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Ectoenzyme regulation by phenotypically distinct fibroblast sub-populations isolated from the human mammary gland

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SUMMARY

Inter- and intralobular mammary fibroblasts have been separated from normal human breast tissue and cultured to study the differential expression of ectoenzymes present within the stroma of the normal gland and associated with breast cancers. Specific ectoenzymes were identified by indirect immunofluorescence and quantified by flow cytometry and semi-quantitative PCR. A consistent difference was noted between the two fibroblast sub-populations at early passage in respect of dipeptidyl peptidase IV (DPP IV) and aminopeptidase N (APN) expression. Early passage intralobular fibroblasts were positive for APN but negative for DPP IV, as seen in the intact tissue. However, with continued sub-culture they gradually began to express DPP IV, until at later passages they became indistinguishable from the interlobular fibroblasts, which were APN and DPP IV-positive at all stages in culture, as they are in intact tissue. Neutral endopeptidase (NEP/CALLA/CD10) is not expressed by normal adult breast fibroblasts but is found in the stroma associated with over 60% of breast cancers. It was up-regulated in vitro on both inter- and intralobular fibroblasts, with final levels that were significantly (<14 times) higher on the former in all pairs of preparations from individual donors analysed. This difference persisted with continued passage, and levels of the ectoenzyme and its messenger RNA were further up-regulated by hydrocortisone in both populations. These results demonstrate that phenotypically distinct cultures of human mammary fibroblast sub-populations can be used to study the regulation of these stromal ectoenzymes. Such cultures also provide a means of investigating the mechanisms and functional significance of changes occurring as the result of neoplasia, as well as enabling the individual roles of inter- and intralobular fibroblasts in epithelial/mesenchymal interactions to be evaluated.

Key words: fibroblast, human breast, ectoenzyme, neutral endopeptidase, CD10, aminopeptidase N, CD13, dipeptidyl peptidase IV, CD26

INTRODUCTION

Ectoenzymes are a large family of membrane-associated metalloproteases that demonstrate cell-type specificity and have a number of potentially important functions. These include the extracellular degradation of peptide hormones (Kenny et al., 1989), degradation of extracellular matrix (Saiki et al., 1993) and facilitation of virus entry into target cells expressing these enzymes (Callebaut et al., 1993). The distribution of three of these enzymes on both stromal and epithelial cells in the human breast has been studied in this laboratory; namely, aminopeptidase N (APN/CD13), dipeptidyl peptidase IV (DPP IV/CD26) and neutral endopeptidase (NEP), also known as CALLA or CD10 (Letarte et al., 1988). Each ectoenzyme has been shown to be expressed by specific cell types in the intact mammary tissue. Thus, APN has been localised to the apical membrane of luminal epithelial cells as well as being present on all stromal fibroblasts, but not on myoepithelial cells or endothelial cells (Atherton et al., 1992b), while DPP IV is found on a sub-set of stromal cells (Atherton et al., 1992a). NEP is expressed only by the myoepithelial cells in the normal adult human mammary gland (Gusterson et al., 1986) although it can be detected on some stromal cells in the developing infant breast (Atherton et al., 1994). It is also found in the adult gland in association with breast tumours. In a series of 32 breast carcinomas, a total of 17 showed significant reactivity for NEP in a sub-population of associated stromal cells whose origin and function is unknown (Atherton et al., unpublished data). NEP has also been seen in a minor stromal cell population associated with fibroadenomas (Mechtersheimer and Moller, 1989).

In the normal human mammary gland two distinct stromal areas can be recognised morphologically. The acini of the lobules are embedded within a loose relatively cellular stroma

(the intralobular stroma), while the lobules and their associated intralobular stroma are surrounded by the interlobular stroma, which is densely collagenous and contains relatively few cells. Functionally different fibroblast sub-populations in the normal human mammary gland, which correspond to these morphological sub-types, have recently been demonstrated using an antiserum to dipeptidyl peptidase IV (DPP IV). This ectoenzyme is present on fibroblasts in the interlobular stroma, while fibroblasts within the lobule are DPP IV negative (Atherton et al., 1992a). In contrast to the ubiquitous distribution of APN on all mammary fibroblasts, this differential expression of DPP IV by inter- and intralobular cells provides a means of immunophenotyping these sub-populations that can be used to assess the purity of preparations obtained from the different areas of mammary stroma. Further support for the concept of functionally different sub-populations of breast stromal cells comes from tumour cell biology. Thus, it has been shown that stromal tumours that are specific to the breast (phyllodes tumours) are derived from intralobular fibroblasts (Atherton et al., 1992a), and that such tumours are associated with a non-random loss of chromosome 6 material, in one case involving a 6p23 translocation (Birdsall et al., 1992).

The aim of the present study was to develop a method to purify normal inter- and intralobular human mammary fibroblasts, to characterise these cells in vitro using antibodies to DPP IV and APN so as to confirm their origin and purity, and to use such cultures to study the functional regulation of the tumour-associated stromal marker NEP at both the protein and mRNA levels.

MATERIALS AND METHODS

Cell culture

Preparations of inter- and intralobular fibroblasts were obtained from a total of 16 separate breast samples from individuals aged 16-36 years. Detailed studies were made using separate cultures of both cell types prepared from the same donor, and in which purity at source had been determined by repeated microscopic examination during establishment from partially digested breast tissue (interlobular cells) and from stromal cells attached to epithelial fragments (intralobular cells).

Preparation of interlobular mammary fibroblasts

Human breast tissue (approx. 4 g) obtained from reduction mammoplasties was finely minced and partially digested, with stirring, for 1 hour at 37°C in 100 ml collagenase (4 mg/ml, type IA; Sigma, Poole, UK) in Leibovitz L-15 medium (Gibco BRL, Paisley, UK) containing 10% (v/v) foetal calf serum (FCS; Imperial Laboratories, Andover, UK) and penicillin/streptomycin (PS, 100 μg/ml). After removing the fat and allowing undigested fragments to settle, the supernatant was aspirated, centrifuged (400 ***g***), and the resulting pellet washed and filtered sequentially through 140 μm, 53 μm and 35 μm sterile nylon filters. The filtrate containing single cells was collected and viable stromal cells were plated at a density of 2×10^5 cells in 25 cm^2 plastic flasks (Nunc, Gibco BRL). The growth medium used was a 1:1 (v/v) mixture of Ham's F12 (Gibco BRL) and DMEM (Imperial Laboratories) with 20% (v/v) FCS and PS. Cells were sub-cultured at a split ratio of 1:3 on reaching confluence. After 3 passages the cultures were routinely fed with DMEM plus 10% FCS (v/v) and PS (basal medium).

