α (HIF-1α), we established HIF-1α-transfectants and

dominant negative HIF-1α-transfectants.   Transfection with

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HIF-1α and dominant-negative HIF-1α enhanced and suppressed

the expression of AMF/PHI/NL mRNA and the random motility,

respectively. These results suggest that hypoxia may promote

the metastatic potential of cancer cells through the enhanced

AMF/PHI/NL mRNA expression and that the disruption of the HIF-1

pathway may be an effective treatment for metastasis.

INTRODUCTION

As metastasis is the major cause of death in cancer patients, control of metastasis is most important in the therapies for cancer patients. In order to control metastasis, we need to understand the details of metastatic process that is now thought to take multiple steps. Although a variety of factors have been documented to play important roles in the metastatic steps (Poste et al., 1980; Liotta, 1986; Liotta, 1988; Nicolson, 1988), many factors are yet to be elucidated. Several clinical investigations demonstrated that patients with hypoxic tumors had poor prognoses and that the incidence of distant metastases was higher in the tumors with low oxygen pressure than in those with high oxygen pressure (Gatenby et al., 1988; Brizel et al., 1996; Hockel et al., 1998; Rofstad, 2000). These studies suggest that the tumor cells exposed to hypoxia at the primary tumor sites acquire aggressive properties including metastatic

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potential more than the well-oxygenated tumor cells do. In fact,

recent reports have demonstrated that hypoxia enhances the

expression of vascular endothelial growth factor (VEGF) and

interleukin-8 (IL-8) in tumor cells, resulting in an increase

of metastatic potential (Claffey et al., 1996; Shi et al., 1999;

Biroccio et al., 2000). However, it remains poorly understood

how hypoxia promotes tumor cells' metastatic potential.

When tumor cells are exposed to hypoxia, hypoxia-inducible

factor-1 (HIF-1), which is a transcription factor composed of

HIF-1α and HIF-1β subunits (Wang et al., 1995; Wang et al.,

1996), is activated and then it promotes the transcription of

several genes such as glucose transporters, glycolytic enzymes,

and angiogenic factors (Dang et al., 1999). A recent report

demonstrates that human common cancer cells over-express

hypoxia-inducible factor-1α (HIF-1α) *in vivo* and that the

expression of HIF-1α is associated with metastasis (Zhong et

al., 1999). Furthermore, infiltration of endothelial cells, which involves various factors, is enhanced when tumor tissues are in hypoxia-induced angiogenesis. These reports have prompted us to hypothesize that hypoxia may also promote the invasion and metastasis of tumor cells by promoting the expression of metastasis-associated genes in addition to angiogenic factors through the activation of HIF-1α. As hypovasculature is an outstanding characteristic of pancreatic cancers with high invasiveness and metastatic potential (Raijiman et al., 1995) and most of pancreatic cancer cells over-express HIF-1α protein (Akakura et al., 2001), we speculate that pancreatic cancer cells may be exposed to severe hypoxia *in vivo* and that pancreatic cancers express higher levels of metastasis-associated genes in addition to angiogenic factors than well-oxygenated tumor cells do. Therefore we examined the expression of more than 9,000 mRNAs in a pancreatic cancer cell

line under hypoxic and non-hypoxic conditions with the use of

a DNA microarray system and compared them to identify the

metastasis-associated genes induced by hypoxia.

We found that autocrine motility factor (AMF) / phosphohexose

isomerase (PHI) /neuroleukin (NL) mRNA was expressed more highly

in the cells under hypoxic conditions than under non-hypoxic

conditions. In this study, we examined the expression of

AMF/PHI/NL mRNA in a variety of cancer cell lines and the random

motility of a pancreatic cancer cell line under hypoxic and

non-hypoxic conditions, because AMF/PHI/NL was reported to

stimulate random motility (Liotta et al., 1986). Furthermore,

we established HIF-1α-transfectants and dominant-negative

HIF-1α-transfectants and examined their expression of

AMF/PHI/NL mRNA and random motility in order to determine the

possible role of HIF-1α in the AMF/PHI/NL mRNA expression and

random motility promoted by hypoxia.

MATERIALS AND METHODS

Cell lines:

Pancreatic ductal adenocarcinoma cell lines (PCI-6, PCI-10, PCI-19, PCI-43 and PCI-66 cells) were established from surgically resected pancreatic cancer tissues. These cell lines were kindly supplied from Dr. Hiroshi Ishikura (The First Department of Pathology, Hokkaido University School of Medicine) and maintained in DMEM/F12 medium supplemented with 10 % fetal calf serum (FCS). TAOV (ovarian cancer), TTOV (ovarian cancer), HepG2 (hepatoma), PC-6 (lung cancer), MiaPaca-2 (pancreatic cancer), BxPC-3 (pancreatic cancer) and KM-12 (colon cancer) cells were maintained in DMEM medium supplemented with 10 % FCS.

