Upregulation of fractalkine contributes to the proliferative response of prostate cancer cells to hypoxia via promoting the G₁/S phase transition

JIEBING TANG¹,²*, YUANYUAN CHEN¹*, RONGJUN CUI³, DONG LI¹, LIJIE XIAO¹, PING LIN¹, YANDAN DU⁴, HUI SUN¹⁴, XIAOGUANG YU¹ and XIULAN ZHENG⁵

¹Department of Biochemistry and Molecular Biology, Harbin Medical University; ²Department of Gastrointestinal Medical Oncology, The Affiliated Tumor Hospital of Harbin Medical University, Harbin, Heilongjiang 150081; ³Department of Biochemistry and Molecular Biology, Mudanjiang Medical University, Mudanjiang, Heilongjiang 157011; ⁴Department of Clinical Laboratory, The Second Clinical Medical School of Inner Mongolia University for the Nationalities, Inner Mongolia Forestry General Hospital, Hulunbuir, Inner Mongolia 022150; ⁵Department of Ultrasonography, The Affiliated Tumor Hospital of Harbin Medical University, Harbin, Heilongjiang 150081, P.R. China

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Abstract. Hypoxia is a common phenomenon in prostate cancer, which leads to cell proliferation and tumor growth. Fractalkine (FKN) is a membrane-bound chemokine, which is implicated in the progression of human prostate cancer and skeletal metastasis. However, the association between FKN and hypoxia-induced prostate cancer cell proliferation remains to be elucidated. The present study demonstrated that hypoxia induced the expression and secretion of FKN in the DU145 prostate cancer cell line. Furthermore, inhibiting the activity of FKN with the anti-FKN FKN-specific antibody markedly inhibited hypoxia-induced DU145 cell proliferation. Under normoxic conditions, DU145 cell proliferation markedly increased following exogenous administration of human recombinant FKN protein, and the increase was significantly alleviated by anti-FKN, indicating the importance of FKN in DU145 cell proliferation. In addition, subsequent determination of cell cycle distribution and expression levels of two core cell cycle regulators, cyclin E and cyclin-dependent kinase (CDK)2, suggested that FKN promoted the G₁/S phase transition by upregulating the expression levels of cyclin E and CDK2. The results of the present study demonstrated that hypoxia led to the upregulation of the secretion and expression of FKN, which enhanced cell proliferation by promoting cell cycle progression in the prostate cancer cells. These findings provide evidence of a novel function for FKN, and suggest that FKN may serve as a potential target for treating androgen-independent prostate cancer.

Introduction

Prostate cancer is one of the most common types of non-cutaneous malignancy in males. In the United States, the incidence of prostate cancer is highest among male malignant tumors (1). In China, increasing evidence suggests that the age of onset of prostate cancer has been decreasing in recent years (2). Although early-stage prostate cancer can be cured by radical prostatectomy, for patients with clinically detectable metastasis, there remains a lack of effective treatment options (3).

Intratumoral hypoxia prevails in several types of fast growing solid tumor and is a marker of poor clinical prognosis in prostate cancer (4). The reaction of tumor cells to hypoxia includes the generation of signal transducing molecules, predominantly growth factors and cytokines, and changes in tumor cell function, which affect cell proliferation, de-differentiation, resistance to apoptosis and metastatic potential (5).
Chemokines are small, chemoattractant proteins, which have the ability to chemoattract cells expressing their cognate G protein–coupled receptors. On the basis of structure, chemokines are divided into four subfamilies: CXC, CC, C and CX3C (6). Several studies have suggested that chemokines and their receptors are involved in a number of disease states, including cardiovascular disease (7), systemic inflammation (8), cancer (9) and infectious disorders (10). Fractalkine (FKN, also known as CX3CL1), is a unique chemokine subclass and the only member of the CX3C chemokine family, existing as a membrane-bound form and a soluble form. The membrane-bound form is synthesized as a transmembrane molecule with an extracellular N-terminal domain attached by a mucin-like stalk to the cell surface (6). Soluble FKN is generated via cleavage at the base of the mucin-like stalk by the metalloproteinases, A disintegrin and metalloproteinase 10 and 17 (11). Several studies have identified that FKN is expressed by a variety of tumors, including prostate cancer, and there has been increasing interest in its involvement in prostate cancer (12-15). FKN-CX3CR1 is reported to be involved in the molecular events that regulate the adhesion, migration and survival of human prostate cancer cells (15). In addition, FKN-CX3CR1 binding has also been observed to be crucial in the progression of prostate cancer and skeletal metastasis (12).