Preparation of intralobular mammary fibroblasts

Fragments of undigested breast tissue obtained by limited enzymatic digestion (see above) were digested further for 3 hours at 37°C in collagenase (2 mg/ml in L-15). The resulting preparations, composed principally of epithelial organoids, were pelleted, washed and filtered through a 140 μm sterile nylon filter that retained the larger fragments, which were discarded. The filtrate was passed through a 53 μm filter and the small/medium-sized organoids that were retained were retrieved, resuspended in L-15 and refiltered using a 35 μm filter to remove all single cells.

Organoids purified in this manner were placed in a Petri dish and, using an inverted phase-contrast microscope in a class II laminar flow hood, lobules that had visible intralobular stroma still attached were picked with a sterile glass pipette. Individual organoids of this type were plated in separate wells in a 24-well culture plate (Gibco BRL) containing growth medium (see above). After 7-14 days the organoids had mobilised and spread, and proliferating intralobular fibroblasts could be seen at the periphery of some explants. Any wells that also contained stromal cells not directly associated with an epithelial outgrowth were rejected.

Fibroblasts were isolated from satisfactory preparations by brief (1-2 minutes) trypsinisation (1 mg/ml type III in 0.02% (w/v) EDTA) and resuspension in growth medium containing 20% FCS (v/v). The harvested cells were filtered through 35 μm filters to remove the largely undisaggregated epithelial fragments. The resulting intralobular fibroblasts from several original wells were pooled, replated into fresh wells and maintained until the cells were near or at confluence. They were then sub-cultured into 25 cm^2 flasks and after a further passage in growth medium containing 20% FCS (v/v) they were routinely sub-cultured in basal medium with 10% FCS (v/v) at a split ratio of 1:3.

Fluorescence staining and flow cytometric analysis

Separate cultures of inter- and intralobular fibroblasts (grown initially in basal medium and then either with or without the further additives described in Results) were analysed for ectoenzyme expression by flow cytometry and indirect immunofluorescence microscopy. Cells grown in 25 cm^2 flasks were harvested by trypsinisation to give single cell suspensions for flow cytometry, while for immunofluorescence microscopy corresponding cells were grown on 13 mm glass coverslips (Chance Propper Limited, Smethwick, UK). In both cases viable cells were stained over ice for the visualisation and analysis of surface-associated ectoenzymes.

Cells were incubated for 40 minutes with 200 μl of primary antibody diluted in L-15 containing 10% (v/v) FCS. For flow cytometric analysis, APN and NEP were detected using the mouse monoclonal antibodies CLB-mon-gran/2 (Janssen Biochimica, Beerse, Belgium) and SS2/36 (DAKO), respectively, both diluted 1:200. DPP IV was localised using the rabbit antiserum RP181 (Dr A. J. Kenny, Department of Biochemistry, University of Leeds, Leeds, UK) used at 1:100. After 3× 5 minute washes in L-15, single-labelled cells were incubated for a further 40 minutes in species-specific FITC-conjugated immunoglobulins (Amersham International, Aylesbury, UK). For simultaneous double-labelling of viable cells for APN and DPP IV, both CLB-mon-gran/2 and RP 181 were added concurrently and visualised separately with species-specific second antibodies, including a sheep anti-mouse FITC and a biotinylated anti-rabbit antiserum (both used at 1:40), followed by a R-phycoerythrin-avidin complex (PE; Vector Laboratories, Peterborough, UK).

Samples stained with either FITC or FITC plus PE were analysed on an Ortho Cytofluorograf 50H flow cytometer and associated Ortho 2150 computer system, using a Spectra-Physics argon-ion laser tuned to 488 nm at 200 mW. A cytogram of forward and orthogonal light scatter was used to identify the region that included only single live cells, and excluded debris and clumps. The fluorescence of gated single viable cells was then recorded and displayed as monovariate histograms (FITC only) or bivariate cytograms (FITC versus PE fluorescence). The small amount of spectral overlap resulting from green fluorescence reaching the orange fluorescence detector was corrected

using control samples from which each primary antibody had been omitted in turn.

Cells grown on coverslips for microscopic immunofluorescence were labelled as described above, except that a Texas Red (TRD)-conjugated donkey anti-rabbit antiserum was used instead of R-PE. They were washed in PBS (3× 5 minutes) and mounted in a 1:1 (v/v) mixture of Hydromount and Citifluor anti-fadents AF1 and/or AF2 (Citifluor Limited, London, UK). The immunostained cells were examined using a Zeiss epi-fluorescence microscope (with narrow band green and red emission filters) using a ×25 water-immersion lens (NA 0.85) or on a MRC-600 confocal imaging system equipped with a krypton-argon laser using a ×20 lens (with the aperture fully open and the gains set at 2.0 for TRD and 5.9 for FITC). The absence of spectral overlap with each of these systems was tested using single-labelled samples.

For analysis of cytoskeletal antigens, cells grown on coverslips were fixed in ice-cold methanol for 4 minutes prior to staining by indirect immunofluorescence. Mouse monoclonal antibodies used to define the phenotype of the mammary stroma preparations further were 1A4 (Sigma), which is specific for smooth muscle α-actin (Skalli et al., 1986), M725 (DAKO) reacting with vimentin, and the monospecific anti-cytokeratin antibodies LL002 (Purkis et al., 1990) and LE61 (Lane, 1982), which visualise cytokeratins 14 and 18, respectively. After staining with primary antibodies for 1 hour in L15/FCS, and washing, antigens were visualised with sheep anti-mouse FITC-conjugated antibody (Amersham International) used at 1:40.

