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Original Paper

Epidermal Growth Factor Receptor Expression by Northern Analysis and Immunohistochemistry in Benign and Malignant Prostatic Tumours

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Epidermal growth factor receptor (EGFR) expression in 44 benign prostatic hyperplasia (BPH) and 55 prostatic carcinoma specimens has been investigated using Northern blot analysis and immunohistochemistry. The values obtained for the EGFR mRNA in the BPH and carcinoma specimens were not significantly different and in the latter there was no correlation with grade. In the immunohistochemical assays, two antibodies to the external and one to the internal domain of EGFR were used. The former ones stained the basal cell membranes intensely whilst cytoplasmic staining of secretory epithelium was seen in BPH specimens with the latter. In the carcinoma specimens, the intensity of membrane staining correlated with the two external domain antibodies, $r = 0.640$, $P<0.001$, but neither of these correlated with the EGFR mRNA results. All three antibodies demonstrated a trend towards elevated expression of EGFR with dedifferentiation which reached signfiicance only with the internal domain antibody results, $P<0.02$. No correlation was observed with tumour EGFR mRNA values and the EGFR immunohistochemical results. The EGFR immunoreaction with the external domain antibody in 14 treated high-grade tumours was comparable to that obtained in 15 untreated anaplastic prostatic tumours. In 5 patients, both pre- and post-treatment samples were available and these exhibited little or no difference in EGFR expression with therapy.

Key words: prostate carcinoma, BPH, immunohistochemical, mRNA analysis, peptide growth factor receptor, EGF receptor

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INTRODUCTION

THE EPIDERMAL growth factor receptor (EGFR) can be activated by the binding of either epidermal growth factor (EGF) or transforming growth factor-α (TGFα), and via its tyrosine kinase activity elicit cellular repsonses including proliferation [1]. Both of these growth factors have been reported to be produced by prostate cell lines [2–4] and to be present in prostatic tissue [5, 6] but their role and that of the receptor in prostatic tumour progression is uncertain. The established prostatic cell lines which express EGFR are responsive, *in vitro*, to EGF and TGFα [3, 7, 8] providing a means of autocrine growth stimulation. These peptides can also modulate the expression of prostate-specific proteins and the androgen receptor [9]. The importance of EGFR, however, in human prostatic carcinoma remains controversial [10–16]. Conflicting reports exist concerning the percentage of tumours exhibiting EGFR positivity within the different prostatic pathologies and the relative amounts present in benign prostatic hyperplasia (BPH) and carcinoma specimens. Similarly, reports on the pattern of EGFR expression accompanying dedifferentiation and within in the various histopathological grades have been at variance. A variety of methods to estimate EGFR have been utilised including ligand-binding assays [10, 11, 13], mRNA analysis [12] and immunohistochemical staining procedures [14–16] and must account in part for the divergent results obtained on such a heterogeneous tissue. Although EGFR appears to have prognostic value in several other malignancies including breast [17, 18], ovary [19] and oral squamous cell carcinomas [20], its predictive value in prostatic cancer is unknown. Using the frozen tissue immunohistochemical assay for EGFR on a small number of patients we were unable to see a survival advantage for prostate cancer patients who were EGFR negative [21]. It was decided to investigate the values of EGFR obtained by mRNA analysis and compare them with the immunohistochemical patterns of expression using three different antibodies to this receptor which

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might indicate the most appropriate procedure for prognostic analysis.

MATERIALS AND METHODS

Prostatic tissue and cell lines

Prostate tissue was obtained from 55 patients with histologically diagnosed prostatic carcinoma, prior to any therapy, and from 44 patients with BPH. 54 patients with carcinoma and 40 patients with BPH underwent transurethral resection (TURP) of their tumours, while the remaining 5 patients had open prostatectomy operations. A representative sample of the TURP curettings was fixed in formal saline followed by paraffin wax embedding, the remainder was snap frozen in liquid nitrogen and stored at −70°C. A portion of the curettings selected for RNA extraction was embedded in OCT cryopreservative mountant for immunocytochemical assay and histological comparison. Grading of the carcinomas was carried out using the Mostofi and colleagues classification [22]. Prostate tissue was obtained from 14 patients who required a further TURP after receiving endocrine therapy (range 2–7 years): orchidectomy (n=9), radiotherapy and Zoladex (n=2), diethylstilboestrol alone (n=1), diethylstilboestrol, cyproterone acetate and orchidectomy sequentially (n=2). Pre- and post-treatment samples were available for 5 of the patients. The DU145 and PC3 cell lines were cultured as described previously prior to RNA extraction [24].