Hypoxia leads to tumor cell proliferation and tumor growth. Emerging evidence has indicated that hypoxia can promote tumor growth by upregulating specific chemokine receptors, including CCR2 (16). Our previous study demonstrated that hypoxia increases the expression of CX3CR1 via the hypoxia-inducible factor and nuclear factor-κB signaling pathway in androgen-independent prostate cancer cells (17). However, the association between FKN and hypoxia-induced prostate cancer cell proliferation remains to be fully elucidated.

The present study aimed to determine the effect of a hypoxic microenvironment on the expression of FKN and the role of FKN in hypoxia-induced prostate cancer cell proliferation. The results showed that FKN expression was upregulated under hypoxic conditions, which resulted in enhanced proliferation of prostate cancer cells.

Materials and methods

Cell lines and reagents. DU145 and PC-3 human prostate cancer cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Human recombinant FKN was purchased from R&D systems, Inc. (Minneapolis, MN, USA). Anti-human FKN antibody was purchased from Abcam (Cambridge, MA, USA). CDK2 primary antibody [mouse mononclonal immunoglobulin (IgG); cat no. sc-6248] was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA) and cyclin E primary antibody (rabbit monoclonal IgG; cat no. 2978s) was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The secondary antibodies for CDK2 (anti-mouse IgG; cat no. A16027) and cyclin E (anti-rabbit IgG; cat no. A16104) were from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All primers used in the present study were synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

Cell culture and hypoxia exposure. Under normoxic conditions the cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA) and 1X penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The cells that were grown under hypoxic conditions were incubated in a hypoxic chamber (Thermo Fisher Scientific, Inc.) containing 1% O₂, 5% CO₂ and 94% N₂ at 37°C.

Cell proliferation assay. The cells were seeded at a density of 3x10⁴ cells/well into a 96-well plate. Cell proliferation was measured following 12, 24, 36, 48 and 72 h of normoxic or hypoxic culture using a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's instructions. Briefly, CCK-8 reagents were added to a subset of wells and incubated for 2 h at 37°C, following which the absorbance was measured at a test wave length of 450 nm on an automated plate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA).

Colony formation assay. The cells were seeded into a 6-well plate at a density of 200 cells/well and incubated for 10 days to allow colony formation. The colonies were fixed with 3% formaldehyde (Tianjin Fuyu Fine Chemical Co., Ltd., Tianjin, China) and stained with 0.1% crystal violet (Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China). The number of colonies containing >50 cells were counted. The colonies were manually counted using a microscope (Olympus TH-200; Olympus, Tokyo, Japan) and images of the colonies were captured.

