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Original Paper

Transcription of the Prorenin Gene in Normal and Diseased Breast

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The angiotensin II type 1 (AT1) receptor is present in a wide variety of human and animal tissues, and is particularly abundant in epithelial cells. Because of this, and because it is known that tissue renin angiotensin systems (RASs) exist that have specific local functions, we investigated the expression and localisation of components of the RAS in normal and diseased breast tissue. Using a monoclonal antibody to the AT1 receptor, immunocytochemistry confirmed that the AT1 receptor was characteristically distributed in ductal epithelial cells in both normal and malignant tissue, and in most, although not all, cells in invasive tumours. Transcription of prorenin mRNA was studied by *in situ* hybridisation, using a DIG-ddUTP tail-labelled probe specific for the human prorenin gene. In normal tissue, and in cases of ductal carcinoma *in situ*, prorenin mRNA was distributed in myoepithelial cells and in a band of connective tissue cells completely surrounding the AT1-containing ductal epithelial cells. This prorenin transcribing tissue was disrupted and attenuated in invasive tumours, and in some of these, prorenin mRNA transcription could not be detected at all. Functions ascribed to the tissue RASs include regulation of mitosis and tissue modelling, as well as fluid and electrolyte transport. The results presented here strongly suggest the possibility that a tissue RAS may also be present in the breast, closely coupled to the provision of angiotensin II to the AT1 receptors in ductal epithelial cells. This mechanism is disrupted in cancer. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: breast cancer, prorenin mRNA, Ang II AT1 receptor, *in situ* hybridisation, immunocytochemistry
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INTRODUCTION

THE RENIN angiotensin system (RAS) has received most attention in relation to its functions in the circulation, in which the generation of the active hormone, angiotensin II (Ang II), is associated with the regulation of aldosterone secretion, salt and water metabolism and blood pressure [1]. More recently, attention has focused on local tissue RASs, particularly in the adrenal, uterus [2–4], gonads, kidney, heart [5], brain and pituitary [6, 7].

Following the development of a highly specific monoclonal antibody to the Ang II type 1 (AT1) receptor [8, 9], we and others have shown that the receptor is present in many tissues, but in particular, in endothelial cells, and in secretory epithelial tissue. Because Ang II and tissue RASs may have an important role in tissue growth and modelling [10–16], it is possible that tissue RASs may be involved in cancer. Using immunocytochemistry and ligand-binding methods, our earlier finding that AT1 receptors are present in breast tissue is consistent with this view [17]. If, as seems likely, the actions of Ang II in breast epithelium are different in nature from its actions in the circulation, then it may be possible that the breast, like other tissues, contains the elements of a discrete RAS, concerned here with the delivery of the hormone specifically to breast epithelium.

This paper describes the use of *in situ* hybridisation to localise sites of prorenin mRNA transcription in normal and

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diseased breast tissue, and to examine their relationship to sites of Ang II action.

MATERIALS AND METHODS

Tissue collection

The tumours were surgically removed at the Breast Unit, Royal Hospital Trust, London, U.K.

Probes

The oligonucleotide probes, 45 bases, were used. The synthesised renin probe is a double-stranded oligonucleotide (55% GC). The sequences are of the antisense (187–142) orientation and the sense (142–187) derived from the translated exon 2 of the human renin gene.

Antibodies

The hybridised DIG-labelled probes were detected with high affinity alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer, Mannheim, Germany; 1:200 dilution).

The 6313/G2 monoclonal antibody was used to detect AT1 receptors in both normal and diseased breast tissue. [8,9]

Anti-vimentin, anti-cytokeratin, and anti-actin antibodies (Sigma, Poole, Dorset, U.K.) were used to discriminate between fibroblasts, epithelial cells, and myoepithelium, respectively. Other reagents were from Sigma.