DNA microarray analysis:

Total RNA was extracted with the use of TRIZOL Reagent (LIFE TECHNOLOGIES, Tokyo, Japan) from the PCI-10 cells that had been incubated for 24 h under non-hypoxic and hypoxic conditions.

The incubation under hypoxic condition (1 % O_2) was done in a

hypoxic chamber gassed with 95 % N_2 and 5 % CO_2 (Wakenyaku Co.

Ltd., Tokyo). mRNA was purified from the total RNA with the

use of a Quickprep mRNA purification kit (Amersham Pharmacia

Biotech, Tokyo, Japan). Differentially expressed genes were

screened with the use of a DNA microarray system, UniGEM human

(KURABO INDUSTRIES LTD, Osaka, Japan). We defined the genes

expressed at more than 2-fold higher levels under hypoxic

conditions than under non-hypoxic conditions as possible

hypoxia-inducible genes in this study.

Establishment of HIF-1α-transfectants and dominant negative

HIF-1α-transfectants:

  Establishment of HIF-1α-transfectants was previously

reported (Akakura et al., 2001). A cDNA for dominant negative

HIF-1α (dnHIF-1α) lacking both the DNA binding and

transactivation domains of HIF-1α was amplified from reverse

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transcription (RT) products of mRNAs purified from the PCI-10

cells and cloned into PCR4-TOPO (Invitrogen, Carlsbad, CA). PCR

primers for dominant negative HIF-1α were selected as previously

described (Halterman et al., 1999) (forward,

ccgctcgagaccatgcgaaggaaagaatctg; reverse,

ggggtacctcatttgtcaaagaggctact). Plasmids were recovered,

purified and sequenced with a DyeDeoxy Terminator kit

(Perkin-Elmer, Urayasu, Japan) on an ABI 377 automated sequencer

(Applied Biosystems, Urayasu, Japan) in the condition described

in the manufacturer's protocol. Cloned fragments were

recovered from the vectors and ligated into PcDNA3.1+ (Invitrogen,

Carlsbad, CA). PCI-43 cells were transfected with the

expression vector for dnHIF-1α with the use of Lipofectamine

(Life Technologies, Tokyo, Japan). Transfectants were cloned

by a limiting dilution method after the selection with G-418

at 800 μg/ml. The transfectants were maintained in the presence

of 400 μg/ml of G-418.

FACS analysis:

Cells were stained with anti-AMFR antibody (rat monoclonal

antibody, IgM) (Niinaka et al., 1998) for 1 h and then stained

with second anti-rat Ig antibody for 1 h. Expression of AMFR

proteins was quantified with the use of FACScaliber (Becton

Dickinson, Mountain View, CA).

Northern blot analysis:

Northern blot analysis was performed by the method described

previously (Choi et al., 2000). Total RNA (20 μg) was separated

by electrophoresis in 1.2 % denaturing formaldehyde-agarose gels.

The RNA was transferred to nylon membrane (Hybond N^{+}, Amersham)

overnight by capillary elution and UV cross-linked. After

prehybridization of the blots for 1-2 hr at 42 ˚C in hybridization

buffer (5XSSPE, 5Xdenhardt's solution, 1% SDS, 50% Formamide

and 0.1 mg/ml of denatured salmon sperm DNA), the membrane was

hybridized overnight at 42 ˚C with the cDNA probes for AMF and

AMF receptor (AMFR). The probed membrane was then washed and exposed to radiographic film. cDNA fragments for AMF and AMF receptor (AMFR) were amplified by RT-PCR, cloned into a TA cloning vector, purified from the vector and then used as probes for Northern blot analysis. PCR primers were as follows: AMF forward, gcttgaccctcaacaccaac; reverse, gaagtgctggtccatccagt; AMFR forward, caggaggaagtgagccagtc; reverse, agcttgctgcctaaccactc.

