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Are Breast Tumours Innervated? Immunohistological Investigations Using Antibodies Against the Neuronal Marker Protein Gene Product 9.5 (PGP 9.5) in Benign and Malignant Breast Lesions

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The aim of the present study was to assess the innervation pattern of benign and malignant breast lesions using the neuronal marker protein gene product (PGP) 9.5. An unlabelled antibody technique (using streptavidin biotin complex formation) was used on paraffin wax sections of tissues fixed in neutral buffered formalin. In 2/4 cases of chronic mastopathy, PGP 9.5 immunoreactivity was seen in relation to blood vessels and the ductal system. No immunoreactivity for PGP 9.5 was seen in the affected tissues of 9/10 cases of fibroadenomata. In 9/16 breast cancers, PGP 9.5-labelled perivascular nerve fibres were detected in connective tissue stroma supporting carcinoma tissue, though not in the immediate vicinity of such tumour tissue. Labelled nerve fibres were detected in large bundles at the periphery of tumours, possibly unrelated to the latter. Our results indicate that the newly formed blood vessels within a tumour are not innervated, though major blood vessels which supply the tumour are innervated.

Key words: PGP 9.5 immunoreactivity, breast cancer
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INTRODUCTION

THE NERVE supply of the breast has been well documented [1, 2], and is thought to be derived from branches of the fourth to sixth thoracic spinal nerves. The principal components of these nerves are somatic afferent fibres supplying the numerous specialised and free nerve terminals, especially in the nipple. In addition, there are sympathetic nerve fibres which supply the smooth muscle in the nipple as well as blood vessels. There are also some sparsely distributed fibres which supply the larger ducts. In some breast disease there is often severe pain, though to what extent this represents involvement of nerves associated with any neoplasm present, or merely is the result of an increased stimulation of the pre-existing nerves, is unknown.

It is well acknowledged that the growth of tumours is in part dependent upon the growth of blood vessels needed to support the neoplastic tissue [3, 4]. Tumour-associated angiogenesis is particularly interesting because the additional blood vessels with which the tumours are associated afford considerable opportunities for the metastasis of neoplastic cells via the vascular system. It has been shown that the process of new blood vessel growth is initiated by factors from the tumour itself [5], as well as by factors derived from macrophages associated with the tumour [6]. The development of new vasculature may be indicative of a new phase in the progression of the tumour, and may be related to the potential for metastasis, such has been reported for melanomas, for example [7–9]. The importance of angiogenesis in tumour fate has been emphasised recently [10] and it was shown that the new vascularisation determines the blood flow, metabolism, growth rate and ATPase kinetics in experimental tumours growing in irradiated murine tumour beds. In humans, details of the molecular determinants of growth, angiogenesis and metastases in breast cancer have recently been published [11], and are in accord with earlier suggestions [12] that the numbers of blood vessels in breast cancers may be reliable indicators of the potential for metastasis. Innervation of tumour blood vessels may be an important factor in the pain resulting from sudden intense vasocongestion in local lymph nodes affected by Hodgkin's disease and other neoplastic diseases [13].

Despite there reportedly being a genetic predisposition to breast cancer [14], the influence of the nervous system, and psychological factors in particular, on tumour progression has been highlighted by a number of studies [15, 16], though a study of the impact of various psychosocial factors on the prognosis of breast cancer concluded that severe life events or social difficulties made no significant contribution to the outcome of breast cancer [17]. Nevertheless, it is of interest to determine whether tumours are innervated and, in particular, whether the new

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blood vessels formed to supply the growing tumour are innervated, bearing in mind their proposed role in metastasis formation.

The innervation pattern of blood vessels supplying normal tissues has been well documented. An authoritative review [18] of vacsular innervation describes the increasing density of innervation along the vascular tree, with little or no innervation of capillaries or venules. Arterioles are densely innervated, with the nerves lying in the adventitial region, but with only the most peripheral smooth muscle cells in direct contact with nerve fibres [19]; those nearest the luminal surface are capable of responding to the mediators in the vessel contents.