Immunocytochemistry

The breast tissue samples were obtained with appropriate informed consent from patients undergoing breast surgery. The samples obtained for the study were divided, and a portion immediately snap frozen in liquid nitrogen for future immunocytochemical and biochemical assays. The remainder was fixed in 10% formalin–saline for 24 h prior to paraffin wax embedding. The sections (8 μm) were dehydrated, incubated in 3% hydrogen peroxide in methanol (v/v) for 15 min, boiled for 16 min in 10 mM citrate buffer, and washed in Tris buffered saline (TBS, pH 7.6, 50 mmol Tris/l, 150 mmol NaCl/l, 2 mmol $MgCl_2$/l). To block non-specific binding, they were then incubated for 20 min with normal rabbit serum (1:5 dilution) in TBS and incubated (60 min) with primary antibody in RPMI 1640 culture medium (ICN-Flow Ltd, High Wycombe, Bucks, U.K.), then washed twice and left to soak in TBS (5 min). The sections were then exposed to biotinylated rabbit antimouse IgG complex (Dako, Ely, U.K.), diluted 1:400 in TBS (30 min), washed in TBS, then incubated for 30 min with avidin–biotin complex (Dako), and washed again in TBS. Visualisation of antigen was achieved through the diaminobenzidine hydrochloride (DAB): hydrogen peroxide chromogen substrate reaction (Sigma) using 10 ml 0.05 M TBS, 6 mg DAB and 0.1 ml fresh 3% hydrogen peroxide for 10 min. The slides were washed in water (10 min), counterstained in Gills haematoxylin (2 min), rewashed in water (5 min), differentiated briefly in acid alcohol (10 ml 1% HCl in 990 ml 70% industrial methylated spirit (IMS); BDH, Poole, Dorset, U.K.), dehydrated in IMS, cleared with xylene twice and mounted in Depex mounting medium.

In situ *hybridisation*

The breast tissue sections (8 μm) were dewaxed in xylene followed by dehydration in ethanol. Dried sections were digested by proteinase K (0–10 μg/ml; Boehringer Mannheim) for 15 min. Control sections were treated with Rnase A (0.1 mg/ml, 20×SSC and 10 mM $MgCl_2$) for 90 min at 37°C. The sections were postfixed in 0.4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4, 20 mmol NaH_2PO_4/l, 80 mmol $NaHPO_4$/l and 100 mmol NaCl/l) for 15 min. The sections were then incubated with 0.25% acetic anhydride (v/v) in 0.1 mol triethanolamine/l and 0.9% (w/v) NaCl solution and incubated for 10 min, followed by washing in 0.05% diethypyrocarbonate. The sections were covered with hybridisation buffer (pH 7.5, 10 mmol Tris/l, 50% (v/v) formamide, 4×SSC (1×SSC contains 150 mmol sodium chloride/l and 15 mmol trisodium citrate/l), 1×Denhardt's solution, 500 μg salmon sperm DNA/ml, 10% (v/v) dextran sulphate) containing digoxigenin 3′-end labelled oligonucleotide probe to human renin and incubated at 37°C overnight. Sense probes were also used as a negative control. The unhybridised probe was washed away with serial washing in 4×SSC, 2×SSC and 1×SSC (35 min at 37°C) and for a further 15 min in modified TBS (pH 7.6, 50 mmol Tris/l, 150 mmol NaCl/l, 2 mmol $MgCl_2$/l and 0.1% (w/v), bovine serum albumin (BSA) with added 0.1% (v/v) Triton X-100 at room temperature. The sections were incubated with blocking reagent (Boehringer Mannheim) 1% (w/v) for 10 min, followed by incubation with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:200 dilution) for 3 h. Excess antibody was removed by washing with TBS (pH 9.5, 100 mmol Tris/l and 100 mmol NaCl/l). The antibody was visualised using 5-bromo-4-chloro-3-indolyl phosphate (BCIP 165 μg/ml) and nitro blue tetrazolium salt (NBT 330 μg/ml) for 2–20 h at room temperature. Levamisole (0.1 mmol/l) was added to the substrate solution to reduce the endogenous alkaline phosphatase activity. Finally, the sections were washed in TE (pH 7.4, 10 mmol Tris/l, 1 mmol ethylene diamine tetraacetic acid (EDTA)/l) buffer for 30 min and mounted with Apathys' mounting medium.

RESULTS

Immunocytochemistry

Breast tissue sections were obtained from 30 patients with malignant cancer, and 23 with benign breast disease. Positive staining for the AT1 receptor was found in all cases except for two malignant tumours, which were negative.

The criteria used for cellular identification, and discrimination between fibroblasts, myoepithelia and epithelia were the staining patterns shown by immunoreactive vimentin, actin and cytokeratin, respectively, and these facilitated the identification of cells exhibiting positive signals for both the AT1 receptor (immunocytochemistry), and for prorenin (*in situ* hybridisation).

