Biochemical and Biophysical Research Communications 455 (2014) 332–338

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Treatment with insulin-like growth factor 1 receptor inhibitor reverses hypoxia-induced epithelial–mesenchymal transition in non-small cell lung cancer

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ARTICLE INFO

Article history:
Received 27 October 2014
Available online 15 November 2014

Keywords:
IGF1R signaling
EMT
NSCLC

ABSTRACT

Insulin-like growth factor 1 receptor (IGF1R) is expressed in many types of solid tumors including non-small cell lung cancer (NSCLC), and enhanced activation of IGF1R is thought to reflect cancer progression. Epithelial–mesenchymal transition (EMT) has been established as one of the mechanisms responsible for cancer progression and metastasis, and microenvironment conditions, such as hypoxia, have been shown to induce EMT. The purposes of this study were to address the role of IGF1R activation in hypoxia-induced EMT in NSCLC and to determine whether inhibition of IGF1R might reverse hypoxia-induced EMT. Human NSCLC cell lines A549 and HCC2935 were exposed to hypoxia to investigate the expression of EMT-related genes and phenotypes. Gene expression analysis was performed by quantitative real-time PCR and cell phenotypes were studied by morphology assessment, scratch wound assay, and immunofluorescence. Hypoxia-exposed cells exhibited a spindle-shaped morphology with increased cell motility reminiscent of EMT, and demonstrated the loss of E-cadherin and increased expression of fibronectin and vimentin. Hypoxia also led to increased expression of IGF1, IGF binding protein-3 (IGFBP3), and IGF1R, but not transforming growth factor β1 (TGFβ1). Inhibition of hypoxia-inducible factor 1α (HIF1α) with YC-1 abrogated activation of IGF1R, and reduced IGF1 and IGFBP3 expression in hypoxic cells. Furthermore, inhibition of IGF1R using AEW541 in hypoxic condition restored E-cadherin expression, and reduced expression of fibronectin and vimentin. Finally, IGF1 stimulation of normoxic cells induced EMT. Our findings indicated that hypoxia induced EMT in NSCLC cells through activation of IGF1R, and that IGF1R inhibition reversed these phenomena. These results suggest a potential role for targeting IGF1R in the prevention of hypoxia-induced cancer progression and metastasis mediated by EMT.

1. Introduction

Insulin-like growth factor 1 receptor (IGF1R) is a transmembrane receptor tyrosine kinase, and is expressed in many types of cancer cells, including non-small cell lung cancer (NSCLC) [1]. IGF1R is activated by binding of its ligand, IGF1, leading to phosphorylation of phosphatidylinositol 3-kinase (PI3K) and phospholipase C gamma (PLCγ) that produces inositol 1,4,5-trisphosphate (IP_3) followed by Ca^{2+} release [2]. Cell signaling via IGF1R is essential in a variety of cellular processes including cancer progression [1].

Advanced NSCLC is the leading cause of cancer-related deaths worldwide [3]. Drug-resistance, metastasis, and invasion are some of the features of fatal cancer, and accumulating evidence has shown that metastasis and invasion are associated with an epithelial–mesenchymal transition (EMT) [4,5].

EMT is characterized by the dissolution of cell–cell junctions and loss of apico-basolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties [6]. During

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; YC-1, 5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol; IGF1R, insulin-like growth factor 1 receptor; IGFBP3, insulin-like growth factor-binding protein 3; phospho-IGF1R, phospho-insulin-like growth factor 1; HIF1α, hypoxia-inducible factor 1α; DMSO, dimethyl sulfoxide; qPCR, quantitative real-time PCR.

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http://dx.doi.org/10.1016/j.bbrc.2014.11.014
0006-291X/

EMT, cells loose expression of the epithelial marker E-cadherin, and gain the mesenchymal markers vimentin and fibronectin. The mesenchymal state is associated with the capacity of cells to migrate to distant organs during the initiation of metastasis [7]. Several factors have been found to be capable of inducing EMT, including transforming growth factor β (TGFβ) [8] and tumor necrosis factor α (TNFα) [9]. Solid tumors often contain regions with insufficient oxygen delivery, a condition known as hypoxia, and several recent reports have suggested that hypoxia might also induce EMT [10]. Hypoxia-induced EMT has been investigated in various cancers, including gastric cancer [11], hepatocellular [12] and renal cell carcinomas [13]. The stabilization of hypoxia-inducible factor 1α (HIF-1α) by hypoxia allows for activation of EMT-inducer. It has been reported that under hypoxic conditions, pancreatic carcinoma cells lost cell–cell adhesion due to down-regulation of E-cadherin and concomitant up-regulation of vimentin [14]. In another study, hypoxic stress induced EMT in colon cancer cells, through β1-integrins and chemokine receptor type 4 (CXCR4) [15].

It has been reported that IGF1R is implicated in EMT-mediated invasiveness in gastric cancer [16]. However, a direct linkage of IGF1R activation with hypoxia and EMT has not been clarified. In the present study, we aimed to investigate whether activation of IGF1R is involved in hypoxia-induced EMT in NSCLC cells and to elucidate whether inhibition of IGF1R could reverse such hypoxia-induced EMT.