The neuronal marker protein gene product marker (PGP 9.5) is regarded as a more sensitive means of labelling nerve fibres, especially their terminal regions, than neuron-specific enolase and neurofibrillary protein [20]. In the present study, we have examined the expression of PGP 9.5 to investigate the pattern of innervation in breast cancers, in which new blood vessel formation has been implicated in possible metastases formation [10], using an indirect immunohistological method for PGP 9.5 expression.

MATERIALS AND METHODS

Tissue samples

Tissues from several random sections of several blocks of each of four chronic mastopathies, 10 benign fibroadenomata and 16 malignant breast tumours were selected from surgical material. Tissues were routinely fixed in 10% neutral-buffered formalin and processed to paraffin wax. Sections (5-μm thick) were cut and mounted on clean glass slides which had previously been coated with 0.5% gelatin. Representative sections from each tumour sample were stained with haematoxylin and eosin for histopathology.

Immunohistology

An indirect peroxidase-labelled antibody (ABC) method was used. Briefly, the procedure was as follows: sections were dewaxed in xylene, after which endogenous peroxidase was blocked using hydrogen peroxide in methanol, before incubation of the sections in non-immune 2% goat serum. After a brief wash in (0.15 M) Tris-buffered saline (TBS) (pH 7.6), sections were incubated in a polyclonal rabbit anti-PGP 9.5 [21] diluted 1:200 (a kind gift of Prof. R.J. Thompson, University of Southampton, U.K.) at 4°C for 24 h. After a brief wash in TBS the sections were incubated for 30 min with a biotinylated goat anti-rabbit IgG (diluted 1:200) for 30 min at room temperature. After washing, the sections were incubated in Vectastain ABC (Vector Laboratories, Peterborough, U.K.), and then after a final wash in buffer, incubated in diaminobenzidine tetrahydrochloride (DAB) for 10 min to reveal binding sites [22]. For each tissue sample a negative control section was examined using non-immune rabbit serum or TBS in place of the anti-PGP 9.5. Purified PGP 9.5 antigen (also a kind gift of Prof. R.J. Thompson) was used to block the immunoreactivity of anti-PGP 9.5 in some instances. Blocking of endogenous peroxidase was checked by reacting a section from each tissue sample with DAB only. Sections of small intestine, prepared in the same way as the tumour tissues, served as controls. Sections were lightly counterstained with Harris's haematoxylin before they were dehydrated, cleared and mounted in DePeX mounting medium.

RESULTS

In 2 out of 4 cases of chronic mastopathy, PGP 9.5-labelled nerve fibres were observed in the affected tissues which were blood vessel-related or related to the ductal system of the breast. In fibroadenomata, no PGP 9.5 immunoreactive nerve fibres were seen in relation to blood vessels in the affected areas of the tissue samples, though in 1 case there were some labelled blood vessels. In the breast cancers, of which there were two intra ductal (*in situ*) carcinomata, 12 invasive ductal carcinomata and two invasive lobular carcinomata, PGP 9.5 immunoreactive perivascular nerve fibres (Figure 1) were detected in connective tissue stroma where it was not in the immediate vicinity of the carcinoma tissues in 9 out of 16 cases. Where there was little stroma related to blood vessels near to the carcinoma tissue the blood vessels were negative for PGP 9.5. The immunoreactive stromal blood vessels were often apparently surrounded by irregular shaped epithelial-derived tumour tissue, such that it often appeared as though the blood vessels were central parts of the tumour. Sometimes there were quite large bundles of immunoreactive nerve fibres (Figure 2), in which case they were more often located at the junction between tumour and normal tissue. There was no evidence of fine nerve fibres passing between the tumour cells, unrelated to vasculature.

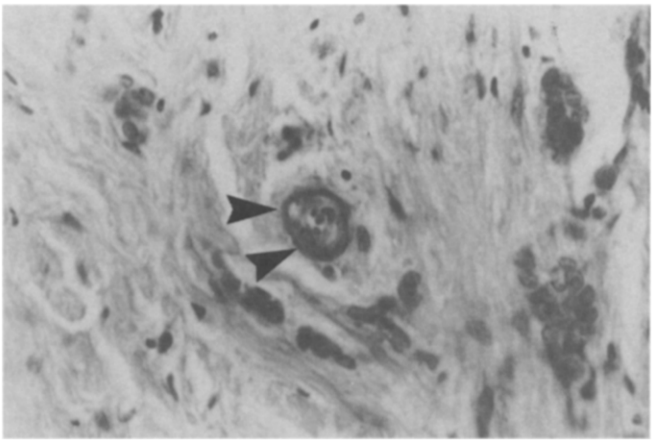

Figure 1. A small blood vessel (arrowed) with immunoreactivity for PGP 9.5 in the connective tissues supporting tumour tissue in an invasive ductal carcinoma of the breast, counterstained with haematoxylin. Original magnification ×370.