RNA extraction and Northern blot analysis

Total RNA was extracted from the prostatic tissue and cell lines using the guanidine isothiocyanate/cesium chloride method [23] followed by purification and Northern blot analysis as described previously [24]. RNA from the prostatic carcinoma cell lines, DU145 and PC3, was included on every blot as a measure of inter blot variation.

Blots were prehybridised (4 h) and then hybridised, overnight at 45°C, with a 2.4-kb EGFR cDNA EcoR1 fragment of pBR322. The probe was labelled with [$^{\alpha 32}$ P]dCTP using the random prime labelling system (Prime-a-gene labelling kit, Promega Corp., Southampton, U.K.) and 3.07×10^7 dpm labelled probe was added to 10 ml of the hybridisation solution. After hybridisation prior to autoradiography on X-ray film, the filters were subjected to stringency washes [23]. The filters were washed in 1% glycerol at 80°C for 2 min to remove the probe before rehybridising filters overnight at 45°C (5×10^5 dpm labelled probe/ml) with a 3.5-kb β-actin cDNA EcoR1/HindIII fragment of pBR322.

Sizes of detectable transcripts were determined with reference to RNA molecular weight markers (Promega Corp.). The density values were adjusted to allow for non-linearity of dpm versus optical density prior to normalisation to the values from the β-actin probe and the results expressed as a percentage of the DU145 mRNA value [23].

EGFR immunohistochemistry

External domain EGFR on frozen sections. Frozen sections of the prostate tumours were immunostained for EGFR using a monoclonal EGFR antibody (Amersham, U.K.) as described previously [25].

EGFR immunoassays on fixed tissue. Immunostaining was performed on formal saline-fixed, paraffin-embedded sections (5 μm) employing two monoclonal antibodies to EGFR, one recognsies residues 985–996 of the intracellular domain of the receptor (Sigma Chemical Co., Poole, U.K.) the other binds to the protein portion of the extracellular domain (Biogenex Laboratories, supplied by Bio Diagnostics, Upton-upon-Severn, U.K.). Mouse immunoglobulin of the same isotype, IgG1, as the above antibodies was used as the negative control. Normal human serum, equivalent to 20% of the final antibody solution, was admixed with undiluted antibody for 30 min followed by the addition of 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Incubations were carried out overnight at 4°C at 1 in 50 dilution for the Sigma EGFR antibody and at room temperature at 1 in 20 dilution for the Biogenex antibody (equivalent to 5 μg/ml). Prior to addition of the primary antibodies, tissue sections were exposed to 0.02% pronase in PBS at 37°C for 20 min for the former and for 10 min for the latter antibody. Immunolocalisation of EGFR was performed using the Strept A-B kit system (DPC Ltd, Caernarfon, U.K.) and was visualised using 3-amino-9 ethyl carbazole for the internal domain and by means of diaminobenzidine (DAB) at neutral pH with 0.05 M imidazole for the external domain antibody.

All tissue sections were lightly counterstained with 10% Ehrlich's haematoxylin. The intensity of staining was scored from 0, +/−, 1+, 2+, 3+ and was carried out by two independent observers.

Statistical analysis

The Mann–Whitney U test was used to compare data obtained for the various pathological groups and carcinoma grades.

RESULTS

Northern blot analysis

Forty-four BPH specimens, 39 carcinoma specimens and 15 specimens containing carcinoma with associated benign epithelial glands were analysed. EGFR message could not be detected in seven carcinoma samples either due to RNA degradation or insufficient RNA being extracted. Northern blot analysis with the EGF receptor probe showed an 8.5-kb transcript and with the actin probe a 2.1-kb transcript in the remaining cases (Figure 1).