PCR analysis of NEP gene expression

NEP mRNA levels in typical human mammary fibroblast cell cultures containing ~10^6 cells were not sufficient to permit direct quantification by northern blotting. NEP gene expression in purified inter- and intralobular populations was, therefore, determined by the polymerase chain reaction (PCR). A 460 bp region, spanning exons 13 to 17 of this gene (D'Adamio et al., 1989), was amplified from randomly primed cDNA made to total RNA. The sequences of the oligonucleotide primers for NEP gene amplification were CALLA F 5′ TTGTAAGCAGCCTCAGCCG 3′ and CALLA R 5′ TTGTCCACCTTTTCTCGGAG 3′. PCR reactions, which contained each primer at 1 μM and $MgCl_2$ at 1.5 mM, consisted of cycles of a DNA denaturation step at 94°C for 1 minute, an annealing step of 50°C for 1 minute and a DNA synthesis step of 72°C for 1 minute. NEP gene expression in different samples was compared using a semi-quantitative PCR transcript assay based on the method of Dallman and Porter (1991). In the present study the amplification of a 150 bp region of the mRNA for the housekeeping gene glucose-6-phosphate dehydrogenase (G6PD) was used to show that each sample had made cDNA to the same efficiency and then undergone equivalent amplification during PCR (Boehm et al., 1991). The relative levels of NEP transcript in samples was also confirmed by following reactions in which G6PD and NEP sequences were co-amplified (Boehm et al., 1991).

RESULTS

Staining patterns in sets of cultures prepared from human mammary inter- and intralobular fibroblasts were determined firstly by indirect immunofluorescence microscopy using both membrane and cytoskeletal antigen markers to establish the identity of the two sub-populations and the persistence, or otherwise, of the markers that distinguish them in vivo. They were further analysed by flow cytometry and semi-quantitative PCR to study their ectoenzyme regulation in vitro at both protein and mRNA levels. In all such sets of cultures no differences could be seen in the phase-contrast morphology of the two sub-fibroblasts populations either during growth or at confluence, and both could be routinely subcultured for between 10 and 12 passages before growth rates declined. Samples were analysed at early (p^{3-4}), mid (p^{7-8}) and late passage (p^{10-12}).

Immunocytochemical analysis of human mammary fibroblast sub-populations

Membrane antigens

When simultaneously analysed for expression of APN and DPP IV at early passage (p^{3-4}) by indirect immunofluorescence the majority (>90%) of interlobular fibroblasts were visibly positive for both antigens, as seen in the intact tissue (Atherton et al., 1992a,b). APN staining was relatively uniform on most cells (Fig. 1A), while DPP IV staining was relatively uniform over the surface of individual cells, but showed significant heterogeneity in intensity between cells (Fig. 1B). Most cells were moderately DPP IV positive, although a few (5-10%) were at the limits of microscopic detection. There was no detectable change in this staining pattern with continued passage.

At early passage the majority (>90%) of intralobular fibroblasts were APN positive (Fig. 1C) but DPP IV negative (Fig. 1D), a staining pattern identical to that seen in the corresponding cells in intact breast tissue. Visibly DPP IV positive cells in intralobular fibroblast preparations varied, but were always less than 10%, and often comprised less than 5%.

A third ectoenzyme, NEP (CALLA/CD10), which is not present on adult human breast fibroblasts in the intact tissue, was found to be consistently expressed in vitro and to different extents by inter- and intralobular mammary fibroblast preparations, and could be detected on such cells after 2-3 days of culture. Readily detectable levels of the enzyme were evident on the cell membranes of the interlobular fibroblasts in the form of uniform, punctate labelling on the surface of individual cells, compared with control cultures from which the primary antibody was omitted. There was significant heterogeneity of expression between individual cells. The levels of staining on the intralobular fibroblasts were much lower than the corresponding interlobular cells in all such preparations, close to the limits of microscopic detection, and for this reason further analysis was carried out by quantitative flow cytometry (see below). However, the difference in levels of NEP in vitro between fibroblasts derived from inter- and intralobular cells was consistently observed by indirect immunofluorescence microscopy in cultures prepared from eight individual breast samples, and was present in early, mid and late passage cultures.

As some endothelial cells stain for DPP IV in intact breast (Atherton et al., 1992a), stromal cultures were examined for their presence as a contaminant. No cells in either type of culture reacted for platelet endothelial cell adhesion molecule (PECAM-1/CD-31) (Newman et al., 1990) when stained using the 9G11 antibody (British Biotechnology Products, Abingdon, UK), thus excluding contamination by mammary microvascular endothelial cells, as these express this antigen in vitro (Hewett et al., 1993).

Cytoskeletal antigens

Fixed preparations of human mammary inter- and intralobular fibroblasts derived from five separate breast samples were immunostained for cytoskeletal antigens including both intermediate (cytokeratin and vimentin) and actin filaments. Cyto-

keratin 14 was weakly expressed by occasional cells of an apparently mesenchymal phenotype in the interlobular fibroblast preparations (<1 cell:1000). It was not detected on cells within the intralobular fibroblast cultures studied, thus excluding significant myoepithelial (basal) cell contamination. Cytokeratin 18 expression was not detected in either fibroblast preparation excluding the presence of luminal epithelial cells. Smooth muscle α-actin filaments were demonstrated in both fibroblast populations, and both sub-populations were also strongly vimentin positive, with no observable differences in intensity or distribution of either antigen between inter- and intralobular cells when these were grown under identical conditions.

Flow cytometric analysis of human mammary fibroblast ectoenzymes

Flow cytometric analysis was used to quantify some of the differences in ectoenzyme expression detected by immunofluorescence microscopy, and to monitor levels as a function of time in culture (DPP IV and APN), and in response to various additives to the culture medium (NEP).