Phagokinetic track assay:

Phagokinetic track assay was performed according to the method reported by Albrecht-Buehler (Albrecht-Buehler et al., 1977). Briefly, gold particle suspension was prepared by mixing 18 μl of 14.5 mM AuCl$_4$ solution and 5 ml of 36.5 mM Na$_2$CO$_3$ solution with 11 ml double-distilled water followed by heating to boiling point and adding 1.8 ml of 0.1 % formaldehyde. Two ml of gold particle suspension was laid on the 18 X 18 mm-square glass coverslips coated with 0.1 % bovine serum albumin. The

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particle-coated coverslips were placed in 35 mm plastic dishes

containing DMEM/F-12 medium supplemented with 5 % FCS. Cells

were seeded in the dishes and incubated for 24 h. After

incubation, the coverslips were removed and fixed in 10%

formaldehyde solution for 30 min and mounted on microscope slides.

Phagokinetic track areas of more than 20 cells that were randomly

selected were observed with the use of a microscope analyzer

(Cosmo zone R500, Nikon, Japan). The experiments were done under

hypoxic (1% O_2/5 % CO_2) and non-hypoxic conditions (20 % O_2/5 %

CO_2). In some experiments, supernatant of the pancreatic cancer

cells PCI-10 cultured in hypoxia was added in the dishes at 0,

12.5, 25, 50, and 100 %. PHI inhibitors described in the previous

report (Watanabe et al., 1996), erythrose 4-phosphate (E4P) and

6-phosphogluconic acid (6PGA) (Sigma) were added into the dishes

at 10, 100, and 1000 μM.

Statistical analysis:

Statistical analysis was done by the Mann-Whitney test.

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RESULTS

Differentially expressed genes under hypoxic conditions:

We defined the genes expressed at more than 2-fold higher

levels under hypoxic conditions than under non-hypoxic

conditions as possible hypoxia-induced genes, which numbered

38. As expected, mRNAs for glycolytic enzymes and angiogenic

factors were expressed at higher levels under hypoxic conditions

than under non-hypoxic conditions. In the glycolytic enzymes,

we found that phosphohexose isomerase (PHI) mRNA was expressed

at a 2-fold higher level under hypoxic conditions than under

non-hypoxic conditions. A recent report has demonstrated that

AMF, which was originally identified as a molecule stimulating

directional motility (chemotaxis) and random motility

(chemokinesis) of melanoma cells (Liotta et al., 1986), was

identical to PHI/NL (Watanabe et al, 1996). As AMF/PHI/NL

secreted by tumor cells acts as a chemokinetic factor (random

motility-stimulating factor) in an autocrine fashion, we focused

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on this molecule that could promote the metastatic potential

under hypoxic conditions.

Expressions of AMF/PHI/NL and AMFR in a variety of cancer cells:

We then examined the expression of AMF/PHI/NL mRNA in several

cancer cell lines, including PCI-10 cells used in the DNA

microarray analysis, before and after exposure to hypoxia in

order to define whether the enhanced expression of AMF/PHI/NL

mRNA in hypoxia was common in a variety of cancer cells. Fig.

1A shows the expression of AMF/PHI/NL mRNA in the pancreatic

cancer cell lines (PCI-6, PCI-10, PCI-19, PCI-43, PCI-66,

MiaPaca-2, and BxPC-3), colon cancer cell line (KM-12), lung

cancer cell line (PC-6), hepatoma cell line (HepG2) and ovarian

cancer cell lines (TAOV and TTOV). All the cell lines expressed

AMF/PHI/NL mRNA at higher levels under hypoxic conditions than

under non-hypoxic conditions, indicating that the increased

expression of AMF/PHI/NL mRNA under hypoxic conditions was common

in a variety of cancer cells. Next we examined the expression

of AMFR. All the cell lines examined, especially PCI-10 cells

used for the examination of motility, expressed AMFR mRNA and

protein (Fig. 1B and 1C), suggesting that the AMF/PHI/NL could

stimulate the random motility of the cells in an autocrine

fashion.

Expression of AMF/PHI/NL mRNA in HIF-1α-transfectants and

dominant-negative HIF-1α-transfectants:

Fig. 2A shows the expression of AMF/PHI/NL mRNA in the

HIF-1α-transfectants and the vector-transfectant under

non-hypoxic conditions. The expression of AMF/PHI/NL mRNA was

higher in the HIF-1α-transfectants than in the

vector-transfectant. Fig. 2B shows the expression of

dominant-negative HIF-1α (dnHIF-1α) mRNA. The

dnHIF-1α-transfectants but not the vector-transfectant

expressed the truncated dnHIF-1α mRNA in addition to the

endogenous HIF-1α mRNA. Expression of AMF/PHI/NL mRNA was

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enhanced after exposure to hypoxia in the vector-transfectant

and the parent cells but not in the dnHIF-1α-transfectants (Fig.