Flow cytometric analysis of cell cycle. Cell cycle analysis was performed using flow cytometry. DU145 cells were pre-incubated with FKN, with 200 pg/ml human recombinant FKN protein added to the medium when the cells were 80% confluent. The cells were harvested and fixed with 70% ethanol prior to being stored at 4°C overnight. The fixed cells were incubated with RNase (25 µg/ml; Sigma-Aldrich, St Louis, MO, USA) at 37°C for 30 min, and the DNA was subsequently stained with propidium iodide (50 µg/ml; Sigma-Aldrich) for 30 min in the dark. The stained cells were then analyzed by fluorescence-activated cell sorting using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells incubated under normoxic or hypoxic conditions using TRIzol® reagent (Invitrogen Life Technologies). RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies), according to the manufacturer's instructions. The cDNA (1 µg in 4 µl buffer) was amplified by qPCR in an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific) using the SYBR Select Master Mix kit (Applied Biosystems) and with gene-specific primers (1 µl). The primers used in the present study were as follows: β-actin sense, 5'-TACCTCATGAGATCCTCACC-3' and antisense, 5'-TTTCTGGATGTCACAGAC-3'; CDK2 sense, 5'-CAGGATGTGACCAAGCGATA-3' and antisense, 5'-CCAACCTCTCCAGCAATA-3'; cyclin E sense, 5'-ACGACGACGACGAAAACCTC-3' and antisense, 5'-GTGTCGACGCTGAAGAAC-3'; and FKN sense,
5'-CTTTCTCATCCACTATAACA-3'; and antisense, 5'-CTCCACTCTTCTTTC-3'. All the primers were purchased from Sangon Biotech Co. Ltd. The thermocycling conditions were initial denaturation at 94°C for 2 min, followed by 30 cycles of 98°C for 10 sec, 60°C for 15 sec and 68°C for 2 min. Each reaction was repeated in triplicate to minimize experimental variation, and the expression levels of β-actin were used as an internal control. The results were quantitated by scanning densitometry using a Bio-Rad 620 Video Densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. The total protein of cyclin E and CDK2 were extracted prior to western blot analysis. Western blot analysis was performed, as described previously (18). Briefly, the cells were washed with PBS and re-suspended in cold lysis buffer with phenylmethanesulfonylfluoride. The cell lysate was incubated on ice for 30 min and centrifuged at 12,000 x g for 15 min at 4°C. The protein concentration of the lysate was determined using a BCA-200 protein assay kit (Beyotime Institute of Biotechnology, Inc., Haimen, China). Equal amounts of protein (40 μg/lane) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes (Pall Corp., Port Washington, NY, USA). Following blocking with 5% fat-free milk for 2 h at room temperature, the membranes were incubated with various primary antibodies (1:500) at 4°C overnight. Subsequently, the bound primary antibody was detected by incubating with appropriate horseradish peroxidase-conjugated secondary antibodies (1:1,000) for 2 h at room temperature, followed by washing with Tris-buffered saline containing Tween 20 three times. The immunoreactive bands were visualized using blot analysis Super ECL Plus Detection Reagents (Applygen Technologies Inc., Beijing, China). The volumes of the protein bands were quantified using a Bio-Rad Chemi Doc™ EQ densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories). The expression levels of tubulin were assessed as an internal control.

ELISA assay. The concentration of soluble FKN in the supernatant was determined using an FKN ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions and in accordance with the procedure of a previous study (19).

Statistical analysis. The results of the present study are expressed as the mean ± standard error of the mean. Each experiment was repeated in triplicate. One-way analysis of variance was used for multiple comparisons. Statistical analyses were performed using SAS software version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Statistical differences between two groups were analyzed using an unpaired two tailed Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Hypoxia induces prostate cancer cell proliferation. To examine the response of prostate cancer cell proliferation to hypoxia, two types of androgen-independent prostate cancer cell lines, DU145 and PC-3, were cultured under normoxic or hypoxic conditions, respectively. Firstly, cell viability was determined following 12, 24, 36, 48 and 72 h of culture using a CCK-8 assay. Morphological changes were also observed using an inverted microscope. DU145 and PC-3 cell proliferation was continuous under the hypoxic and normoxic conditions. The cell proliferation rates of the DU145 and PC-3 cells exposed to hypoxia were significantly higher, compared with those under normoxia at 24-48 h (P<0.05; Fig. 1A and B). Furthermore, microscopic observation revealed markedly increased cell density in the two cell lines following 24 h culture under hypoxic conditions, compared with normoxic conditions, and cell growth under hypoxic and normoxic conditions remained normal as observed by inverted microscopy (Fig. 1C and D). Subsequently, the colony-forming ability of the DU145 and PC-3 cells under the two conditions were determined in order to further confirm the effect of hypoxia on prostate cancer cell proliferation. The clone clusters of the two cell lines under hypoxic conditions were larger and more numerous, compared with those under normoxic conditions for 10 days (Fig. 2). The results suggested that hypoxia induced DU145 and PC-3 cell proliferation. In addition, the DU145 cell proliferative response to hypoxic treatment was more marked, compared with that of the PC-3 cells. Therefore, DU145 cells were selected as the experimental cells for subsequent investigation of the underlying molecular mechanisms.

