MOLECULAR ONCOLOGY XXX (2015) 1–10

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Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer

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ARTICLE INFO

Article history:
Received 18 March 2015
Received in revised form
1 May 2015
Accepted 4 May 2015
Available online ■

Keywords:
Nerve fibers (axons)
Tumor microenvironment
Nerve growth factor
Breast cancer

ABSTRACT

Infiltration of the tumor microenvironment by nerve fibers is an understudied aspect of breast carcinogenesis. In this study, the presence of nerve fibers was investigated in a cohort of 369 primary breast cancers (ductal carcinomas in situ, invasive ductal and lobular carcinomas) by immunohistochemistry for the neuronal marker PGP9.5. Isolated nerve fibers (axons) were detected in 28% of invasive ductal carcinomas as compared to only 12% of invasive lobular carcinomas and 8% of ductal carcinomas in situ ($p = 0.0003$). In invasive breast cancers, the presence of nerve fibers was observed in 15% of lymph node negative tumors and 28% of lymph node positive tumors ($p = 0.0031$), indicating a relationship with the metastatic potential. In addition, there was an association between the presence of nerve fibers and the expression of nerve growth factor (NGF) in cancer cells ($p = 0.0001$). *In vitro*, breast cancer cells were able to induce neurite outgrowth in PC12 cells, and this neurotrophic activity was partially inhibited by anti-NGF blocking antibodies. In conclusion, infiltration by nerve fibers is a feature of the tumor microenvironment that is associated with aggressiveness and involves NGF production by cancer cells. The potential participation of nerve fibers in breast cancer progression needs to be further considered.

Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; EGF, epidermal growth factor; IHC, immunohistochemistry; NGF, nerve growth factor; PGP9.5, protein gene product 9.5; TMA, tumor microarrays.

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http://dx.doi.org/10.1016/j.molonc.2015.05.001
1574-7891/

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

1. Introduction

The role of the nervous system in cancer etiology, and in particular the influence of nerve fibers in the tumor microenvironment, is an understudied aspect of cancer biology (Ondicova and Mravec, 2010). It is well established that cancer cells can grow around existing nerves and eventually invade them in a process called perineural invasion (Marchesi et al., 2010). This is generally associated with a poor prognosis and can cause pain as demonstrated in pancreatic cancer (Bapat et al., 2011). Conversely, the infiltration of tumors by growing nerves, or tumor axonogenesis, has only recently been suggested to actively participate in cancer progression. Indeed, a recent report (Magnon et al., 2013) has revealed that tumor infiltration by nerve fibers is essential for prostate cancer progression from early initiation to metastasis. The mechanism remains unclear but includes liberation of catecholamines and acetylcholine in the vicinity of cancer cells, resulting in the stimulation of tumor growth and invasion. In addition, another recent study has shown that denervation suppresses gastric tumorigenesis (Zhao et al., 2014a), further pointing to the role of the nervous system in carcinogenesis. These pioneering studies in prostate and gastric cancer suggest a potential value of anti-neurogenic therapies (Jobling et al., 2015) and raise the possibility that tumor infiltration by nerve fibers may also be important in other types of cancer.

In breast cancer, little is known about nerve fibers in the tumor microenvironment. A study in mice has shown that nerve infiltration in bone metastases participates in the stimulation of metastatic growth (Campbell et al., 2012) and recent investigations have evidenced the presence of nerves in human primary breast tumors (Zhao et al., 2014b; Huang et al., 2014). Anatomical and histological studies have shown that the normal breast is innervated by both sympathetic and sensory fibers from the 4–6th thoracic nerves (Sarhadi et al., 1996). Sensory fibers supply the nipple and skin, whereas sympathetic fibers innervate blood vessels and ducts. Therefore, it is conceivable that nerve fibers could be attracted into breast tumors, especially in light of the evidence that breast cancer cells can produce neurotrophic growth factors such as the neurotrophins (Hondermarck, 2012). In particular, nerve growth factor (NGF), which can stimulate the growth of sympathetic and sensory nerves, is produced and secreted by breast cancer cells (Adriaenssens et al., 2008). During embryonic development, NGF plays a major role in directing nerves to their correct targets (Skaper, 2012) and similarly it can be hypothesized that the production of NGF by breast cancer cells may result in the attraction of nerve fibers into primary breast tumors.