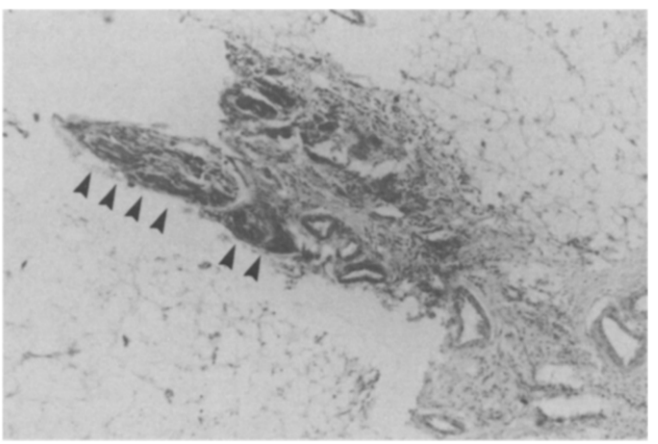

Figure 2. A large bundle of PGP 9.5 immunoreactive nerve fibres (arrowed) passing through fat tissue towards the tumour (out of picture) in an invasive ductal carcinoma of the breast, counterstained with haematoxylin. Note the slight immunoreactivity of the glandular tissue. Original magnification ×100.

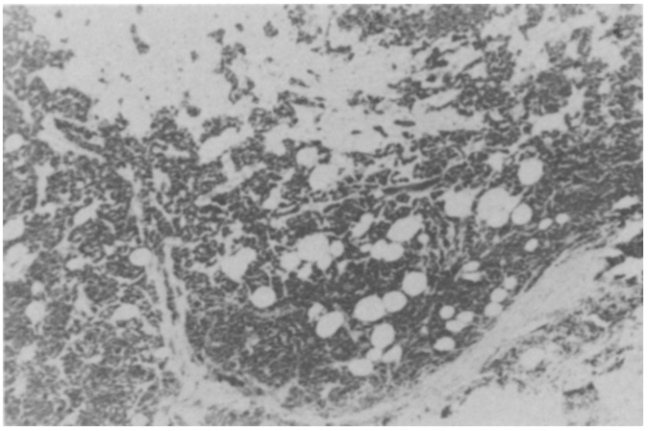

Figure 3. A dense population of PGP 9.5 immunoreactive malignant cells in an invasive ductal carcinoma of the breast, counterstained with haematoxylin. Original magnification ×100.

As an incidental finding it was also observed that in the more differentiated tumours (5 out of 16 cases) large numbers of the tumour cells were strongly immunoreactive for PGP 9.5 (Figure 3). In other locations the tumour cells were less intensely immunoreactive. Normal glandular tissue was also slightly immunoreactive for PGP 9.5 (Figure 2).

Control slides in which the primary antibody was omitted, and substituted with non-immune rabbit serum, or TBS, or blocked with purified antigen showed no significant immunoreactivity. In sections of small intestine nerve fibres and ganglia in the wall of the viscus were intensely immunoreactive.

DISCUSSION

Normal breast tissue is innervated in three main ways. There are somatic sensory nerves conveying impulses from tactile receptors in the skin, sympathetic motor nerves mainly innervating blood vessels and a sparse innervation of the larger ducts [1, 2]. The sparse innervation of the latter is in accord with the results of the present study which have demonstrated an association of a few nerve fibres with some of the larger duct systems in two of the cases of chronic mastopathies examined in the present study. In the benign fibroadenomata, despite the numer of cases examined, no immunoreactivity for PGP 9.5 was observed (with one exception) such that it may be concluded that nerve fibres only rarely penetrate the relatively thick connective tissues in these benign tumours. In the breast cancers examined in the present study a small proportion of blood vessels were immunoreactive for PGP 9.5, and were found in connective tissue stroma supporting the carcinoma, though not close to the tumour.