Hypoxia upregulates the mRNA expression and secretion of FKN. To assess the effects of hypoxia on mRNA expression, the mRNA expression levels of FKN in DU145 cells was examined 0, 6, 12 and 24 h following hypoxic treatment. RT-qPCR demonstrated that FKN was expressed at low levels in the DU145 cells. However, with increasing duration of hypoxic treatment, the mRNA expression of FKN increased in a time-dependent manner, with significant differences at 12 and 24 h (P<0.05; Fig. 3A and B), which resembled the response of cell proliferation to hypoxia. These results suggested that hypoxia-upregulated the mRNA expression of FKN in a time-dependent manner in the prostate cancer cells. In addition, the concentration levels of soluble FKN in the supernatant, determined using an ELISA kit, revealed that the protein secretion levels of FKN in the DU145 cells increased significantly following 24 h of hypoxic treatment, compared with those following normoxic treatment (P<0.05; Fig. 3C). Therefore, the results suggested a positive correlation between FKN and hypoxic prostate cell proliferation.

Hypoxia-induced prostate cancer cell proliferation is mediated by FKN. To investigate the role of FKN in hypoxia-induced prostate cancer cell proliferation, FKN-specific antibody anti-FKN or isotype IgG were added to the hypoxic DU145 cells, and cell proliferation was determined following 24 and 48 h of incubation using a CCK-8. As shown in Fig. 4A, DU145 cell proliferation was markedly inhibited by anti-FKN pretreatment (P<0.05). To further verify the role of FKN in prostate cancer cell proliferation, the proliferation of the cells was also examined following exogenous administration of human recombinant FKN protein (100 pg/ml or 200 pg/ml) under normoxic conditions. As shown in Fig. 4B, DU145 cell proliferation was markedly enhanced by treatment with 200 pg/ml exogenous FKN protein (P<0.05), and this increase was significantly alleviated by anti-FKN (P<0.05). These data suggested that FKN was involved in the regulation of hypoxic prostate cancer cell proliferation.
Figure 1. Hypoxia increases prostate cancer cell proliferation in androgen-independent prostate cancer cell lines. Proliferation of (A) DU145 and (B) PC-3 cells cultured in hypoxic and normoxic conditions for different durations were determined using a Cell Counting kit-8 assay. Data are expressed as the mean ± standard error of the mean. Microscopic observation of (C) DU145 and (D) PC-3 following 24 h of culture under hypoxic or normoxic conditions (magnification, x100). Cell density significantly increased in the two cell lines following 24 h of hypoxic culture, compared with normoxic culture. *P<0.05, vs. normoxic conditions. OD, optical density.

Figure 2. Hypoxia increases colony-forming ability. The colony-forming ability of the (A) DU145 and (B) PC-3 cells (crystal violet stained) were determined using a colony formation assay. The cells were seeded into a six-well plate and incubated for 10 days to allow colony formation. The clone clusters of the two cell lines under hypoxic treatment were larger and more numerous, compared with those under normoxic treatment for 10 days.

