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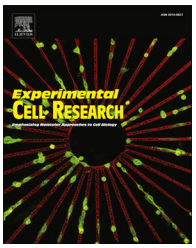

Research Article

Snail/beta-catenin signaling protects breast cancer cells from hypoxia attack

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ABSTRACT

The tolerance of cancer cells to hypoxia depends on the combination of different factors – from increase of glycolysis (Warburg Effect) to activation of intracellular growth/apoptotic pathways. Less is known about the influence of epithelial–mesenchymal transition (EMT) and EMT-associated pathways on the cell sensitivity to hypoxia. The aim of this study was to explore the role of Snail signaling, one of the key EMT pathways, in the mediating of hypoxia response and regulation of cell sensitivity to hypoxia, using as a model in vitro cultured breast cancer cells. Earlier we have shown that estrogen-independent HBL-100 breast cancer cells differ from estrogen-dependent MCF-7 cells with increased expression of Snail1, and demonstrated Snail1 involvement into formation of hormone-resistant phenotype. Because Snail1 belongs to hypoxia-activated proteins, here we studied the influence of Snail1 signaling on the cell tolerance to hypoxia. We found that Snail1-enriched HBL-100 cells were less sensitive to hypoxia-induced growth suppression if compared with MCF-7 line (31% MCF-7 vs. 71% HBL-100 cell viability after 1% O_2 atmosphere for 3 days). Snail1 knock-down enhanced the hypoxia-induced inhibition of cell proliferation giving the direct evidence of Snail1 involvement into cell protection from hypoxia attack. The protective effect of Snail1 was shown to be mediated, at least in a part, via beta-catenin which positively regulated expression of HIF-1-dependent genes. Finally, we found that cell tolerance to hypoxia was accompanied with the failure in the phosphorylation of AMPK – the key energy sensor, and demonstrated an inverse relationship between AMPK and Snail1/beta-catenin signaling.

Totally, our data show that Snail1 and beta-catenin, besides association with loss of hormone dependence, protect cancer cells from hypoxia and may serve as an important target in the treatment of breast cancer. Moreover, we suggest that the level of these proteins as well the level

Abbreviations: HRE, hypoxia-responsive element; HIF-1, hypoxia-inducible factor-1; MTT, 3-[4,5–dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA; AMPK-5′, adenosine monophosphate-activated protein kinase; PBS, phosphate buffered saline; PFA, paraformaldehyde; TRITC, tetramethylrhodamine isothiocyanate mixed isomers; EMT, epithelial-mesenchymal transition; MTA3, metastasis-associated protein 3; ER alpha, estrogen receptor alpha; FCS, fetal calf serum; TCF, T-cell-specific transcription factor; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, DL-dithiothreitol; TBS, Tris-buffered saline

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of AMPK phosphorylation may be considered as predictors of the tumor sensitivity to anti-angiogenic drugs.

Introduction

Tumor cells are characterized with the increased tolerance to hypoxia and an effective adaptation to hypoxic conditions *in vivo* and *in vitro*. The hypoxic response is mediated via the hypoxia-inducible factor-1 (HIF-1) which regulates the expression of numerous genes involved in different cellular pathways – from glucose metabolism to cell proliferation/survival and angiogenesis [1–4]. An important part of hypoxia response is the stimulation of epithelial–mesenchymal transition (EMT) mediated, at least in a part, via the HIF-1-dependent activation of Snail family proteins, and resulting in a formation of mesenchymal-like cell phenotype, loss of cell contacts and increase in cell motility [5–7]. As shown, HIF-1 induces direct activation of Snail expression – through interaction with canonical hypoxia-responsive element (HRE) in Snail promoter [8], and/or stimulates Snail indirectly – via activation of TGF-beta and Notch signaling [7,9]. The experiments with different breast cancer cells revealed the increase in Snail expression with correspondent decline in the epithelial markers under hypoxia whereas the cell migratory activity was not completely affected [5]. The more poorly differentiated and higher proliferated breast tumors were found to be characterized with increased Snail expression [10]; the association between mammary tumor recurrence and the level of Snail protein has been shown [11].