Dipeptidyl peptidase IV and aminopeptidase N

A total of 4 separate preparations of inter- and intralobular fibroblasts were analysed for expression of APN and DPP IV at early (p^4), mid (p^8) and late passage (p^{11}) using double-labelling (Fig. 2). At early passage, flow cytometry on freshly harvested

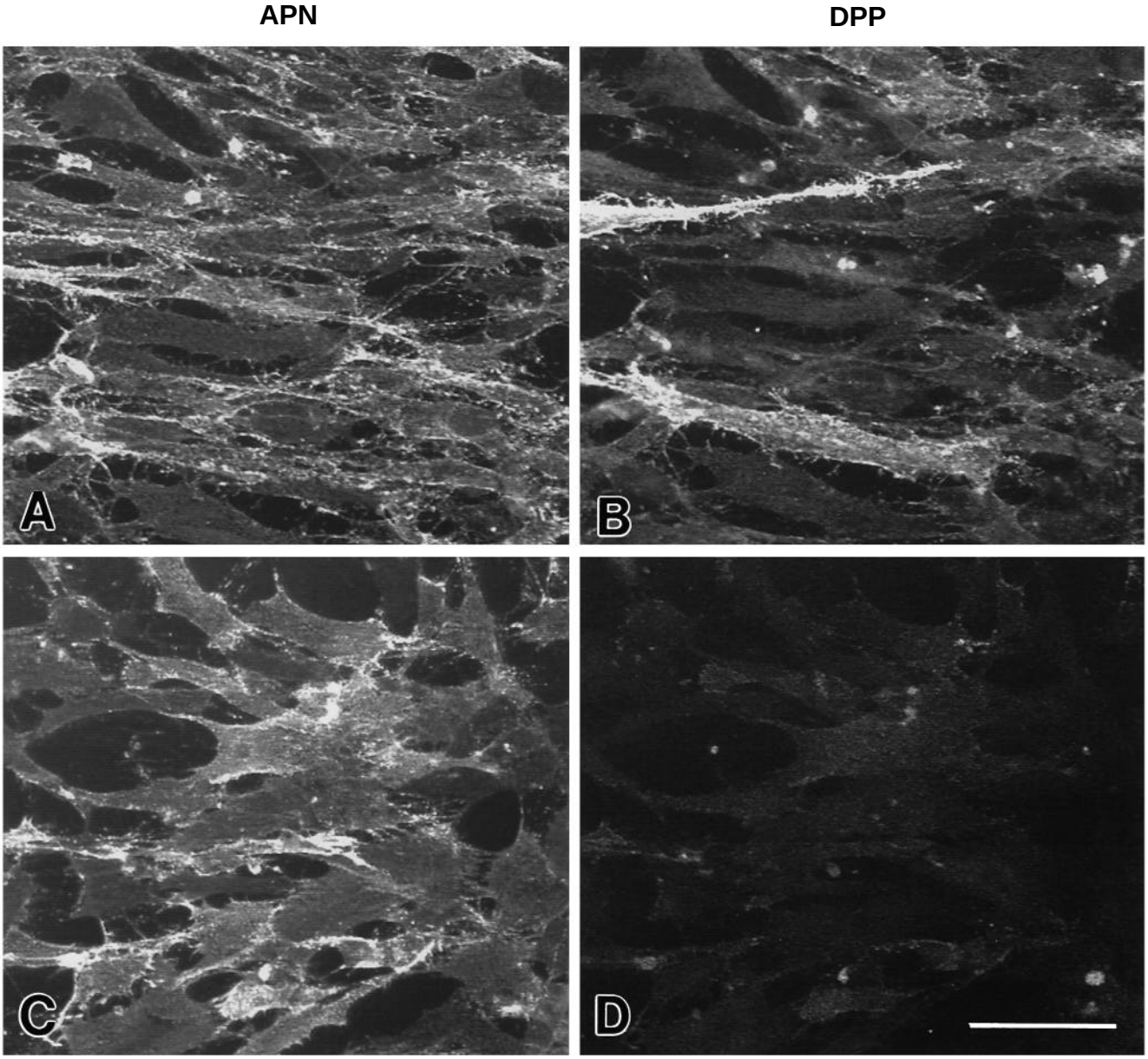

Fig. 1. Indirect double-immunofluorescence of human mammary fibroblast sub-populations. Live cells at early passage (p^4) grown separately in culture with DMEM plus 10% (v/v) FCS were simultaneously stained for expression of cell surface-associated APN and DPP IV, with mouse and rabbit primary antibodies visualised with FITC- and Texas Red (TRD)-conjugated species-specific antibodies, respectively. Separate fluorescence images were recorded from the same field of inter- (A,B top) and intralobular cells (C,D bottom) for APN-FITC (A,C left and DPP IV-TRD (B,D right), using a confocal microscope with fluorochrome-specific filters. Bar, 100 μm.