In seemingly normal tissue, or in benign breast disease, which may be found with tumours in the same mastectomy samples, epithelial cells lining the acini stained uniformly for the AT1 receptor (Figure 1a), whereas fibroblasts and stromal tissue were negative. The positive staining was retained in tumours, both in ductal carcinoma *in situ* (Figure 1b), and in infiltrating ductal carcinoma (Figure 1c), although in malignant tumours some negative cells were observed adjacent to strongly positive cells. Samples treated similarly, but in the absence of primary antibody, showed no immunostaining (Figure 1d). As in 'normal' ducts, fibroblasts and stromal connective tissue surrounding the tumours were negative. Overall, however, the pattern of staining index for the two groups of patients was not appreciably different.

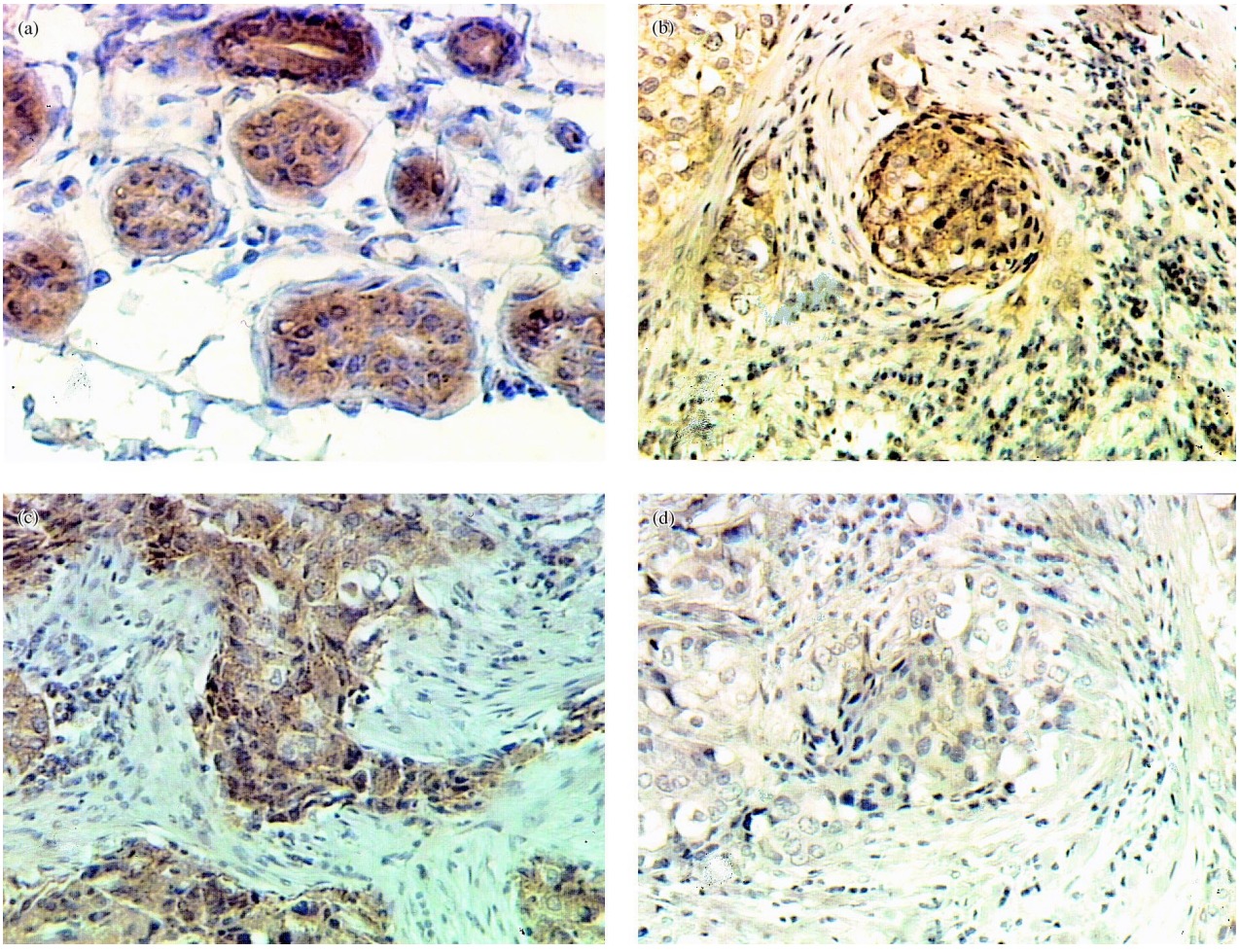

Figure 1. Immunocytochemistry showing the distribution of angiotensin II type 1 (AT1) receptors in (a) normal breast ducts, (b) ductal carcinoma *in situ*, (c) infiltrating ductal carcinoma. Receptors (brown stain) were present both in normal breast duct epithelium, and in cancer. They were absent from stromal fibroblasts and connective tissue. Control sections, exemplified here for ductal carcinoma *in situ* (d), omitting primary antibody, showed no staining. Magnification ×200.

