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Fibroblasts induce epithelial to mesenchymal transition in breast tumor cells which is prevented by fibroblasts treatment with histamine in high concentration

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ABSTRACT

Epithelial to mesenchymal transition (EMT) of cancer cells is an essential process in cancer progression. Cancer cells that undergone EMT loose cell–cell contacts, acquire mesenchymal properties and develop migratory and invasive abilities.

In previous studies we have demonstrated that histamine may modify the invasive phenotype of pancreatic and mammary tumor cells. In this work we proposed to investigate whether histamine may also influence the interaction between tumor cells and normal fibroblasts. The potential activation of normal CCD-1059Sk fibroblasts by histamine and EMT phenotypic changes induced in MCF-7 and MDA-MB-231 breast tumor cells by the conditioned media (CM) derived from fibroblasts were evaluated. Initially, we determined the presence of H1, H2 and H4 histamine receptors and matrix metalloproteinase 2 (MMP2) mRNA in CCD-1059Sk fibroblasts. MMP2 gelatinolytic activity, cell migration and alpha-smooth muscle actin expression were increased in fibroblasts by low doses (<1 μM) and decreased by high doses (20 μM) of histamine. MCF-7 cells cultured with CM from fibroblasts exhibited spindle-shaped morphology, cell spreading and cytoplasmic expression of β-catenin but there was no change in MMP2 activity and cell migration. MDA-MB-231 cells cultured with CM from fibroblasts showed a more elongated phenotype, cell spreading, cytoplasmic β-catenin, increased MMP2 activity and endogenous TGF-β1 expression, and enhanced cell migration and invasion. Notably, all these features were reversed when mammary tumor cells were cultured with CM from fibroblasts treated with 20 μM histamine. In conclusion, high doses of histamine may prevent the activation of fibroblasts and also avert the EMT related changes induced in epithelial tumor cells by fibroblasts CM.

1. Introduction

Epithelial to mesenchymal transition (EMT), a critical normal process during embryogenesis, organ development and wound healing, is also present in fibrosis and cancer (Kalluri and Weinberg, 2009). During EMT epithelial cells can undergo impressive phenotypic changes that reflect their "transformation" to mesenchymal cells. Epithelial cells lose their apico-basal polarity and their cell–cell contacts. Down-regulation of the epithelial marker E-cadherin usually highlights the beginning of EMT (Baranwal and Alahari, 2009). Cytoplasmic and nuclear accumulation of β-catenin and enhancement in the expression of EMT related genes (Slug, Snail, Twist) are observed in epithelial cells during this process (Andrews et al., 2012). Moreover, the expression of mesenchymal markers like N-cadherin, vimentin and alpha-smooth muscle actin (α-SMA) as well as the secretion and activity of MMP2 and MMP9 are augmented during EMT (Kalluri and Weinberg, 2009).

Metastasis is the major cause for cancer related mortalities. It depends on the ability of tumor cells to change their epithelial phenotype to a migratory and invasive or mesenchymal-like phenotype. Carcinomas arising from epithelial tissues represent 90% of human neoplasias and the inappropriate activation of EMT in epithelial cells allows benign tumors to progress into invasive and metastatic cancers (Acloque et al., 2009; Gavert and Ben-Ze'ev, 2008). It is recognized that the induction of EMT requires cells to be

Abbreviations: CM, conditioned media; CM(−), unconditioned media; CM(+), CM from CCD-1059Sk fibroblasts; CM(20μM HA), CM from fibroblasts treated with 20 μM histamine; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; HA, histamine; MMP, matrix metalloproteinase; α-SMA, alpha-smooth muscle actin.

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competent for undergoing EMT and an EMT-permissive microenvironment to exist. Interactions of malignant cells with tumor stroma are crucial to increase the malignancy of neoplastic cells and change the phenotype of normal fibroblasts in activated fibroblasts. In turn, activated fibroblasts secrete a variety of factors that influence neighboring cells in a paracrine manner to promote tumor growth and invasion (Kalluri and Zeisberg, 2006; Räsänen and Vaheri, 2010; Xouri and Christian, 2010).

Histamine is a biogenic amine whose actions mainly comprise allergic and inflammatory responses through the activation of four G protein-coupled receptors (H1, H2, H3 and H4). However during the last decades accumulating evidence supports histamine actions in proliferation of normal and tumor cells with different responses depending on histamine receptor subtype activated and cells or tissues where receptors are expressed. A stimulatory effect on cell growth is commonly observed in epithelial cells at doses lower than 1 μM while histamine over 10 μM reduces cell proliferation (Cricco et al., 2006a, 2008; Francis et al., 2009; Massari et al., 2011; Medina et al., 2006; Rivera et al., 2000). In previous studies we have demonstrated that histamine may also modify the invasive phenotype of pancreatic and mammary tumor cells modulating the expression and activity of MMPs and cell migration in a dose-dependent manner (Cricco et al., 2006b, 2011; Genre et al., 2009).

In this work we proposed to investigate whether histamine may also influence the interaction between tumor cells and normal fibroblasts. To better understand the role of histamine in tumor biology we evaluated the potential activation of normal fibroblasts by histamine and the EMT phenotypic changes induced in breast tumor cells by conditioned media (CM) derived from histamine-treated fibroblasts. An extensive knowledge of multiple interactions between tumor and stromal cells in human cancer will help to delineate more effective strategies for therapeutic intervention.