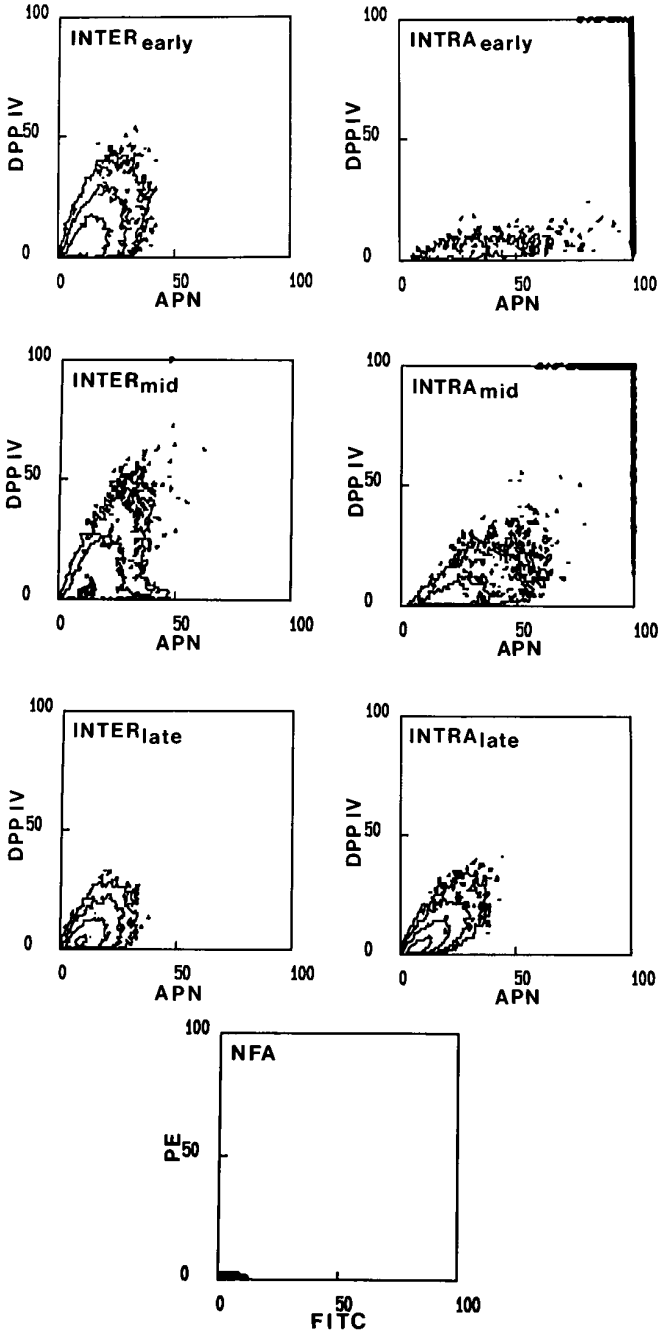

Fig. 2. Flow cytometric analysis of expression of APN and DPP IV by human mammary fibroblast sub-populations. After staining of cell suspensions with primary unconjugated antigen-specific antibodies, these were simultaneously visualised with FITC and PE, respectively, using species-specific second antibodies. 2-D contour cytograms of inter- (left) and intralobular cells (right) are shown for early (p^4), mid (p^8) and late-passage cells (p^{11}), grown under the same conditions as described for Fig. 1, and analysed at the same time, using cells retrieved from frozen stocks. Contour lines represent a pre-determined number of gated single viable cells as a fraction of the total analysed, with respect to different levels of green and orange fluorescence indicated on the two axes, with at least 10^4 cells measured in each analysis. The fluorescence of a mixture of inter- and intralobular mid-passage cells when no first antibodies were included in the staining procedure (NFA) is shown in the bottom panel.

single cell suspensions confirmed that the two sub-populations of cells differed in a manner that was consistent with immunofluorescence microscopy on cells in monolayer (see Fig. 1). Thus, at least 95% of interlobular fibroblasts were double labelled, while the majority (>90%) of the corresponding intralobular fibroblasts stained exclusively for APN, with only a few DPP IV positive cells being detected. Corresponding staining profiles at mid-passage (p^8) were still different, with the interlobular fibroblasts similar to the early-passage cells. While the majority of intralobular fibroblasts had now become double-labelled (i.e. expressing both DPPIV and APN) their mean level of DPP IV fluorescence was still lower than their interlobular counterparts. By late passage (p^{11}) the staining profiles recorded by flow cytometry were essentially identical for both types of mammary fibroblasts, with almost all cells double-labelled, to an equal degree, for both APN and DPP IV in both types of preparation (Fig. 2). The initial marked difference, followed by a gradual shift towards a similar pattern of double staining at late passage, was seen in all sets of inter- and intralobular mammary fibroblasts analysed in this manner.

Neutral endopeptidase

Flow cytometric analysis of 5 separate inter- and intralobular fibroblast preparations grown in basal medium (DMEM + 10% FCS) confirmed that there was a substantial difference in mean levels of NEP expression by these sub-populations in vitro (Figs 3 and 4). The interlobular fibroblasts consisted of a heterogeneous population with the majority of cells expressing significantly greater amounts of NEP than their intralobular counterparts, in which low, but nonetheless above control, levels were detected in all preparations (Fig. 3). This difference in NEP expression was seen at all passages examined ($p^{3\text{-}10}$) and in every pair of inter/intralobular fibroblasts analysed, with differences in mean values ranging from 3- to more than 14-fold in individual sets of cultures (Figs 3 and 4).

The consistent difference in NEP expression in basal medium allowed the functional regulation of ectoenzyme levels, in response to various additives including hormones and peptide growth factors, to be quantitatively analysed by flow cytometry. When the mammary fibroblasts were cultured in a medium originally designed for the maintenance of mammary epithelial cells and containing hydrocortisone (HC; 5 μg/ml), insulin (I; 5 μg/ml) and cholera toxin (CT; 100 ng/ml) (O'Hare et al., 1991), NEP levels increased over a 48-72 hour period in both sub-populations when compared to cells grown in medium without these additives. After a 72 hour incubation in the presence of HC/I/CT, levels of cell-surface associated enzyme were increased on interlobular fibroblasts by an average of 2.1 times (±0.05, s.d., *n*=3) (Fig. 4). The rise in NEP levels measured on intralobular fibroblasts in response to the same additives was higher, averaging a 7.0 times (±1.7 s.d., *n*=3) increase over mean basal levels (Fig. 4), with final stimulated levels being similar in both cell types. No difference in the time-course of response between the two cell types was observed.

The factors responsible for this increase were identified using the same method of analysis on cells treated individually with each additive. Levels of NEP detected on both fibroblast populations increased in response to both HC and CT, while I had no effect. This pattern of response was obtained with cells prepared from three different breast tissue samples (Fig. 4). Combinations of additives were also studied, and when HC and I were added

concurrently to the cells final NEP levels were similar to those seen with HC alone. This pattern of a combined response to HC and CT, with I having no effect either on its own or in combination with other additives, was seen in both inter- and intralobular fibroblasts. A dose response of NEP to HC alone showed a significant response at levels as low as 0.05 μg/ml (Fig. 5).