In situ *hybridisation*

Messenger RNA coding for prorenin was seen in all the sections of normal or benign breast. Using the antisense hybridisation probe, positive prorenin mRNA staining was characteristically distributed in fibroblasts surrounding the breast acini (Figure 2a, b), and in the myoepithelial layer, while hybridisation with the sense probe gave no staining (Figure 2c). There was considerable variation in the prorenin mRNA staining in malignant tissue. In ductal carcinoma *in situ*, the structure of the pre-existing duct was reflected in the more or less continuous surrounding band of positively staining fibroblasts (Figure 3a, c), as in benign samples. Control sections, here omitting the antisense probe, were unstained (Figure 3b, d). In infiltrating ductal carcinoma, the completeness of this surrounding band of positively staining cells was seemingly disrupted (Figure 3e, g). Again, controls treated with the sense probe were negatively stained (Figure 3f, h). Finally, in some large infiltrating tumours, no positive staining for prorenin mRNA could be seen at all.

DISCUSSION

The functions of the RAS have been considered largely in terms of its roles in sodium and potassium homeostasis and the regulation of blood pressure. The continued acquisition of information on the distribution of angiotensin receptors and components of tissue located RAS emphasise that our interpretation needs to be widened. The identification of the cell types in which the AT1 receptor is localised, and their close relationship to probable sites of angiotensin generation has led to the identification of new organ-specific functions [18–21]. In particular, the use of the monoclonal antibody 6313/G2, directed against the N-terminal extracellular domain of the AT1 receptor, in immunocytochemical studies, suggests that Ang II has a widespread role in the maintenance of epithelial structure and function [21]. Such functions may include the regulation of water and electrolyte transport [15, 21–25], cilia beat activity [26], and mitosis and tissue differentiation [11–16, 26]. In particular, there is evidence for the trophic effects of Ang II on vascular smooth muscle [27–30], skeletal muscle and connective tissue [31], the adrenal cortex [32] and kidney [33], and in addition, the induction of 'early–intermediate' genes associated with mitogenesis [34].

The data here demonstrate the presence of Ang II AT1 receptors in both benign and malignant breast conditions. Emphasising its epithelial role, the AT1 receptor was