EGFR mRNA values were normalised to the values for actin and expressed as a percentage of the mRNA value obtained for DU145 which acted as the control on all blots (Figure 1). The relative abundance of EGFR mRNA in samples of frozen prostate tissue is shown in Figure 2. Mann–Whitney analysis demonstrated no significant difference between BPH tissue (mean 0.59), samples in which both carcinoma and benign glands were evident (mean 0.56), and those in which only carcinoma was seen (mean 0.58). EGFR mRNA expression in the various histopathological grades of prostatic carcinoma was not significantly different, well differentiated tumours n=6, median 0.58; moderate n=16, median 0.52; anaplastic carcinoma n=25, median 0.53. In the BPH tissues regression analysis of mRNA value obtained versus the percentage of either stroma or epithelium present gave values of r=0.124, P>0.05 and r=0.037, P>0.05, respectively. In the samples containing only carcinoma, regression analysis of mRNA EGFR values versus percentage of stroma present gave r=0.386, P<0.01.

Immunohistochemical analysis

Positive membranous immunostaining for EGFR was observed in the majority of the basal cells seen in frozen BPH tissues (Figure 3b). Membrane staining was absent or of low intensity in the secretory cells of the acini (Figure 3).

A similar distribution of membrane immunoreactivity was

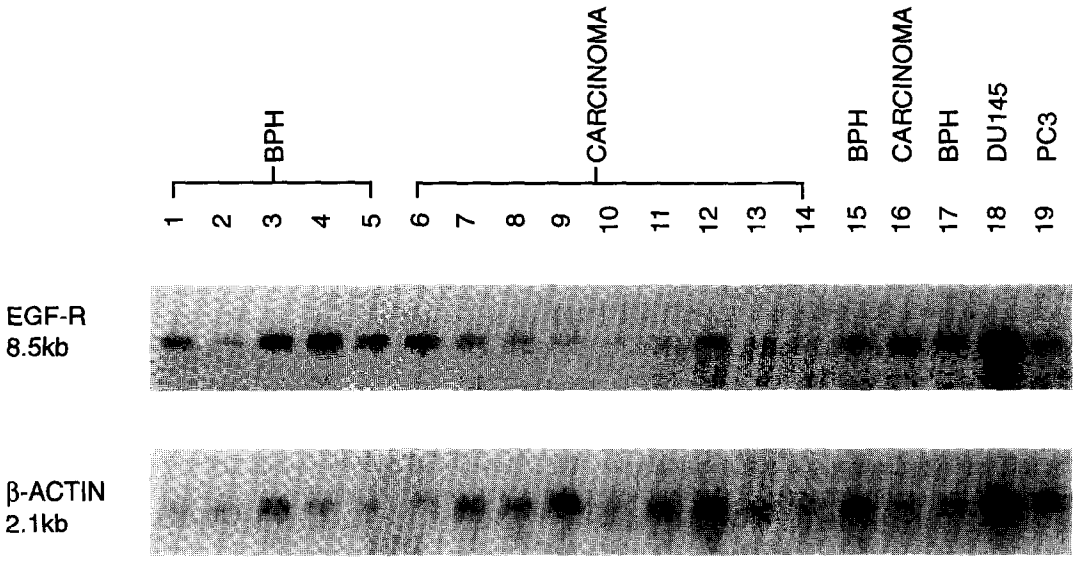

Figure 1. Northern blot analysis of EGFR and actin expression in a group of prostatic tumour specimens. RNA from the prostatic cell lines were included on every blot as positive controls. RNA was electrophoresed and transferred to nylon filters followed by sequential hybridisation with each probe. Exposure times for EGFR and actin were 40 and 20 h, respectively.