In the present study, the hypothesis that nerve fibers are a significant component of the breast tumor microenvironment has been explored by analyzing a cohort of breast cancers. Nerve fibers were detected in a significant proportion of invasive breast cancers and their presence was associated with lymph node invasion, suggesting a relationship with metastatic potential. An association was found between the presence of nerve fibers and the expression of NGF by cancer cells, and in co-culture with neuronal cells, breast cancer cells were able to induce neuronal outgrowth via the release of NGF.

2. Materials and methods

2.1. Breast cancer tissue samples and cell lines

High-density tumor microarrays (TMAs) of invasive ductal carcinomas (IDC), invasive lobular carcinomas (ILC) and ductal carcinomas in situ (DCIS) of the breast were obtained from Biomax (Maryland, USA, catalog number BR1921, BR1921a and BR8011). They included 159 IDC, 160 ILC and 50 DCIS. Histopathological subtypes were reviewed by a pathologist (MMW). Clinical annotation included age at diagnosis, tumor size, lymph node status, estrogen receptor, progesterone receptor, human epidermal growth factor (EGF) receptor 2 (HER2). MDA-MB-231, MCF-7, BT-474, and SKBR-3 breast cancer cell lines were from the American Type Culture Collection. JIMT-1 cells were from DSMZ (Germany). Brain metastatic 231-BR cells and HME human mammary epithelial cells (transformed but non-tumorigenic) were a generous gift from Barbara Steeg (Bethesda, USA) and Robert Weinberg (Boston, USA), respectively. Individual cell line authentication was performed after DNA extraction (Promega genomic purification kit, catalog number A1120) and using the GenePrint 10 PCR amplification kit (Promega catalog number B9510). All breast epithelial cell lines were maintained in RPMI-1640 with 10% fetalfetal calf serum (JRH Biosciences) and 2 mM L-glutamine. The neuron-like PC12 cell line was from Ralph A. Bradshaw (University of California San Francisco). They were maintained in Dulbecco's modified eagle medium (DMEM) from Life Technologies (Australia) with 5% foetal calf serum, 10% horse serum (Sigma), and 2 mM L-glutamine. All cell lines were grown in 75 cm^2 tissue culture flasks in a humidified incubator at 37 °C with 5% CO_2. The study was approved by the Human Research Ethic Committee of the University of Newcastle, Australia.

2.2. Immunohistochemistry

After deparaffinization and rehydration of the TMAs following standard procedures, heat induced epitope retrieval was carried out in a citrate based low pH buffer (Vector Laboratories) using a decloaking chamber (Biocare) at 95 °C for 20 min. Immunohistochemistry (IHC) was then performed using an ImmPRESS detection kit (Vector Laboratories) as per the manufacturer's recommendations. Briefly, after inactivation of endogenous peroxidases with H_2O_2, and blocking with 2.5% horse serum, rabbit PGP9.5 antibody (Abcam, catalog number ab15503), or rabbit NGF antibody (Abcam, catalog number ab52918), or non-immune rabbit IgG control (Alpha Diagnostic, catalog number 20009-1-200) were applied at a 1:200 dilution. ImmPRESS HPR anti-rabbit IgG (peroxidase) was then applied to the sections and revealed with DAB peroxidase substrate solution (Vector laboratories). Finally, TMA slides were counterstained with hematoxylin (Gill's formulation, Vector laboratories), dehydrated and cleared in Xylene before mounting in Ultramount #4 mounting media (Thermo Scientific). Imaging was performed using an Axioplan-2 microscope fitted with an AxioCam Mrc5 digital camera (Carl Zeiss AG). The presence or absence of nerve fibers was recorded for

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