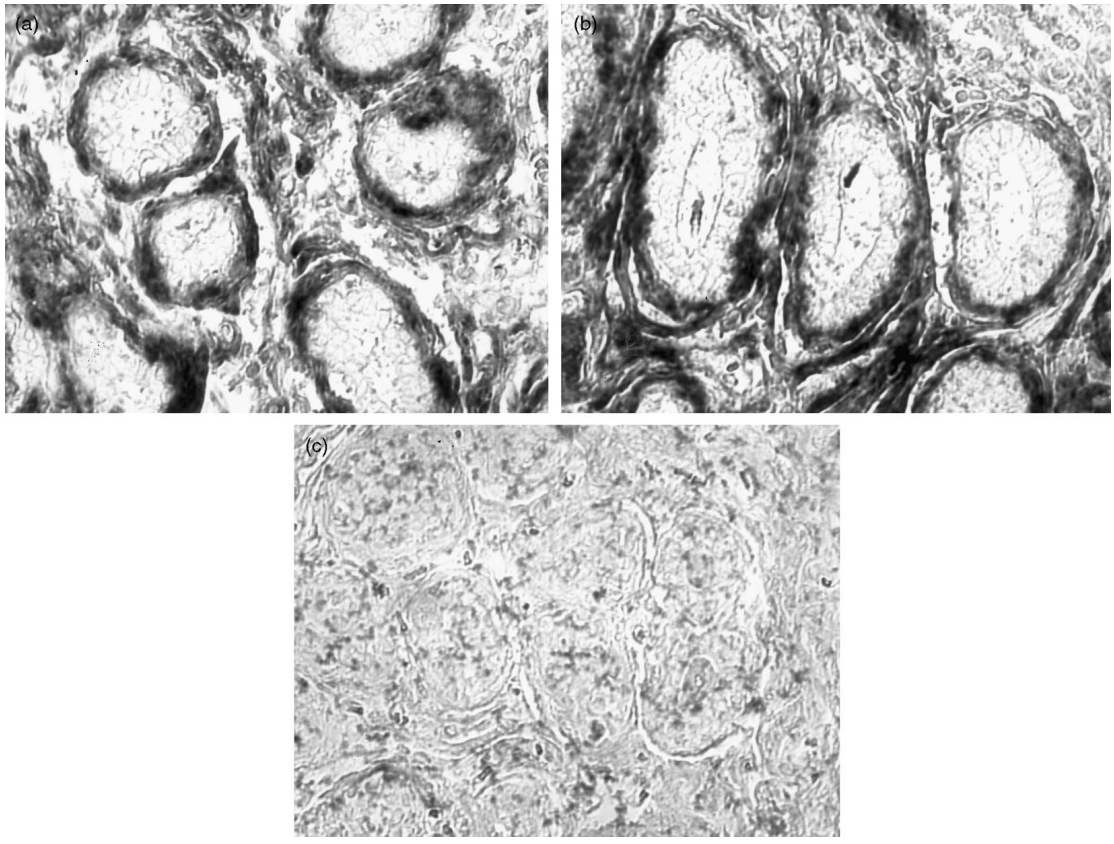

Figure 2. Immunocytochemistry for prorenin mRNA in normal breast tissue. Message (dark stain) was confined to myoepithelial cells, fibroblasts and connective tissue surrounding the ducts (a, b). It was absent from epithelial cells. Control sections (sense probe) gave no staining (c). Magnification ×200.

expressed strongly in the epithelial cells in both ducts and lobules of normal tissue (Figure 1a). It was also present in malignant tumours (Figure 1b, c).

If Ang II has a specific role in the breast epithelium, then it is reasonable to suppose that the availability of systemic Ang II to breast ducts is strictly limited, and that, in view of the now widespread evidence for the existence of tissue RASs, there may be a specific localised tissue source of Ang II concerned only with the supply of the hormone to breast epithelium. In the systemic RAS, Ang II concentrations closely parallel plasma renin activity, and changes in plasma renin activity are considered to be the rate limiting step in Ang II generation [35–38]. Because of these considerations, we used *in situ* hybridisation to examine the cellular localisation of mRNA coding for the renin precursor, prorenin.

The results showed that prorenin mRNA was transcribed in most of the samples examined, and that positive signals were invariably found in close proximity to the ductal epithelium. Prorenin mRNA was particularly evident in the fibroblasts of the connective tissue that surrounds the acini, and in the myoepithelial layer, thus seemingly encircling the Ang II responsive cells (Figures 2a, b and 3a, c, e, g). This relationship was maintained in earlier stages of tumours (Figure 3a, c). Here too the breast ducts were surrounded by prorenin gene transcribing fibroblasts, and most of the epithelial cells of the ducts were AT1 positive. However this relationship was not evident in invasive disease, in which the more patchy expression of AT1 receptors was coupled with a reduction in the numbers of cells transcribing prorenin mRNA. This was only partially attributable to the loss of myoepithelial cells, and prorenin transcription in fibroblasts was also greatly reduced. Initially, this shows that the tumour is no longer completely encircled by potential sites of angiotensin production and in some cases the transcription of prorenin is no longer evident at all.

The presence of prorenin mRNA alone does not prove the existence of a complete RAS in the breast, although, as in the systemic system, it is probably a key component: evidence of the presence of other RAS components, including angiotensinogen, and angiotensin-converting enzyme are currently lacking. Alternatively, it may be argued that localised production of angiotensinogen is not necessarily a prerequisite for local Ang II production, given a highly localised source of renin. Angiotensin-converting enzyme is known to be widely distributed throughout the vasculature, but it may not, in fact, be essential for Ang II production, since at least in some tissues, chymase has this role [39].

It is the apparently close coupling of prorenin expression with sites of angiotensin action that is so compelling about the data presented here. Since Ang II has numerous functions in epithelial and other tissues, including the regulation of mitosis and tissue differentiation, the observation that prorenin transcription apparently fails in invasive carcinoma, crucially suggests that here Ang II is not available to maintain these functions. This has profound implications for our understanding of cancer.