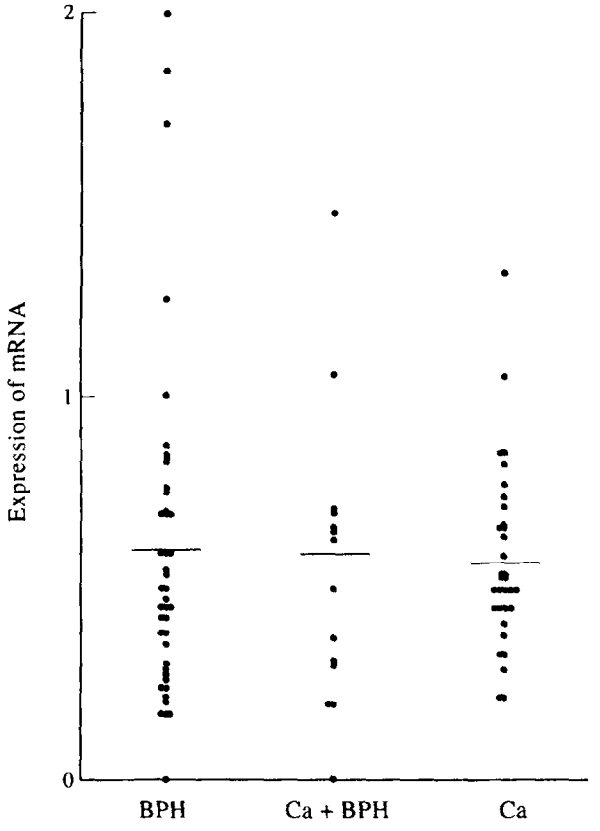

Figure 2. Relative abundance of EGFR mRNA in the three specimen groups: BPH specimens (n=44), samples in which both carcinoma and benign glandular tissue were present (n=14), samples containing only carcinoma (n=33). mRNA is expressed in arbitrary units represented as a percentage of the mRNA level detected for DU145 which was included on every blot as a control. Individual and mean values for each group are plotted.

observed with the antibody to the external domain of EGFR on fixed BPH tissues. Intense staining (3+) of basal cell membranes of 95% of the acini was observed whilst expression was absent or of low intensity (+/−) on secretory cell membranes (Figures 3a and 4). Using the antibody to the internal domain of EGFR (Sigma) cytoplasmic staining was seen in the secretory epithelium whilst the basal cells were unstained (Figures 3c and 4).

Immunohistochemical data obtained with the three antibodies on the prostate carcinoma specimens are plotted in Figure 3 and examples of the staining patterns observed are shown in Figure 4 c–h. Again, the two antibodies directed to the external domain of EGFR exhibited similar immunoreactivity in the carcinoma cells (Figure 4 g and h). In samples in which the frozen and formal saline-fixed tissue histology were identical (n=41), significant correlation was found in the intensity scores, r=0.6399, P<0.001. The internal domain antibody (Sigma) gave mainly cytoplasmic, often heterogeneous staining (Figure 4 d and f). No significant correlation was found with the cytoplasmic intensity scores for this antibody and the membraneous staining expression seen with the other antibodies. Although in some tumours, EGFR positivity was comparable in intensity (Figure 4 c and d), in other specimens intense cytoplasmic staining was observed with the internal antibody whilst the membrane staining was weak or absent with the external antibody (Figure 4 e and f).

The trend to increased EGFR expression accompanying dedifferentiation was seen with all three antibodies and is illustrated in Figure 3. Statistical significance (P<0.02) was, however, reached only when intensity scores for the internal domain antibody were compared for the well (n=12) and poorly differentiated (n=29) cancer groups.

Comparison of the mRNA and immunohistochemical results

Regression analysis of the EGFR mRNA values for the three pathological groups, BPH, carcinoma and associated BPH and carcinoma only, and their respective immunostaining intensity scores for either the internal or external domain antibodies did not demonstrate any correlation.