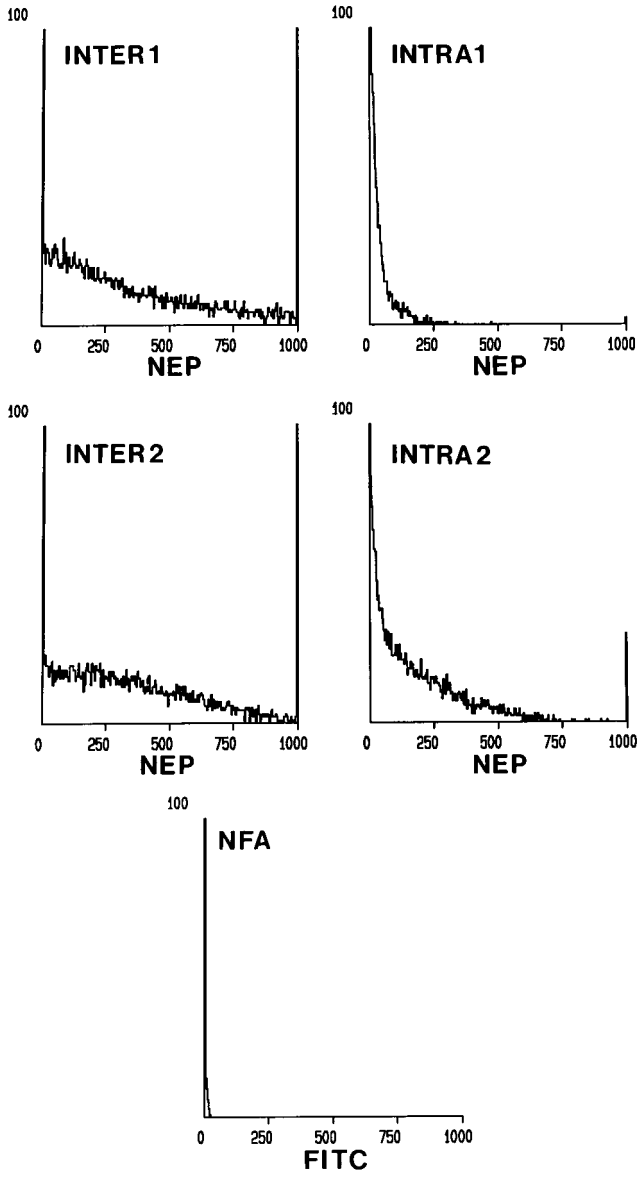

Fig. 3. Flow cytometric analysis of NEP expression by human mammary fibroblast sub-populations. Inter- (left) and intralobular cells (right) were grown in basal medium (DMEM plus 10% (v/v) FCS) and analysed at mid-passage (p^{6-8}). Each monovariate histogram shows the number of cells (*y* axis) expressing a given level of fluorescence (FITC, *x* axis) measured on a linear scale. Results from two sets of preparations are illustrated, both analysed at the same photomultiplier settings. Those on the top (set 1) show a 14-fold difference (mean value of interlobular cells being 421 with intralobulars measuring 31), while that on the bottom (set 2) a 2.3-fold difference (corresponding mean fluorescence values being 381 and 161), these being the greatest and the least differences recorded in sets from individual donors (see also Fig. 4). Mean fluorescence in a sample labelled with FITC-congugated second antibody in the absence of the anti-NEP first antibody (NFA) was 5 units (bottom panel).

Other potential physiological stimuli were also screened by flow cytometry for their effect on NEP levels on mammary intralobular fibroblast preparations. However, neither testosterone, dehydroepiandrostene (DHA), androstendione, oestrone, oestradiol or progesterone at 1 μg/ml resulted in any significant change in NEP levels (i.e. ± 10% of controls). Known modulators of NEP expression in fibroblasts from other sources include both phorbol esters, e.g. TPA (Werb and Clark, 1989) and transforming growth factor-β (Casey et al., 1993). Both were tested on cultured mammary fibroblasts and a small reduction in mean NEP levels (–24%) was obtained with TGF-β (R&D Systems, porcine platelet-derived) after 3 days of treatment at 10 ng/ml in medium with 2% (v/v) foetal calf serum. However, the effects of TPA/PMA (phorbol 12-myristate 13-acetate, Sigma) on NEP were minimal on these cells, with mean fluorescence values of 109% and 112% of control values at 10 ng/ml and 1 μg/ml, respectively (duplicate determinations on intralobular mammary fibroblasts at mid-passage), despite obvious morphological effects.

PCR analysis of NEP gene expression in mammary fibroblasts

In order to confirm that the changes measured using antibody staining were due to changes in NEP, and to determine whether these were due to changes in gene expression as distinct from translation, post-translational degradation or modulation of the protein that has been observed in lymphocytes (Pulczynski et al., 1989), levels of NEP mRNA were also assessed in a semi-quantitative manner in cultures of inter- and intralobular mammary fibroblasts, using PCR (Fig. 6), as northern blotting was not sufficiently sensitive to detect NEP mRNA directly in the relatively small numbers of cells (~10^6) routinely available for assay from these preparations.

Equal amounts of total cellular RNA harvested from confluent cultures were subjected to cDNA synthesis and the resulting RNA/DNA hybrids were amplified by PCR. Similar levels of the control PCR product (G6PD) were identified in

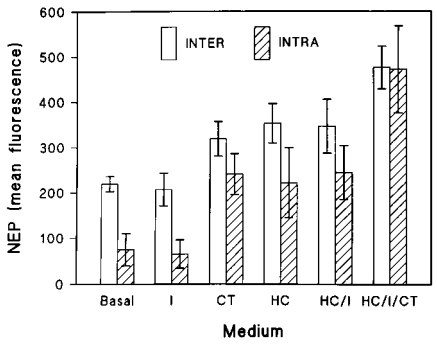

Fig. 4. Effect of medium additives on human mammary fibroblast sub-populations. Sets of replicate cultures of inter- and intralobular fibroblasts from 3 separate donors (not including those illustrated in Fig. 3) were established on basal medium (DMEM plus 10% (v/v) FCS). At mid-passage (p^{6-8}) they were treated for 72 hours with either hydrocortisone (HC, 5 μg/ml), insulin (I, 5 μg/ml), cholera toxin (CT, 100 ng/ml) or combinations thereof. Harvested cells were stained for NEP, visualised with FITC, and mean fluorescence levels (± s.d.) quantified by flow cytometry (as for Fig. 3).

