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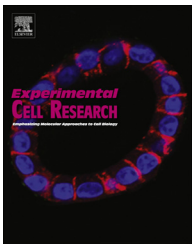

Research Article

Induction of pancreatic cancer cell migration by an autocrine epidermal growth factor receptor activation

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ABSTRACT

Pancreatic cancer is characterized by aggressive local invasion and early metastasis formation. Active migration of the pancreatic cancer cells is essential for these processes. We have shown previously that the pancreatic cancer cells lines CFPAC1 and IMIM-PC2 show high migratory activity, and we have investigated herein the reason for this observation. Cell migration was assessed using a three-dimensional, collagen-based assay and computer-assisted cell tracking. The expression of receptor tyrosine kinases was determined by flow-cytometry and cytokine release was measured by an enzyme-linked immunoassay. Receptor function was blocked by antibodies or pharmacological enzyme inhibitors. Both cells lines express the epidermal growth factor receptor (EGFR) as well as its family-member ErbB2 and the platelet-derived growth factor receptor (PDGFR)α, whereas only weak expression was detected for ErbB3 and no expression of PDGFRβ. Pharmacological inhibition of the EGFR or ErbB2 significantly reduced the migratory activity in both cell lines, as did an anti-EGFR antibody. Interestingly, combination of the latter with an anti-PDGFR antibody led to an even more pronounced reduction. Both cell lines release detectable amounts of EGF. Thus, the high migratory activity of the investigated pancreatic cancer cell lines is due to autocrine EGFR activation and possibly of other receptor tyrosine kinases.

Introduction

The difficult diagnosis of pancreatic cancer at a localized resectable stage makes the prognosis of patients with this disease extremely poor. Another reason of the poor prognosis is the early metastasis formation of the tumor to the regional lymph nodes and liver [1,2]. An essential prerequisite for metastasis formation is the active migration of malignant cells from the primary tumor via lymphatic or blood vessel routes. This complex process of cancer cell migration is regulated by many different signalling pathways as well as substances of various classes and origins [3]. The most important migrational regulators can be divided into two major groups: On the

Abbreviations: cAMP, cyclic AMP; EGF(R), epidermal growth factor (receptor); EMT, epithelial to mesenchymal transition; FITC, fluorescein isothiocyanate; FAK, focal adhesion kinase; GPCR, G protein-coupled receptor; mAb, monoclonal antibody; PI3, phosphatidyl-inositide 3; PLC, phospholipase C; HB-EGF, heparin-binding EGF-like growth factor; PDGF(R), platelet-derived growth factor (receptor); PKA/C, protein kinase A/C; TGF, transforming growth factor; VEGF(R), vascular endothelial growth factor(R)

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one hand there are cytokines and growth factors, which regulate receptor kinases and related receptors with associated kinases [4,5]. The second group includes chemokines and neurotransmitters, which are able to activate members of the huge family of G protein coupled receptors (GPCRs) [6,7]. One example of these GPCR ligands is the stress catecholamine hormone norepinephrine. In previous in vitro cell migration studies we have observed that norepinephrine induces diverse effects on the migratory activity of different human tumor cells: some cell lines increase their migratory activity in response to norepinephrine, some do not show any significant norepinephrine-induced effects and others reduce their migratory activity after norepinephrine treatment [8–12].

The pancreatic cancer cell lines CFPAC1 and IMIM-PC2 are part of the last named group. The inhibitory effect of norepinephrine on the migration of these cells appears to be due to an imbalance of two cell signaling pathways. The protein kinase C/phospholipase C (PKC/PLC) pathway is already activated in the absence of norepinephrine, leading to an activation of the motor protein non-muscle myosin II and thus to a high spontaneous migratory activity. Therefore, norepinephrine does not cause further activation of this PLC/PKC pathway, but instead activates the second cyclic AMP/protein kinase A (cAMP/PKA)-dependent pathway and finally leads to an inhibitory effect on pancreatic cancer cell migration [10].

The signalling pathway or signalling event, which is responsible for the constitutive PLCγ activation in pancreatic cancer cells, is not clear. We hypothesize that an autocrine activation of receptor tyrosine kinases might be the reason for the constitutive PLCγ activation. Ligand binding induces dimerization and autophosphorylation of receptor tyrosine kinases and results in phosphorylation of various SH2 domain-containing signal transduction molecules such as PLCγ, PI3-kinase, GAP and cSrc [13,14]. Examples of these receptor tyrosine kinases are the platelet-derived growth factor receptors α and β (PDGFRα and PDGFRβ) and the four members of the ErbB receptor family: the epidermal growth factor receptor (EGFR/ErbB1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). The expression of the PDGF receptors and at least one of the members of the EGFR family has been proven in a large proportion of pancreatic cancers [15–17].