EGFR expession in tumours after therapy

Formal saline-fixed tumour tissue from 14 patients, who had received therapy for their prostatic cancer 2–7 years previously and required TURP for local tumour regrowth, was assayed for membrane EGFR expression using the external domain antibody. All of these tumours were grade III, 43% of which

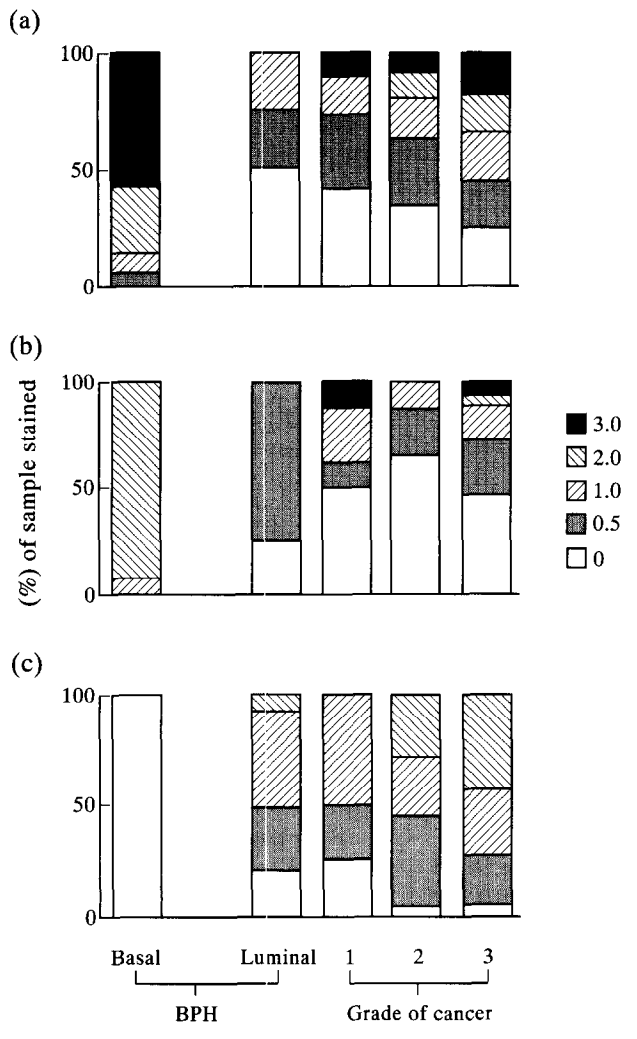

Figure 3. Histographic comparison of the immunostaining in the BPH, basal and secretory components, and carcinoma specimens with the two external domain antibodies (a) Biogenex and (b) Amersham and the internal domain antibody (c) Sigma. The percentage of specimens at the various intensity scores is plotted, from 0 to 3+. The carcinoma specimens have been graded depending on their differentiation [22].

were unstained, 7% were assigned a score +/−, 21% 1+, 22% 2+ and 7% 3+ for EGFR expression. EGFR expression was absent in 28% of the pretreatment group of grade III tumours and the intensity scores of the remainder were 14% +/−, 25% 1+, 15% 2+ and 18% 3+. In 5 patients where pre- and post-treatment samples were available either little or no consistent change in EGFR expression was detected in the carcinoma cells. In this group, 3 patients had identical EGFR scores pre- and post-therapy, in 1 patient the EGFR scores increased from +/− to 1+ and in another decreased from 2+ to 1+.

DISCUSSION

The role of EGFR and its ligands in the proliferation of prostatic cell lines suggests that it may modulate prostatic growth but its expression in adenocarcinomas remains controversial. This is undoubtedly due to factors such as the biopsy techniques and the methodologies used to measure expression and the inherent tissue and tumour heterogeneity. This study indicates that those techniques requiring homogenisation and extraction of tumours do not correlate well with those where individual cells are assessed immunohistochemically. Also, it has shown that antibodies specificities are crucial to the evaluation. Consideration of these factors is important when interpreting clinical data.

Positive EGFR immunostaining in the basal cell membranes of the glandular tissue was observed in 100% of BPH specimens with the two external domain antibodies which is in agreement with that reported previously for EGFR immunohistochemistry on frozen tissue sections [14–16]. It is this basal cellular component which appears to proliferate in response to damage to the glandular epithelium and stains intensely for EGFR. Additionally, when BPH epithelial monolayers are established in explant culture by a process akin to wound healing these cells too are strongly EGFR positive and have basal cell characteristics [25].