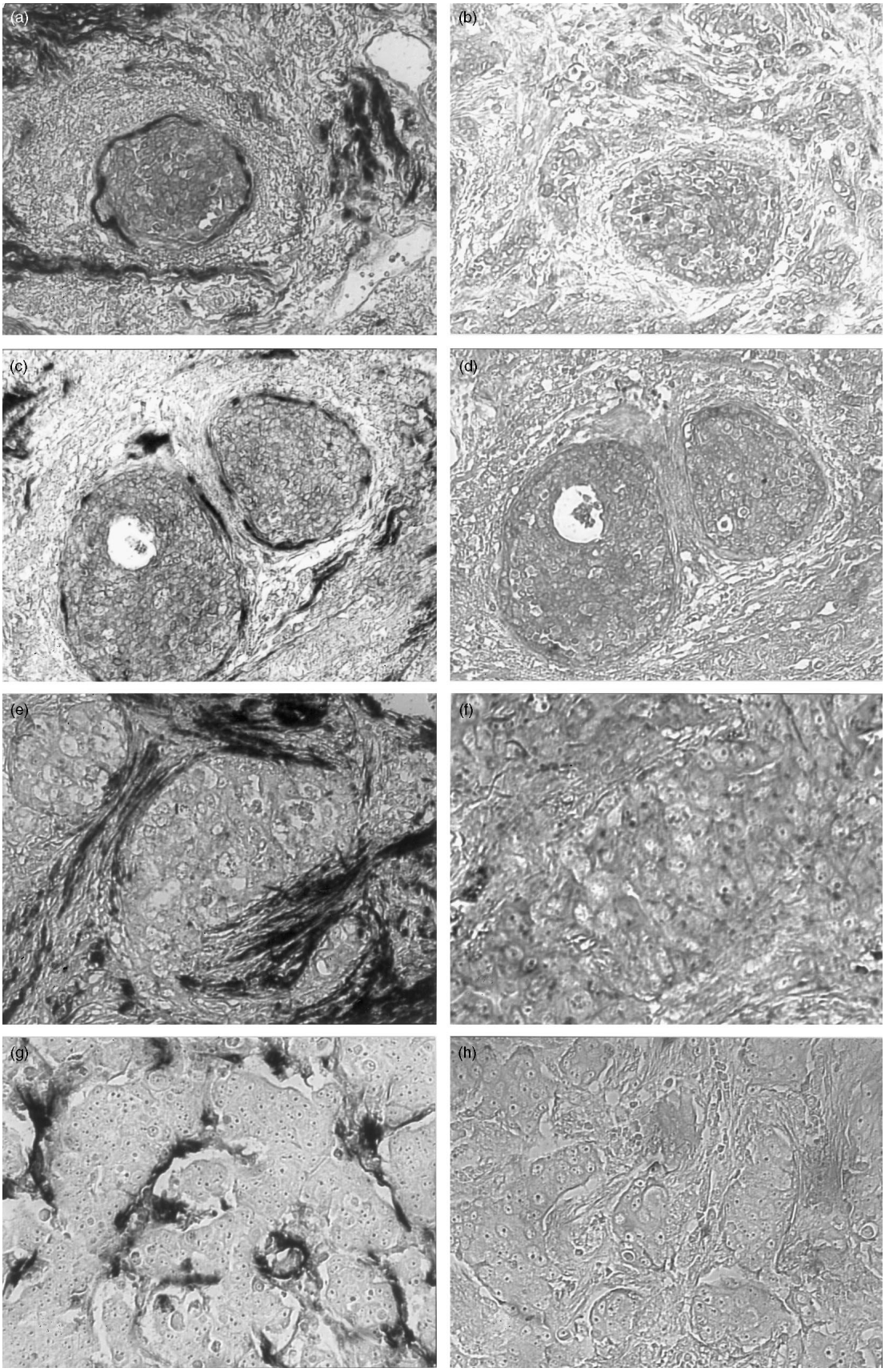

Figure 3. Immunocytochemistry for prorenin mRNA in ductal carcinoma *in situ* (a, c) and infiltrating ductal carcinoma (e, g). Positive staining was confined to the myoepithelial cells, fibroblasts and connective tissue surrounding the tumour in (a) and (c), and to disrupted sheets of mostly fibroblasts lying between the cancer cells in (e) and (g). Control sections (sense probes) are shown for each sample (b, d, f, h), and were unstained. Magnification ×200.

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