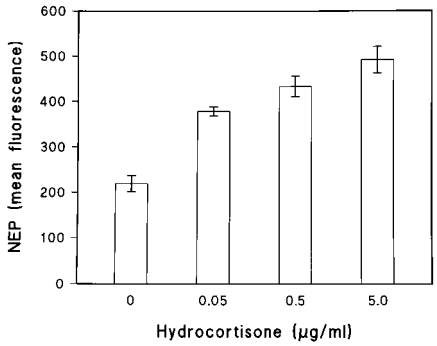

Fig. 5. Dose-response of NEP expressed on human mammary fibroblasts to hydrocortisone. Mid-passage interlobular cultures were treated for 72 hours, harvested, stained for NEP-FITC, and analysed by flow cytometry. Results are means (± s.d.) of the mean fluorescence levels in triplicate cultures at each concentration. The basal medium was DMEM plus 10% (v/v) FCS.

all samples. After 20 cycles of amplification, levels of NEP mRNA detected (represented by the expected 460 base pair PCR product) were higher in HC/I/CT-treated cells than in the control samples grown in basal medium. They were also higher in samples from the interlobular fibroblasts compared with the corresponding intralobular cells from the same donor (Fig. 6). Thus, although this method only gives a semi-quantitative estimate of relative mRNA levels in the two populations, the differences and the changes observed were consistent with the changes in cell-surface protein levels measured by flow cytometry.

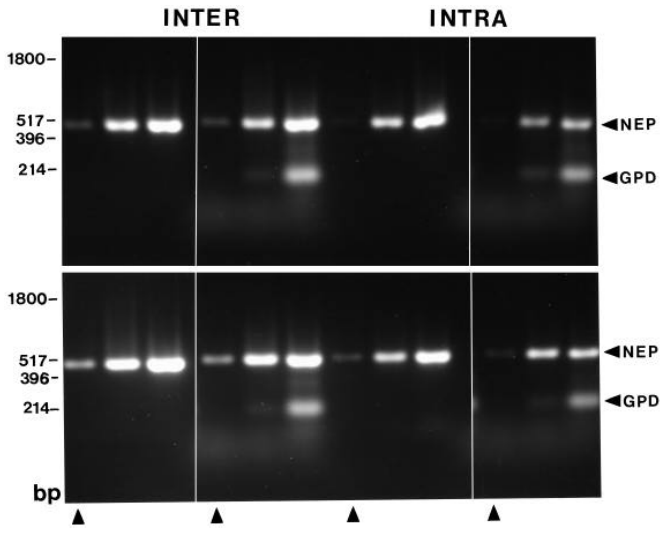

Fig. 6. Comparison of NEP and G6PD mRNA in human mammary fibroblast sub-populations using semi-quantitative PCR. For each sample a 500 μl PCR reaction containing the appropriate primer pairs of cDNA was divided into 100 μl volumes. Reactions were removed from the programmable heating block after completion of 20, 25 and 30 cycles, respectively, and the relative amounts of PCR product in 10 μl volumes of each determined by agarose gel electrophoresis. The profile of amplification shows that 20 cycles of PCR (arrowhead) is informative for levels of NEP mRNA, and comparison of the relative amounts of product shows that NEP gene expression is higher in cultures grown in HC/I/CT medium (bottom) than in basal medium (top), and in interlobular fibroblasts (left) compared with intralobular cells (right). The second set of 20, 25 and 30 cycle reaction products in each case contains co-amplified G6PD, and shows the same differences in NEP. The mobilities of pUC19/*Hin*fI restriction fragments (bp) are marked.

DISCUSSION

To investigate the functional importance of the different types of mammary stromal fibroblasts and the role of cell-type-specific ectoenzymes in mammary tissue homeostasis and tumorigenesis, methods have been developed for the separation of human inter- and intralobular fibroblasts. Using a panel of cell-surface-associated ectoenzymes as phenotypic markers, it has been shown that purified populations of these two cell types can be prepared from human breast tissue, using differential enzymatic disaggregation in combination with culture of selected fragments of parenchyme with associated stroma. Immunocytochemical and flow cytometric analysis of such cultures has demonstrated consistent initial differences in their expression of the ectoenzyme DPP IV that correspond with the distribution of this marker in the intact stroma. These methods are, therefore, capable of providing human breast fibroblast sub-populations of specific phenotype and origin, and thus complement previously developed techniques for the separation of luminal and myoepithelial cells from its parenchyme (O'Hare et al., 1991; Clarke et al., 1994).

A differential expression of NEP was also detected in the cultured breast fibroblast sub-populations, using indirect microscopic immunofluorescence, flow cytometry and PCR. This ectoenzyme was significantly up-regulated in culture, and to a consistently greater degree in fibroblasts derived from the interlobular compartment when cells were cultured in a basal medium without hormonal additives. While the difference in DPP IV expression was maintained only for a limited period of time in vitro, NEP expression was found to differ in the inter- and intralobular cultures in a consistent and persistent manner. Differences in the ratio of NEP expression between different sets (see Figs 3 and 4) may be due to the success with which pure cultures of each sub-population have been established, or may reflect intrinsic donor-related variation. NEP was further up-regulated in both populations by both physiological (hydrocortisone) and non-physiological (cholera toxin) stimuli, under which conditions NEP levels tended to converge.

Within the normal adult human breast expression of NEP is restricted to the myoepithelial cells (Gusterson et al., 1986), and it is not found on any cells within either the inter- or intralobular stroma. However, it has been detected on stromal cells within the developing rodent gland (Gusterson et al., 1986) and in the infant human breast (Atherton et al., 1994). NEP expression has been detected in a sub-population of stromal cells associated with a significant proportion (~60%) of human breast carcinomas, where it is clearly up-regulated. The up-regulation of NEP in cultures of both normal inter- and intralobular human mammary fibroblasts observed in this study is consistent with its reported in vitro expression on cultured fibroblasts from adult human skin (Lorkowski et al., 1987) and bone marrow (Braun et al., 1983). Modulation of NEP levels in vitro has also been demonstrated in response to several

factors. GM-CSF has been shown to be a powerful inducer of NEP on neutrophils (Connelly et al., 1993), and similar effects of this and other cytokines on lung fibroblasts have recently been reported (Kondepudi and Johnson, 1993). In cultured human osteoblast-like cells it has recently been shown to be up-regulated by calcitonin and 1,25-dihydroxyvitamin D3, and down-regulated by phorbol ester (Howell et al., 1993). In so far as stromal cells are concerned, treatment of rabbit synovial fibroblasts with the phorbol ester TPA caused a rapid decrease in levels of NEP mRNA (Werb and Clark, 1989), while treatment of human skin fibroblasts with TGF-β1 resulted in a substantial decrease (60-90%) in both NEP mRNA and protein levels (Casey et al., 1993).