2. Materials and methods

2.1. Cell culture

CCD-1059Sk fibroblasts derived from normal skin of human mammary gland (ATCC CRL-2072) and the breast cancer cells MCF-7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS, Gibco, CA, USA), 0.3 g/l L-glutamine and 40 mg/l gentamicine. 1 mM sodium pyruvate was added to fibroblasts cultures. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Treatments

Histamine dihydrocloride and histamine H1 receptor agonist 2-[3-(trifluoromethyl)phenyl]histamine dimaleate were from Sigma–Aldrich (St Louis, MO, USA). Histamine H2 receptor agonist Amthamine dihydrobromide, histamine H3 receptor agonist R-(α)-methyl-histamine dihydrobromide and histamine H4 receptor agonist Clobenpropit dihydrobromide were from Tocris (MO, USA). H1, H2 and H3 histamine receptor agonists are specific (Leschke et al., 1995; Eriks et al., 1992; Leurs et al., 1995). Clobenpropit is an H4 histamine receptor agonist and H3 histamine receptor antagonist (Liu et al., 2001).

2.3. Generation of conditioned media

Fibroblasts grown up to 60% confluence in RPMI supplemented with 1 mM sodium pyruvate and 10% FBS. Subsequently fibroblasts were incubated with histamine or not for 24 h at 37 °C, 5% CO_2. Then media were discarded, fibroblasts were rinsed with phosphate buffered saline (PBS) and FBS free RPMI was added to cultures. Fibroblasts were incubated for additional 24 h and then media were collected, centrifuged at 13,000 × *g*, 4 °C for 5 min and frozen at −70 °C for further use. Treatments did not modify the number of fibroblasts during the experiment, as evaluated by counting adherent cells at the end of the experiment.

Conditioned media [CM(+) and CM(20 μM HA)] were prepared by mixing media collected from fibroblasts (not treated or treated with histamine respectively) and fresh RPMI in a ratio 1:2. The mixture was supplemented with 10% FBS.

CM(−) was prepared trying to resemble CM(+) and CM(20 μM HA) preparation as much as possible by incubating RPMI at 37 °C, 5% CO_2 for 24 h and posterior centrifugation at 13,000 × *g*, 4 °C for 5 min. This medium was frozen at −70 °C and then diluted in a ratio 1:2 with RPMI and supplemented with 10% FBS to be used as CM(−).

2.4. Cell-scatter assay

2×10^3 MDA-MB-231 or MCF-7 cells were seeded onto six-well plates, allowed to adhere overnight and then switched to CM(−), CM(+) or CM(20 μM HA) for 7 days. Media were changed twice during incubation time. Cell scattering was evaluated microscopically following staining with 1% toluidine blue solution.

2.5. Immunocytochemical/Immunofluorescence analysis

Fibroblasts were grown onto glass coverslips and treated with different doses of histamine for 24 h. Tumor cells were seeded onto glass coverslips and incubated with CM(−), CM(+) or CM(20 μM HA) for 24 h.

Immunocytochemistry: cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 in 1% FBS/PBS. Endogenous peroxidase activity was blocked with 3% H_2O_2. Cells were incubated overnight at 4 °C with rabbit anti MMP2 and anti MMP9 antibodies (1:100, Calbiochem, La Jolla, CA, USA). Immunoreactivity was detected by using peroxidase-conjugated anti-rabbit IgG and visualized by diaminobenzidine staining (Sigma). Finally, cells were counterstained by immersion in hematoxylin. Light microscopy (Axiolab Karl Zeiss, Göttingen, Germany) was performed.

Immunofluorescence: cells were fixed, permeabilized, blocked in PBS with 1% bovine seroalbumine and incubated overnight at 4 °C with anti E-cadherin (1:50, Santa Cruz Biotechnologies, CA, USA), anti β-catenin (1:100, Invitrogen, NY, USA), anti α-SMA (1:50, Abcam, Cambridge, UK) antibodies and for 1 hr at room temperature with Alexa Fluor 488 dye conjugated anti-mouse (1:400, Invitrogen) or FITC-conjugated mouse anti-rabbit (1:100, Sigma) antibodies. Cells were stained with 0.25 μg/ml propidium iodide or 0.1 μg/ml DAPI to visualize nuclei. Coverslips were mounted with FluorSave Reagent (Calbiochem), and immunoreactivity was visualized by a laser confocal microscope (Olympus Fluo View FV1000). Signal specificity was controlled by replacing the first antibody with PBS or Mouse Isotype Control (purified normal mouse immunoglobulin, Invitrogen, Camarillo, USA) in the case of rabbit or mouse antibody respectively.

2.6. Gelatin zymography

Cells were seeded and treated for 24 h. Then media were replaced by fresh serum-free RPMI. After 24 h supernatants were collected, mixed with non-reducing buffer and electrophoresed on 7% sodium dodecyl sulfate-polyacrilamide gels with 0.1% gelatin. Gelatinolytic activity was visualized by staining zymograms with Coomassie Brilliant Blue G250 (Sigma) and destaining in acetic acid–methanol–H_2O (1:3:6). Densitometric analyses were performed using the software ImageJ 1.42q (NIH, USA).