Are the EMT-associated pathways involved in the cell adaptation to hypoxia and respondent for support of cell growth under hypoxia, what pathways are involved, and what is the mechanism of such protection – there are still open questions.

Intriguingly, the tolerance to hypoxia in breast tumors is often accompanied with the loss of hormonal dependency and increase in the cell resistance to (anti)estrogens [12,13]. The numerous studies demonstrate the inverse correlation between estrogen receptor (ER) status of breast cancers and expression of HIF-1 and hypoxia-related genes [14–16]. Among the latter, the important role belongs to Snail family proteins which negatively control the ER expression/activity in breast cancers (see Ref. [17]). As revealed, Snail directly inhibits ER alpha expression, and overexpression of Snail protein was found to be associated with ER alpha negative status of breast tumors [18]. ER alpha in turn inhibits Snail expression – partially, via activation of MTA3 (metastasis-associated protein 3) pathway [18,19]. We have shown that estrogen resistance of breast cancer cells correlated with increase in Snail1 expression and activity, and demonstrated that Snail1 inhibition partially restored the sensitivity of the resistant cells to antiestrogen tamoxifen [20].

Totally, the mechanism of the interrelations between hypoxia-dependent and estrogen-dependent pathways is still unclear. We propose that Snail family proteins may serve as possible candidates for such interrelations – being involved in the support of growth of estrogen-dependent cells, from one side, and controlling the hypoxic response, from another side.

Here we showed that Snail1 – the key protein of epithelial-mesenchymal transition, may serve as the protective factor supporting the growth and survival of breast cancer cells under hypoxic conditions. The protective effect of Snail1 was found to be mediated via activation of beta-catenin – the transcription cofactor which regulates the expression of hypoxia-related genes. The inhibition of Snail signaling results in the increase in cell sensitivity to hypoxia demonstrating that Snail1 may be considered as an important target in the treatment of breast cancer.

Materials and methods

Cell cultures

The human breast cancer cell line MCF-7 and human breast epithelial cell line HBL100 (immortalized by SV40 large T antigen) were cultured in standard DMEM medium supplemented with 7% fetal calf serum (FCS) (HyClone) at 37 °C and 5% CO_2. The cell growth was evaluated by the MTT-test based on the accumulation of a MTT reagent (3-[4,5–dimethylthiazol–2–yl]-2,5-diphenyltetrazolium bromide) by living cells.

Transient transfection and measurement of reporter gene activity

To determine the transcriptional activity of Snail1, beta-catenin and HIF-1, the cells were transfected with the plasmids containing luciferase reporter gene controlled by Snail-binding element of E-cadherin promoter, beta-catenin/TCF-responsive promoter or canonical hypoxia-responsive element (HRE), respectively. The plasmids used in this work were kindly provided by Dr. Antonio García de Herreros [21], Dr. Giovanni Melillo [22] and Dr. Victor Tatarskii Jr. The transfection was carried out for 4 h at 37 °C using Metafectene PRO (Biontex). To control the efficiency and potential toxicity of the transfection, the cells were transfected with the β-galactosidase plasmid. All subsequent experiments were performed during 24 h after transfection. The luciferase activity was measured according to a standard protocol (Promega) using a Tecan Infinite M200 Pro, and calculated in arbitrary units as the ratio of the luciferase/galactosidase activity.

Small interfering RNA oligonucleotides

Scrambled nonspecific siRNA (sense 5′-CAGUCGCGUUUGCGACUGGdTdT-3′), Snail1 specific siRNA (sense 5′-AGGCCUUCAACUGCAAAUAdTdT-3′) and beta-catenin specific siRNA (sense 5′-AGCUGAUAUUGAUGGACAGdTdT-3′) along with their corresponding antisense RNA oligonucleotides were purchased from Syntol (Russia). These RNAs were dissolved in annealing buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA) as 10 μM solutions and annealed at room temperature following heating to 95 °C.

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