Figure 3. Hypoxia increases the mRNA expression and secretion of FKN in DU145 cells. (A) RT-qPCR analysis of the mRNA expression of FKN. The intensity of each band was quantified using densitometry, and data were normalized to those of actin. (B) ELISA analysis of the protein secretion of FKN in the supernatant. *P<0.05, vs. 0 h. Data are expressed as the mean ± standard error of the mean. FKN, fractalkine; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
FKN promotes G₁/S phase transition by upregulating the expression levels of cyclin E and CDK2. Cell proliferation is closely associated with cell cycle regulation. In order to determine whether hypoxia-induced prostate cancer cell proliferation was associated with cell cycle regulation by FKN, the cell cycle distribution in the DU145 cells was measured under normoxic or hypoxic conditions using flow cytometric analysis (Fig. 5). Compared with normoxic conditions (Fig. 5A and B), hypoxic treatment decreased the proportion of cells in the G₁ phase, between 73.31 and 54.21%, and increased the proportion of cells in the S phase, between 23.00 and 44.63%. However, anti-FKN pre-treatment increased the proportion of cells in G₁ phase from 54.21 to 75.59% and decreased the S-phase population from 44.63 to 19.80%. FKN, fractalkine; aFKN, anti-FKN; rFKN, human recombinant FKN protein.
human recombinant FKN protein under normoxic conditions was analyzed. As shown in Fig. 5A and B, exogenous FKN protein administration decreased the proportion of cells in the G1 phase between 73.31 to 52.63%, and increased the proportion of cells in the S phase between 23.00 and 44.59%. These results suggested that FKN promoted the G1/S phase cell cycle transition.

Cell cycle progression depends on the formation of CDK/cyclin complexes, and cyclin E and CDK2 are G1/S phase-specific regulators (20). Therefore, the present study determined the mRNA and protein expression levels of cyclin E and CDK2 using RT-qPCR and western blot analysis. As shown in Fig. 6A-D, the mRNA and protein expression levels of cyclin E and CDK2 in the DU145 cells exposed to hypoxia were significantly higher than those in the cells exposed to normoxic conditions (P<0.05), and anti-FKN pre-treatment markedly attenuated the increased expression levels of cyclin E and CDK2 (P<0.05). To further verify the role of FKN in the regulation of cyclin E and CDK2, the expression levels of cyclin E and CDK2 in DU145 cells exposed to normoxia with anti-FKN or exogenous FKN protein pre-treatment were also examined. The mRNA and protein expression levels of cyclin E and CDK2 were significantly elevated by 200 pg/ml exogenous FKN protein administration (P<0.05), and anti-FKN pretreatment markedly alleviated this increase (P<0.05; Fig. 6C and D).
Discussion

Prostate cancer is the most frequently diagnosed type of malignancy and is the second leading cause of cancer-associated mortality in males (21). Early-stage prostate cancer depends on androgens for growth and survival, and androgen ablation therapy causes them to regress (22). However, cancer, which is not cured by surgical procedures eventually become androgen-independent, and androgen ablation fails to target androgen-independent cells (23). Therefore, examining the underlying molecular mechanisms and potential therapeutic targets in androgen-independent prostate cancer is essential for improving clinical outcomes. The present study presented data, which demonstrated that hypoxia, a common phenomenon in prostate cancer, induced the expression and secretion of FKN, and that antagonizing hypoxia inhibited hypoxic prostate cancer cell proliferation.