Membrane staining of carcinoma cells was observed in 45% of the tumours with the Amersham antibody, although the majority expressed very low amounts, and in 67% with the Biogenex antibody where the staining was more intense. The number of carcinomas expressing EGFR was higher than reported in other studies [11, 14, 15] and appeared to be more prevalent in the higher grade tumours, suggesting a role in progression, which would be consistent with the findings of Idikio [26]. Higher amounts of EGFR mRNA per total RNA have been found in the prostatic cell lines PC3 and DU145, both derived from metastatic prostatic adenocarcinomas, again suggesting that EGFR is involved in progression but also that it may be a prerequisite for *in vitro* culture of prostatic tumours.

The antibody to the internal domain bound to cytoplasmic regions of the secretory epithelial cells but was not observed in the basal cells. It is possible that the specific antigenic site of the EGFR molecule to which this antibody binds is masked because of conformational changes when expressed within basal cell membranes. The majority of adenocarcinomas (89%) exhibited cytoplasmic staining with this antibody although often heterogeneous in distribution. No agreement was found in the internal domain antibody results and EGFR mRNA values for the tumours. Similarly, no correlation was found in these two parameters in a range of prostatic cell lines (unpublished data).

In this study, EGFR mRNA values from BPH and carcinoma samples were not significantly different. A previous report found significantly higher EGFR mRNA levels in carcinoma than in BPH samples [12]. It was suggested that stromal areas within their BPH samples may account for lower levels. Although we were unable to find a correlation in BPH tissue between the percentage of stroma and the EGFR mRNA values, a negative correlation was observed in the carcinoma specimens. The lack of correspondence of the mRNA results and the immunohistochemical assays is probably due to tumour tissue heterogeneity, as good correlation was found with the mRNA levels and the external domain antibody assays in a range of prostatic and breast cell lines (data not shown). Additionally, RNAases in the tissues and the biopsy technique used (cauterisation) may have significantly influenced the mRNA values determined. Ligand binding assays have reported that BPH had lower levels of EGFR than carcinoma and additionally that the concentration of receptor increased from well differentiated to anaplastic [10], whereas another group found the reverse situation [11].

In several other malignancies EGFR expression is associated with poor prognosis [17–20] but to date, this has not been shown in prostatic cancer. Using the frozen tissue immunoassay for EGFR we found no significant difference in the survival of prostatic cancer patients categorised according to EGFR positiv-