2. Materials and methods

2.1. Cell culture and reagents

NSCLC cell lines, A549 and HCC2935, were purchased from the American Type Culture Collection (Rockville, MD, USA). HCC2935 cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan), while A549 was maintained in DMEM medium (Invitrogen, Carlsbad, CA, USA); both media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Cell lines were verified to be mycoplasma-free using the MycoAlert Kit (Lonza, Amagazaki, Japan). Cells were grown in a humidified 5% CO_2 atmosphere at 37 °C in an incubator, in which the oxygen tension was held at either 21% (normoxia) or 1% (hypoxia). AEW541 was purchased from Selleck Chemicals (Houston, TX, USA) and YC-1 was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Morphological analysis

Cells were visualized at 200× magnification with an Olympus light microscope (Olympus, Tokyo, Japan). Digital images of the A549 and HCC2935, in both normoxic and hypoxic conditions were randomly captured and examined for morphologic characteristics consistent with EMT. Spindle-shaped cells were counted and divided by the total cell number from each image to obtain the spindle-shaped cell percentage. Results were displayed as the means of spindle-shaped cell percentages from 5 images.

2.3. Wound healing scratch assay

A549 and HCC2935 cells were seeded in 6-well plate and allowed to attach to the plate surface for 24 h. A scratch was made in the center of the culture well using a sterile 200-μL micropipette tip and images were captured immediately after the scratch at 0 h and again after 24 h incubation at 37 °C in both normoxia and hypoxic conditions.

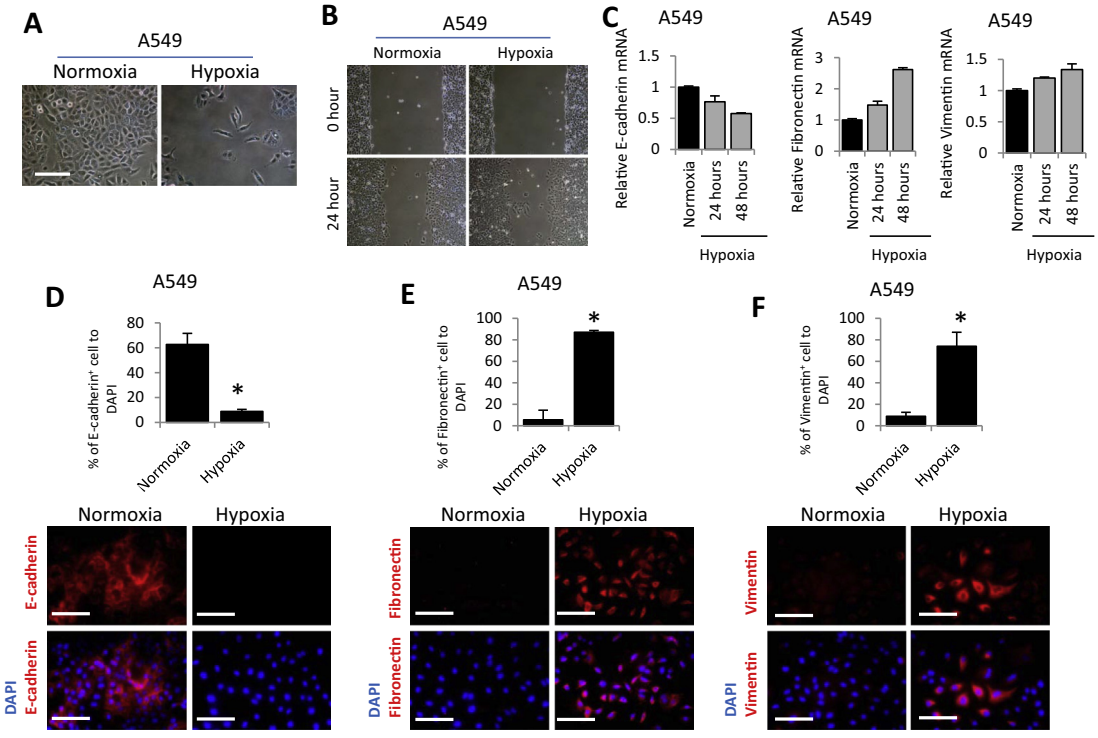

Fig. 1. Hypoxia induced EMT in A549 cells. (A) Hypoxia altered cell morphology to irregular and spindle-shaped. (B) Scratch was generated in the center of the culture well at 0 and after 24 h in normoxic or hypoxic conditions. (C) Quantitative real-time PCR was performed with primers specific for E-cadherin, fibronectin, and vimentin in A549 parental and hypoxic cells (24 and 48 h), and data were normalized to actin expression. (D–F) A549 cells, grown on Lab-Tek chamber slides, were exposed to normoxia or hypoxia for 24 h, fixed, and incubated with primary antibodies against E-cadherin (D), fibronectin (E), or vimentin (F) followed by secondary antibodies labeled with Alexa Fluor 488 anti-rabbit IgG (red). Cell nuclei were stained with DAPI (blue). Images were obtained using Axioplan 2 system. Five images were captured from each group and E-cadherin$^+$, vimentin$^+$, and fibronectin$^+$ cells numbers were divided by the corresponding DAPI numbers. Data are expressed as the means ± standard deviation. $^*P < 0.05$ indicates a significant difference from control group. Scale bar indicates 200 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)