Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro

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Abstract

Inhibition of angiogenesis has become a major target in experimental cancer therapies. Vascular endothelial growth factor (VEGF) and its receptors are essential for breast cancer progression and relevant targets in experimental anti-angiogenesis. VEGF, produced by carcinoma cells, acts in a paracrine fashion on endothelial cells and displays autocrine activity on carcinoma cells. Breast cancer cells express VEGF-A, VEGF-B, VEGF-C and their receptors VEGFR-1 (Flt-1), VEGFR-2 (Flik-1/KDR) and neuropilin (NP-1/NP-2). VEGF-A triggers cellular signalling, an invasive phenotype of certain breast cancer cell lines and influences cell survival. However, such an autocrine VEGF/VEGFR signalling loop remains to be established. We demonstrate production of VEGF-A in cell lines MDA-MB-468, T47d, MCF-7, HBL-100 and in a primary breast cancer culture. Moreover, these cells showed baseline VEGFR-2 tyrosine-phosphorylation that could be enhanced by VEGF-A stimulation. In addition, VEGF-A leads to increased phosphorylation of ERK1/2 and Akt indicating that VEGF-A stimulation plays a crucial role in the regulation of cell growth, apoptosis, survival and differentiation. Moreover, we have established a novel breast cancer cell culture MW1 that expresses high levels of VEGF-A. We demonstrate that VEGFR-2 on the surface of breast cancer cells is functional and is capable of being stimulated by external VEGF-A. VEGF-A production by and VEGFR-2 activation on the surface of breast cancer cells indicates the presence of a distinct autocrine signalling loop that enables breast cancer cells to promote their own growth and survival by phosphorylation and activation of VEGF-2. Moreover, this autocrine loop represents an attractive therapeutic target.

Abbreviations: ATCC – American Type Culture Collection; EGFR – epidermal growth factor receptor; IGFR – insulin-like growth factor receptor; PI3-kinase – phosphatidylinositol 3’-kinase; VEGF – vascular endothelial growth factor; VEGFR – vascular endothelial growth factor receptor

Introduction

Angiogenesis and angiogenic factors are crucial for promoting growth and dissemination of solid tumours. Vascular endothelial growth factor (VEGF) is a potent stimulator of tumour angiogenesis and represents an important prognostic marker. Moreover, the inhibition of VEGF activity using the monoclonal antibody bevacizumab represents the first successful antiangiogenic treatment strategy as recently shown for colon cancer [1, 2]. PTK787, an orally active inhibitor of VEGF receptor tyrosine kinases, exhibited stable disease response in advanced colorectal cancer patients [3]. VEGF expression has been correlated with poor prognosis of breast cancer [4]. VEGF-A exists in different isoforms with VEGF-A121 and VEGF-A165 being the most common splice variants secreted by tumour cells in general and by breast cancer cells in particular. High levels of VEGF-A mRNA can be detected in breast cancer cell lines [5]. High expression levels of VEGF-A exceeding the levels in adjacent non-neoplastic tissue [6] have been demonstrated in breast carcinomas. The functional significance of VEGF-A in the development of tumour vasculature is well documented, and recently, an autocrine action of VEGF on tumour cells has been suggested. Initial evidence for
VEGF signalling has been documented in melanoma cells [7, 8], in leukaemia cells [9] in prostate carcinoma cells [10], in colon carcinoma cells [11], in bladder tumour [12] and in breast carcinoma cells [13--19]. Immunohistochemistry was used to detect VEGF as well as VEGF receptors in breast cancer, and possible autocrine and paracrine effects of VEGF-2 on tumour cells were distinguished [13] by means of double staining for receptor/ligand combinations. Immunohistochemical staining of primary breast cancer highlighted the expression of VEGF-2 in breast carcinomas not only on endothelial cells, but also on epithelial cells of mammary ducts [15]. The production of VEGF-A mRNA as well as mRNAs of VEGF-1 and VEGF-2 has been demonstrated in primary cultures of epithelial and stromal cells derived from breast tumours [14]. It has been shown that VEGF stimulation of T47d breast cancer cells results in changes in cellular signalling and invasion, possibly by activation of MAP kinases, phosphatidylinositol 3'-kinase (PI 3-kinase) and Akt [16]. Suppression of VEGF expression promotes apoptosis of breast cancer cells by inhibition of PI 3-kinase activity associated with the involvement of the VEGF receptor neuropilin. This autocrine VEGF/neuropilin loop does not only enhances survival, but also facilitates invasion of breast carcinoma cells [17]. These findings suggest the importance of VEGF for breast carcinoma cell survival independent of angiogenesis by autocrine stimulation of survival. In addition, neuropilin was found to be a critical coreceptor for VEGF-2 in endothelial cells [20] pointing out that VEGF-2 might play a role in this autocrine signalling loop, possibly with neuropilin as a VEGF-2 coreceptor that facilitates VEGF-mediated signalling [17].