The activation of the different ErbB receptors can be induced by the following ligands: EGF, transforming growth factor alpha (TGF-α), amphiregulin, betacellulin, heparin-binding EGF-like growth factor (HB-EGF), epiregulin, epigen and neuregulins 1–6 [18,19]. Among these ligands, TGF-α, amphiregulin, betacellulin, epiregulin and EGF are specific activators of EGFR [20]. A study of Murphy et al. revealed that autocrine EGFR activation stimulates the proliferation of these cells [21]. Furthermore, different studies indicated that EGF and PDGF initiate the association of their respective receptors with PLCγ and induce the PLCγ phosphorylation [22,23].

In the context of these findings the present study focuses on the role of EGFR, ErbB2 and PDGFR in pancreatic cancer cell migration and provides EGF as an important autocrine factor for the regulation of the migratory activity of these cells.

Materials and methods

Cell culture

The pancreatic cancer cell line CFPAC1 was purchased from American Type Culture Collection (Manassas, VA, USA) and the pancreatic cancer cell line IMIM-PC2 was a gift from Francisco X Real (Spanish National Cancer Research Centre, Madrid, Spain). The cells were grown in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, Steinheim, Germany), supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 2% penicillin/streptomycin (Sigma-Aldrich), 1 mM sodium pyruvate (Biochrom) and 2 mM L-glutamine (PAA Laboratories GmbH, Linz, Austria) at 37 °C in a humidified atmosphere of 5% CO_2.

For the pancreatic cancer cell line CFPAC-1 reference STR data are available The GenomeLab Human STR Primer Set (Beckman Coulter, Krefeld, Germany) was used and analyzed on a CEQ8800 sequencer (Beckman Coulter) according to the manufacturer's protocol to control for the cell line identity. STR data were submitted to on-line verification tool of DSMZ (German Collection of Microorganisms and Cell Lines) to confirm identity (http://old.dsmz.de/human_and_animal_cell_lines/main.php?contentleft_id=101). For IMIM-PC2, there is no reference available.

Cell migration assay

The migratory activity of the two cell lines was analyzed as described in detail previously [24]. In short, 5×10^4 cells were mixed with carbonate-buffered collagen solution (Advanced BioMatrix, San Diego, CA, USA) containing minimal essential medium (Sigma-Aldrich) as well as one or two of the following substances: the EGFR kinase inhibitor AG1478 (300 nM, Merck KGaA, Darmstadt, Germany), EGF (100 ng/ml, Sigma-Aldrich), the ErbB2 inhibitor 4-(3-Phenoxyphenyl)-5-cyano-2*H*-1,2,3-triazole (10 μM, Merck KGaA), a neutralizing anti-EGFR antibody (10 μg/ml, Merck KGaA) and a neutralizing anti-PDGFRα antibody (10 μg/ml, Abcam, Cambridge, UK). After polymerization of the collagen, the migration of the cells was recorded by time-lapse video microscopy for 15 h. Subsequently two-dimensional projections of the paths of 30 randomly selected cells were digitized in 15 min intervals. The migratory activity was calculated for each step as the portion of cells, which was locomotory active. The average of the activity was calculated for all steps of the entire observation period. The graphs show the mean values and standard deviations of the migratory activity of at least three independent experiments. In minimum 90 cells were analyzed per sample. Statistically significant changes ($p < 0.05$) were calculated using Student's *t*-test (two-tailed and unpaired).

Flow cytometry

A FacsCalibur flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany) was used to investigate the expression of EGFR, ErbB2, ErbB3, PDGFRα and β in pancreatic cancer cells. 1×10^5 cells were fixed with 1% paraformaldehyde (Sigma-Aldrich), washed with 0.5% Triton X-100 (Sigma-Aldrich) and incubated with one of the following antibodies (Ab): anti-EGFR (Ab-1) mouse mAb (528) IgG2a (200 ng/ml, Merck KGaA), isotypic control IgG2a (1:250 dilution, Beckman Coulter, Marseille, France), anti-c-ErbB2/c-Neu (Ab-2) mouse mAb 9G6 (200 ng/ml, Merck KGaA), ErbB-3 (298) mouse monoclonal IgG1 (200 ng/ml, Santa Cruz Biotechnology, Heidelberg, Germany), isotypic control IgG1 (400 ng/ml, Beckman Coulter), PDGFRα (D13C6) XP™ rabbit mAb (1:50 dilution, New England Biolabs GmbH, Frankfurt), PDGFRβ (C82A3) rabbit mAb (1:50 dilution, New England Biolabs GmbH) or normal rabbit IgG (1:50 dilution, Santa Cruz Biotechnology). After 30 min incubation one of the following secondary