A hypoxic microenvironment contributes to the progression of cancer (19). Under certain conditions, the interaction between tumor cells and their microenvironment is facilitated by a variety of soluble factors, including growth factors and chemokines (24). Hypoxia has been suggested to promote cell migration and invasion in various types of tumor by regulating specific chemokine receptors (25-27). FKN is the only member recognized so far, which belongs to the CX3C chemokine subfamily. FKN exists as a membrane-bound form functioning as an adhesion molecule, and as a soluble form acting as a potent chemoattractant for monocytes, natural killer cells and T cells (28). Therefore, FKN is important in several disorders comprising imbalance of the immune response, including arthritis (29), asthma (30), human immunodeficiency virus (31) and Crohn’s disease (32). In addition, the involvement of FKN/CX3CR1 in the pathogenesis and progression of multiple malignant diseases has also been a focus of interest. For certain tumor entities, FKN was demonstrated to be correlated with a higher local recurrence risk and metastatic potential (33,34). In epithelial ovarian cancer cells, FKN functions as an important regulator of malignant cell proliferation via binding to CX3CR1 and consequently activating AKT signaling (35). In prostate cancer, neutralizing antibodies against FKN markedly repress the ability of prostate cancer cells to adhere to the bone marrow endothelium (15). In the present study, the results demonstrated that hypoxia promoted cell proliferation in two types of androgen-independent prostate cancer cell lines, DU145 and PC-3. Subsequently, the effects of hypoxia on FKN synthesis and secretion were investigated in the prostate cancer cells. The results demonstrated that hypoxia induced the secretion and mRNA expression of FKN in a sustained manner. Based on these observations, it was hypothesized that FKN was associated with prostate cancer cell proliferation. The present study subsequently determined DU145 cell proliferation rates following FKN inhibition with the anti-FKN FKN-specific antibody under hypoxic conditions. The results demonstrated that anti-FKN pre-treatment markedly inhibited DU145 cell proliferation. To further investigate the role of FKN under normoxic conditions, DU145 cell proliferation was examined following the administration of exogenous human recombinant FKN protein. The results revealed that DU145 cell proliferation markedly increased, and this increase was significantly alleviated by treatment with anti-FKN, which indicated that DU145 cell proliferation was mediated by FKN. These data suggested that upregulation of the expression and secretion of FKN was involved in hypoxia-induced prostate cancer cell proliferation.

Cellular proliferation follows an orderly progression through the cell cycle. The traditional subdivisions of the standard cell cycle include the G1, S, G2, and M phases (36). Accumulating evidence has suggested the relevance of cell cycle deregulation in human cancer. The majority of types of human cancer exhibit deregulated control of G1 phase progression, a period during which the cells either initiate proliferation or remain quiescent (37). The present study examined the role of FKN in prostate cancer cell cycle regulation using flow cytometry, and the results revealed that either exogenous FKN protein administration or hypoxic treatment notably increased the proportion of cells in the S phase, and anti-FKN pre-treatment attenuated hypoxia-induced cell accumulation in the S phase. These data indicated that FKN regulated the G1/S checkpoint, resulting in promotion of the G1 to S phase transition. The transition between one cycle phase and another occurs in order and is regulated by a variety of cell cycle regulators, the most prominent of which are cyclins and their associated CDKs. Of these, cyclin E is important for initiation of the S phase, and its association with CDK2 regulates the progression between the G1 and S phase (38). The results of the present study demonstrated that antagonizing FKN attenuated the hypoxia-induced mRNA and protein expression levels of cyclin E and CDK2 in the prostate cancer cells. By contrast, prostate cancer cells, exposed to exogenous FKN protein pre-treatment under normoxic conditions exhibited elevated mRNA and protein expression levels of cyclin E and CDK2, and anti-FKN pre-treatment markedly alleviated this elevation. These findings suggested that FKN promoted G1/S cell cycle progression through upregulating the expression levels of cyclin E and CDK2, resulting in hypoxic prostate cancer cell proliferation.

Aberrant epidermal growth factor receptor (EGFR) signaling is associated with characteristics of aggressive malignancies, including increased proliferative potential, nitric oxide synthesis and accelerated G1/S cell cycle progression. White et al (39) reported that EGFR activation is involved in the proliferation of human vascular smooth muscle cells in response to FKN treatment. Therefore, hypoxia may induce the secretion of FKN and subsequent EGFR signaling activation, which promotes cell cycle progression, resulting in increased cell proliferation.

In conclusion, the results of the present study provided evidence of a novel function for FKN, which enhances cell proliferation by promoting cell cycle progression in hypoxic prostate cancer cells. Further investigations are required to further expand on these findings, with the goal of identifying a potential therapeutic target.

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