Most studies rely on established human breast cancer cell lines as experimental models. However, little attention had been given to the fact that many of these cell lines had originally been described more than 20 years ago and that they are more or less dedifferentiated. Therefore, we focused on establishing short-term and long-term cultures from breast carcinomas as well as from pleural effusions of patients with metastasised breast carcinoma in order to generate a more representative in vitro model. Our study for the first time demonstrates expression of VEGF-2 and presence of tyrosine-phosphorylated, i.e., activated VEGF-2 in various breast carcinoma cell lines and in a primary culture of breast carcinoma cells. Furthermore, we show that VEGF-A stimulation results in increased levels of tyrosine-phosphorylated VEGF-2, induction of Akt phosphorylation and activation of ERK1/2.

Materials and methods

Cells

MDA-MB-468, MCF-7 and T47d cell lines were obtained from the American Type Culture Collection (ATCC) and cultivated as indicated by ATCC. The immortalised breast cell line HBL-100 was obtained from Dr Robert Zeillinger [5] and cultivated in RPMI medium. The porcine aortic endothelial cell line PAEC/KDR overexpressing VEGFR-2 was used as previously described [21].

Primary breast cancer cultures

Surgically removed breast cancer tissue was cut into 1 mm³ pieces and incubated in 200 U/ml collagenase type III (Gibco) in culture medium 199 (Sigma) for up to 20 h. Epithelial cell aggregates were separated from single cells by differential sedimentation and centrifugation as described by Speirs [14] and by Forozan [22]. To isolate cancer cells from pleural effusion, cells were centrifuged at 1200 rpm for 5 min and resuspended in medium. Red blood cells were lysed by adding 9 ml of sterile deionised water to the pellet. The pellet was quickly resuspended and 1 ml 10× Hank's balanced salt solution (Life Technologies) was added to re-adjust the salt concentration. Cells were centrifuged; pellets were plated in CRML medium (Gibco) containing 10% foetal bovine serum, 5 μg/ml insulin, penicillin and streptomycin. Cells were grown until subconfluent, after which they were trypsinised and replated. The primary breast cancer culture MW1 was established from pleural effusion of a 39-year-old patient with metastasised breast cancer. The patient had primary surgery for breast cancer in July 1998 (pT1c multifocal, pN2, G2, ductal-invasive). First diagnosis of the metastatic disease was in September 2000 with bone metastasis, and the patient first suffered from malignant pleural effusions from March 2001. The pleural effusions were drained in January 2002, collected and treated as mentioned above. The cells were passaged several times and showed doubling times of about 24 h. The primary breast cancer culture MW2 was also established from a patient with pleural metastasis from breast cancer and passage two and three were used for immunostaining and flow cytometry. The primary breast cancer cultures MW3 and MW4 were derived from primary surgery of patients with breast cancer (both pT2 and ductal-invasive), and respectively, three and four passages were possible.

Immunohistochemistry

Immunohistochemistry was performed on primary breast cancer cell cultures after 6--8 weeks in culture using anti-cytokeratine antibodies (clone AE1/AE3, DAKO, reacting with the 65--67, 64, 59, 58, 56.5, 56, 52, 50, 48 and 40 kDa cytokeratines), rabbit polyclonal antiserum recognising VEGF-2 (C1158, Santa Cruz) or a phospho-VEGFR-2-antibody (Tyr 996, Cell Signaling). Streptavidin--peroxidase immunostaining kit (Immunotech) was used for detection. Negative controls included omission of the primary antibody.