As a tumour grows it depends upon its blood supply for nutrition etc, and the innervation may facilitate appropriate vasomotor activity. It is, therefore, most likely that the innervated blood vessels are those which are original pre-existing blood vessels which have come to be in the vicinity of the tumour as growth increases. In contrast, the newly formed blood vessels within the tumour stroma, closest to the carcinoma, are devoid of innervation. The demonstration of nerve fibres related to blood vessels close to, though not within, tumour tissue in the malignant breast tumours may be related to the suggestions of LeShan [15] and Ramirez and colleagues [16] that psychological factors may operate in the susceptibility of some individuals to increased growth rate and metastasis formation. Barraclough and colleagues [17], however, found no relationship between the patient's prognosis and psychosocial events in the patient's life. The functional role of these innervated blood vessels which are not within the cancer tissue itself is unclear. It seems more likely that factors other than local innervation are the possible substrate for pyschological influences.

The immunoreactivity of some of the tumour cells in the breast cancers requires further discussion. The marker PGP 9.5 has been used to identify neuroendocrine tumours of the dispersed neuroendocrine system [23] so a cytoplasmic immunoreaction, such as in the present study, is not unique, although the PGP 9.5 labelling of breast cancer cells is unlikely to relate to neuroendocrine tumours [23]. It does, however, extend the work carried out by Nesland and colleagues [24] who found that breast cancer cells expressed neuron-specific enolase immunoreactivity. In this work, despite the suggestion that the cells were neuroendocrine in nature, convincing demonstration of hormonal content was not forthcoming. Recently, immunoelectron microscope investigations of the chromogranin content of granules in various breast cancers have been carried out [25]. The heterogeneity of the results discouraged acceptance of the concept of a neuroendocrine component of such cancers. The immunoreactivity in our studies could simply be a non-specific cross-reaction of some unrelated series of antigenic determinants, or it could represent the content of ubiquitin carboxyl-terminal hydrolase (the PGP 9.5 antigen [26]) in the breast cancer cells. Immunocytochemical and immunoblotting experiments in our laboratory indicate that the immunoreactivity for PGP 9.5 is genuine since it is also present in breast cancer cell lines [27]. Furthermore, it has been shown that the PGP 9.5 antigen is expressed in normal breast epithelium [27], as well as in epididymis epithelium [28].

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Induction of Tumour Cell Lysis by a Bispecific Antibody Recognising Epidermal Growth Factor Receptor (EGFR) and CD3

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A bispecific antibody construct (bAb) recognising CD3 and epidermal growth factor receptor (EGFR) was studied *in vitro*. Human peripheral blood lymphocytes (PBL), pre-activated with monoclonal antibody OKT-3 or with irradiated tumour cells, were armed with the bAb construct and targeted to autologous and allogeneic tumour target cells in culture. bAb EGFR×CD3 promoted significant cytolysis even at a concentration of 1 ng/ml. The specificity of target cell lysis was provided by the EGFR specificity of the bAb, as tumour cells negative for EGFR were not lysed. However, not only EGFR-positive tumour cells but also EGFR-positive normal cells were killed. Human renal cancer cell lines and the normal autologous kidney cell cultures expressing the same level of EGFR molecules were lysed to a similar extent. These results may contribute toward the planning of future clinical trials with such bAb.

Key words: bispecific antibodies, epidermal growth factor receptor, cytotoxic T lymphocytes
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INTRODUCTION

T LYMPHOCYTES ARE known as potent effectors in the immune response to cancer cells [1]. However, the role of cytotoxic T lymphocytes (CTL) in cancer patients is not well defined and remains a target for further research. Bispecific antibodies (bAb) have potential for tumour therapy since they can activate the lytic potential of a broad spectrum of CTL. In this regard, bAb recognising tumour-associated antigens and T cell markers were reported to bridge CTL and malignant cells *in vitro*, independent of CTL lytic potential or specificity [2, 3]. With the help of this