2C). These results suggested that hypoxia might enhance the

expression of AMF/PHI/NL mRNA through the activation of HIF-1.

Random motility:

As shown in Fig. 3A, the random motility of PCI-10 cells

increased under hypoxic conditions by more than 4-fold compared

with that under non-hypoxic conditions. Furthermore, the

random motility of the HIF-1 α-transfectants under non-hypoxic

conditions was higher than that of the vector-transfectant (Fig.

3B). Fig. 3C shows the random motility of the

dnHIF-1α-transfectants under hypoxic and non-hypoxic

conditions. The random motility of the vector-transfectant but

not of the dnHIF-1α-transfectants was enhanced under hypoxic

conditions. These results in combination with the changes in

the AMF/PHI/NL mRNA expression suggested that the random motility

was closely correlated with the levels of AMF/PHI/NL mRNA

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expression.

Next, as AMF/PHI/NL has been reported to act in an autocrine fashion, we examined the effects of supernatant of PCI-10 cells cultured under hypoxic conditions on their motility. Fig. 3D shows that the supernatant enhanced the random motility of PCI-10 cells under non-hypoxic conditions in a dose-dependent manner, indicating that the random motility-stimulating activity in the supernatant stimulated it in an autocrine fashion. In order to determine whether the presumed random motility-stimulating factor in the supernatant was identical to AMF/PHI/NL, we examined the effects of two carbohydrate phosphates, 6-phosphogluconic acid (6PGA) and erythrose 4-phosphate (E4P), which have been reported to inhibit the PHI enzymatic activity and the AMF-induced cell motility (Watanabe et al., 1996). Fig. 3D shows that 6PGA and E4P almost completely abrogated the motility-stimulating activity of the supernatant.

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Figure 3E shows that 6PGA and E4P directly reduced the random

motility of PCI-10 cells enhanced under hypoxic conditions.

DISCUSSION

A number of reports have demonstrated that the probability

of distant metastases correlates with oxygen pressure in the

tumor tissues (Gatenby et al., 1988; Brizel et al., 1996; Hockel

et al., 1998; Rofstad, 2000), suggesting that hypoxia may promote

metastatic potential. However, it remains poorly understood

how hypoxia promotes the metastatic potential of tumor cells.

In this study, we clearly demonstrated that hypoxia enhanced

the AMF/PHI/NL mRNA expression in a variety of cancer cells and

it also enhanced the random motility in an autocrine fashion.

In accordance with our results, a recent report demonstrated

that AMF/PHI/NL was identified as a hypoxia-inducible gene by

a representational difference analysis using mRNA extracted from

hypoxic and normoxic Capan-2, a human pancreatic cancer cell

line (Yoon et al., 2001). However, it was not determined whether

the enhanced expression of AMF/PHI/NL resulted in the enhanced

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motility. As hypoxia is frequently observed in tumor tissues

in vivo (Guillemin et al., 1997; Blancher et al., 1998), we

suspected that AMF/PHI/NL might be expressed in various cancer

cells *in vivo*. In accordance with this speculation, previous

reports demonstrated that the expression of AMF/PHI/NL was found

in human colorectal, bladder, esophageal, and gastric cancers

and that the expression of AMF/PHI/NL correlated with the disease

progression (Watanabe et al., 1991; Nabi et al., 1992).

Furthermore, we found that AMF/PHI/NL was expressed also in the

tumor cells of human pancreatic cancers *in vivo* (data not shown).

Recently we have reported that most of pancreatic cancer cells,

which are known to show high invasiveness and high metastatic

potential *in vivo*, over-expressed HIF-1α proteins

constitutively (Akakura et al., 2001). Those results in

combination with the findings demonstrated in this study suggest

that high expression of AMF/PHI/NL may be attributable to the

high invasiveness and high metastatic potential of pancreatic

cancers.

It is well-known that metastasis requires coordinated

activation of various factors involved in proliferation,

motility, cell-to-cell and cell-to-substrate contacts,

degradation of extracellular matrix, inhibition of apoptosis,

and adaptation to an inappropriate tissue environment (Poste

et al., 1980; Liotta et al., 1986). Our DNA microarray study

showed that mRNA expressions of various angiogenic factors and

glycolytic enzymes were enhanced in hypoxia in accordance with

the previous reports (Vaupel et al., 1989; Semenza, 2000).