In the present study the effects of TPA were minimal when the surface antigen NEP was measured by flow cytometry, although TGF-β did cause a small decrease in NEP levels, indicating that stromal cells from different tissues (and possibly species) may differ to some extent in the manner and extent to which NEP levels are modulated. In the human mammary fibroblasts it was found that hydrocortisone was a powerful inducer of NEP, at both the mRNA and protein levels, with detectable responses at concentrations within the physiological range (>0.05 μg/ml). Concurrent changes in both mRNA and protein showed that the effect was probably a direct transcriptional one and not due to changes in the distribution of the enzyme. Glucocorticoids have also been shown to induce NEP in transformed human tracheal epithelial cells (Borson and Gruenert, 1991). Although it has been reported that progesterone will modulate NEP in human endometrium (Casey et al., 1991), no response to sex steroids was observed in the present study of human breast fibroblasts. This study does not, therefore, establish precisely why NEP is upregulated in vitro and in vivo in association with breast cancers. It does, however, show that cultures prepared as described will: (a) differ consistently in their base-line NEP levels, depending on their specific stromal origin; and (b) respond consistently to a variety of exogenous agents. Such cultures provide, therefore, a suitable system with which to investigate in vivo-derived factors that might suppress NEP, and cancer-derived factors that might induce it.

In accordance with results obtained from the immunocytochemical labelling of normal human breast sections (Atherton et al., 1992a,b), interlobular fibroblasts grown in vitro continued to express both APN and DPP IV. The phenotype of the cells, with respect to expression of these ectoenzymes, was maintained over a number of passages, as demonstrated by flow cytometric analysis. In contrast, the staining profile of the intralobular fibroblasts changed with continued passage. Initially, the phenotype of these cells (APN positive but DPP IV negative) matched that seen in the intact breast (Atherton et al., 1992a,b). As these cells were sub-cultured, however, their staining profile altered through weak to moderate DPP IV expression, until finally the cells came to exhibit an interlobular-like phenotype, that is co-expression of both APN and DPP IV. One explanation for the changing marker profile seen within the intralobular fibroblast cultures could be overgrowth of these preparations by contaminating interlobular fibroblasts. However, if this were the case both a single-labelled and a double-labelled population would be expected to co-exist temporarily during the transition phase. On the contrary, all cells in this sub-population began to express DPP IV in gradually increasing amounts (see Fig. 2). The acquisition of DPP IV by the intralobular fibroblasts probably, therefore, represents a gradual modulation in the phenotype of the cells. The continued low expression of NEP by intralobular fibroblasts in culture (see Fig. 3) is further evidence that overgrowth by the more strongly NEP-positive interlobular cells does not account for progressive changes in DPP IV expression seen in intralobular cell preparations. Thus, while modulation of NEP levels by mammary fibroblasts can be linked to factors such as hydrocortisone, the changes in DPP IV occur under basal conditions, possibly in response to as yet unidentified factors in the medium used for culture.

The normal role of NEP in the mammary gland in vivo, and the mechanism of its regulation in stromal fibroblasts during development and in association with breast cancers remains speculative at present. In vitro experiments have shown that NEP is capable of inactivating several regulatory peptides. These include atrial natriuretic peptide (Kenny and Stephenson, 1988), enkephalins, angiotensins, bradykinin and oxytocin (Stephenson and Kenny, 1987), as well as calcitonin (Howell et al., 1993), endothelins (Vijayaraghavan et al., 1990) and chemotactic peptides (Connelly et al., 1993). In the present context it has recently been reported that oxytocin, which is a good substrate for NEP, may be mitogenic for, as well as stimulating, differentiation of myoepithelial cells (Sapino et al., 1993). The role of NEP in breast cancer has yet to be defined, but the potential significance of this ectoenzyme in tumour cell biology is demonstrated by the fact that bombesin-like peptides, which are involved in autocrine growth stimulation in many small cell lung cancers, are also major substrates for NEP, which can regulate the growth of these tumours in vitro (Shipp et al., 1991). Several functions have been postulated for DPP IV, including the induction and inactivation of cytokines (Schön et al., 1989), while other studies have shown that substance P is hydrolysed by this enzyme (Heymann and Mentlein, 1978). In the intestine and nervous system DPP IV may serve to inactivate substance P. Its specific role in the mammary stroma is, however, not known.

Until recently the function of such ectoenzymes, including APN, was considered to lie exclusively in the hydrolysis of small peptides. Recent results, however, have delineated a potentially broader role. Of particular importance in the context of the present results is the evidence that APN (CD13) may be important in the activation of type IV collagenolysis (Saiki et al., 1993). If such a role in extracellular matrix modulation extends to other mammary ectoenzymes, then it is possible that the up-regulation of NEP, seen in normal breast stromal cells in vitro and in association with tumours in vivo, may be involved as a factor in breast cancer invasion and metastasis. Such an hypothesis is open to test as inhibitors of these ectoenzymes are available (Kenny et al., 1987; Stephenson and Kenny, 1987).

In summary, the methods described here have enabled two different types of stromal fibroblasts to be isolated from the human mammary gland and studied separately. The results with a panel of markers have shown that while each cell type can retain a distinctive immunophenotype in vitro, and that some ectoenzymes (e.g. DPP IV and NEP) are subject to modulation. These sub-populations provide a tool for the further analysis of stromal factors that influence normal mammary epithelial functions, as well as providing a test system for

potential stromal responses to factors emanating from the normal parenchyme or from breast tumours.

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