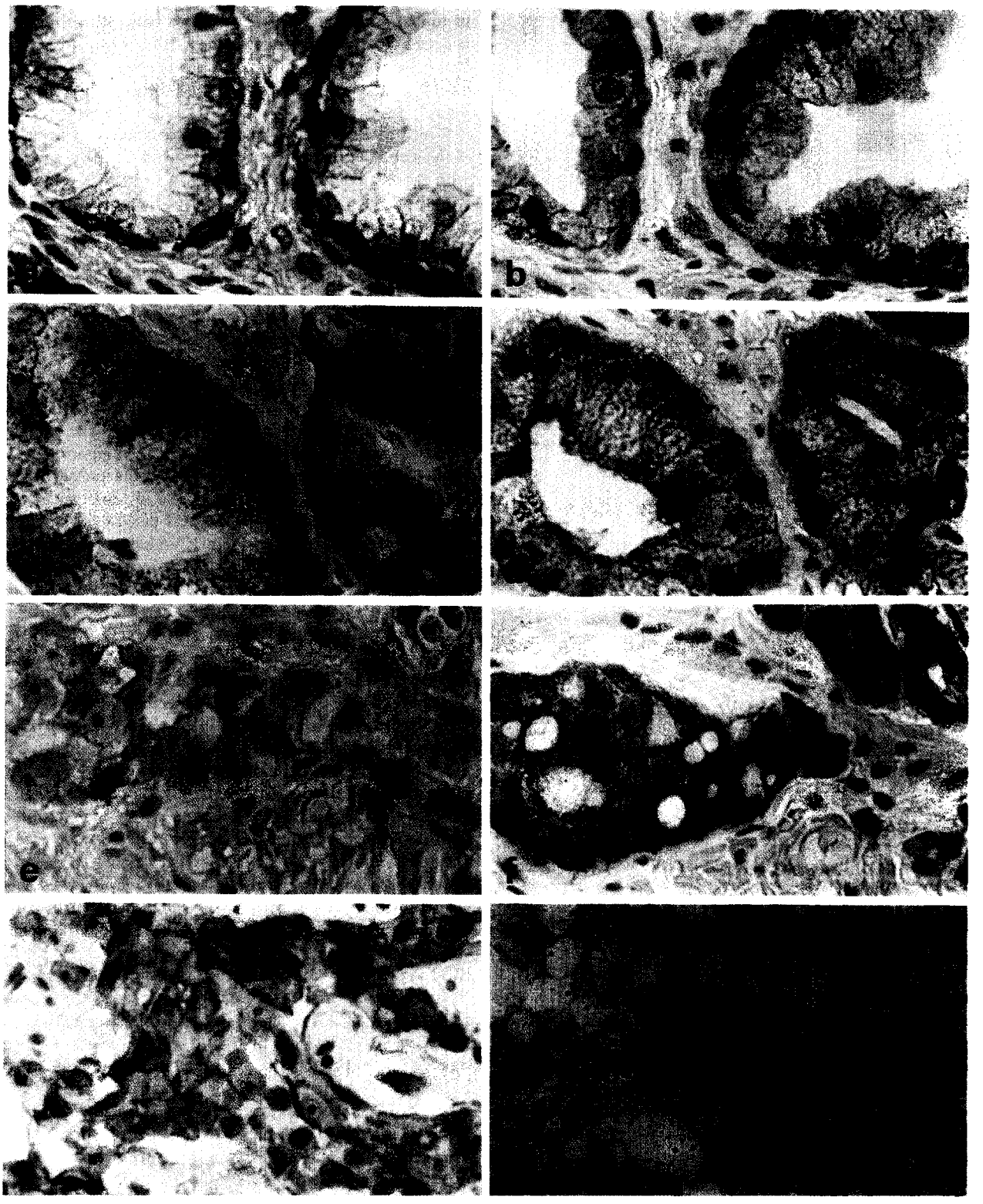

Figure 4. Immunohistochemical distribution of EGFR in formal saline-fixed sections of BPH (a, b), and three prostatic carcinoma specimens (c–g). Consecutive sections were incubated with the external domain antibody (Biogenex) in (a), (c), (e) and (g) and with the internal domain antibody (Sigma) in (b), (d) and (f). Immunolocation of EGFR on a frozen section of carcinoma incubated with the external antibody (Amersham) is shown in (h). Intense membrane staining of the basal cells is seen in (a) and absent in (b), the latter section, however, showing positive cytoplasmic staining of secretory epithelium. All three carcinomas exhibited EGFR-positive membranes (c, e and g) although the intensity varied. Cytoplasmic staining was detected in the more differentiated tumour (d) being stronger at luminal margins, whilst a more intense product distributed throughout the cytoplasm was seen in (f).

ity [21]. Currently, the fixed tissue histochemical assay for the external domain which allows easier morphological assessment and the use of archival material is being examined for its prognostic value.

The results obtained with the prostatic tumour tissue from patients who had been on therapy for 2 or more years indicated that there was no increase in clones of cells expressing EGFR accompanying local progression. Also, this subset of tumours (locally progressive) did not express characteristically high or low EGFR levels prior to therapy. Interestingly, it has been reported that in the rat prostate, EGF binding is increased after castration and that this can be inhibited by dihydrotestosterone [27] and, conversely, that in the hormone-dependent Dunning R3327 G tumour that the EGFR levels are decreased following castration [28]. We have not been able to analyse metastatic tumour tissue after relapse on hormonal therapy, however, it would appear that in local progression of the primary tumour EGFR is not involved.

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