Increased expression of angiogenic factors under hypoxic

conditions enhances the angiogenesis that supports the survival

and growth of tumor cells in the metastatic sites as well as

in the primary sites (Claffey et al., 1996; Rofstad et al., 1999).

Likewise increased expression of glycolytic enzymes supports

the survival and growth of tumor cells under hypoxic conditions

(Malhotra et al., 1999). Accordingly, these angiogenic factors

and glycolytic enzymes induced by hypoxia could enhance the

metastasis in cooperation with AMF/PHI/NL. Namely, hypoxia

promotes the infiltration of endothelial cells into tumor tissues

in its inducing angiogenesis; the hypoxia may also induce the

activation of various factors other than angiogenic factors and

AMF/PHI/NL in cancer cells. We now search the possible

metastasis-associated genes, which should have

hypoxia-responsive elements (HRE) in the promoter region.

All together, our present results provide a new insight into

the mechanisms and a possible means for control of metastasis.

We now propose that the enhancement of metastatic potential may

be one of hypoxic responses of tumor cells exposed to hypoxia.

The findings of dominant-negative HIF-1α-transfectants suggest

that the disruption of the HIF-1 pathway may be an effective

treatment for metastasis, in addition to the treatment of primary

tumors through the inhibition of various genes necessary for

the growth and metastasis of tumor cells *in vivo*, in accordance

with the previous report (Kung et al., 2000).

ACKNOWLEDGEMENTS

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Figure Legends

Figure 1. **Expression of AMF/PHI/NL and AMFR mRNAs**.

A: AMF/PHI/NL mRNA expression. RNAs were extracted from the

cells incubated under non-hypoxic (N) and hypoxic (H) conditions

(20 % and 1 %, respectively) for 24 h and then Northern blot

analysis was performed. B: AMFR mRNA expression. RNAs were

extracted from the cells incubated under non-hypoxic (N) for

24 h and then Northern blot analysis was performed. A

representative result of two different experiments is shown.

C: FACS analysis of AMFR expression. Cells were stained with

anti-AMFR antibody (rat monoclonal antibody, IgM) for 1 h and

then stained with second anti-rat Ig antibody for 1 h. A

representative result of two different experiments is shown.

Figure 2. **Expression of AMF/PHI/NL mRNA in the transfectants**:

A: Northern blot analysis of AMF/PHI/NL mRNA in the

HIF-1α-transfectants (PCI-10/H2 and PCI-10/H3) and vector-transfectant (PCI-10/V2) under non-hypoxic conditions. AMF/PHI/NL mRNA expression in PCI-10 cells under non-hypoxic (N) and hypoxic (H) conditions were shown as controls. B: Northern blot analysis of dominant negative HIF-1α (dnHIF-1α) mRNA in the transfectants. C: Northern blot analysis of AMF/PHI/NL mRNA expression in the dnHIF-1α -transfectants (PCI-43/dnH3 and PCI-43/dnH7) and the vector-transfectant (PCI-43/vV3) under non-hypoxic (N) and hypoxic (H) conditions. RNAs were extracted from the cells incubated under non-hypoxic (N) and hypoxic (H) conditions for 24 h and then Northern blot analysis was performed.

Figure 3. **Random motility**:

Data present the mean ± SD of three different experiments. A: Random motility of the PCI-10 cells under hypoxic and normoxic

conditions. *P<0.01; significantly different compared with the

relative motility of the PCI-10 cells under normoxic conditions.

B: Random motility of the HIF-1α-transfectants under non-hypoxic

conditions. *P<0.01; significantly different compared with the

vector control. C: Random motility of the

dnHIF-1α-transfectants and the vector-transfectant under

hypoxic and non-hypoxic conditions. *P<0.05; significantly

different compared with the relative motility of the PCI-43 cells

under normoxic conditions. D: Random motility of PCI-10 cells

under normoxic conditions in the presence or absence of the

supernatant of PCI-10 cells cultured under hypoxic conditions.

*P<0.01, **P<0.05; significantly different compared with the

relative motility of PCI-10 cells under normoxic conditions in

the absence of supernatant. †P<0.05; significantly different

compared with the relative motility of PCI-10 cells in the

presence of 100 % supernatant. E: Random motility of the PCI-10

cells under hypoxic conditions in the presence of

AMF/PHI-inhibitors. *P<0.01; significantly different

compared with the relative motility of PCI-10 cells under

normoxic conditions. † P<0.01; significantly different

compared with the relative motility of PCI-10 cells under

normoxic conditions in the absence of inhibitor.