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Human breast epithelium transplanted into nude mice

Proliferation and milk protein production in response to pregnancy

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Summary. Epithelial components of the normal human breast and their response to hormonal manipulation have been studied in the nude mouse. Six to eight week-old female athymic nude mice were used as the recipients of enzymatically prepared breast organoids, composed of ductal and lobuloalveolar structures. After 12 weeks *in situ* in the mouse mammary fat pad the human breast tissue retains its normal morphology as demonstrated by the presence of myosin positive myoepithelial cells and keratin positive luminal cells. Monoclonal antibodies M8 and M18 raised to components of the human milk fat globule membrane give a similar staining pattern in the xenografted organoids to that seen in the donor tissue. On mating the recipient female nude mice, the human breast tissue responds with both an increased ^{3}H-thymidine labelling index and α-lactalbumin production. This model in conjunction with in vitro studies is, therefore, suitable for the study of extrinsic and intrinsic factors controlling differentiation and morphogenesis in the human breast.

Key words: Human breast – Nude mice – α-lactalbumin

The immunodeficient athymic nude mouse affords an in vivo culture environment for studies of the control of growth and the effects of hormonal manipulation on human tissues. In order to carry out a detailed analysis of the functional potential of individual cell types within a complex tissue such as the breast it is, therefore, probably necessary to use a combination of in vitro and in vivo systems. There is, for example, evidence, from studies comparing normal (Doran et al. 1980; Sun T-T et al. 1978) and malignant (Cowley et al. 1983; Monaghan et al. 1983) human keratinocytes in vitro and in vivo that the animal environment provides "permissive" conditions for the expression of the differentiated phenotype. Thus, in order to develop

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adequate culture systems for human breast tissue it is necessary to determine whether the constituent cells still retain their full differentiative potential in terms of both structure and function after removal from the donor site and implantation as a xenograft.

Several investigators (Hillman et al. 1983; Jensen and Wellings 1976) notably McManus and Welsch (McManus and Welsch 1980 and 1981) have carried out studies of human breast biopsy xenografts from both normal and dysplastic tissues. These studies have demonstrated that when the epithelial component and the stroma is present various hormonal manipulations in the animal can induce proliferation. Although 'lactogenesis' has been induced in xenografted bovine tissue (Welsch et al. 1979) it has not been reported with human breast tissue under these conditions (McManus and Welsch 1981). The experiments of Hillman et al. (Hillman et al. 1983) involving implantation of long-term explant cultures of normal human mammary tissue into nude mice do, however, demonstrate that some normal structured elements can be retained by xenografts maintained for prolonged periods.

We, in common with others (Gaffney et al. 1976) developed in vitro systems for studying normal epithelial cells prepared from reduction mammoplasty material (Easty et al. 1980), essentially free from the connective tissue elements. In these cultures some differentiated features such as recognisable myeoepithelial cells are rapidly lost (Easty et al. 1980), although some epithelial markers demonstrated with monoclonal antibodies to the milk fat globule membrane are retained for prolonged periods by some, but not all of the cells (Edwards et al. 1984). In order to study the effects of hormonal manipulation and a foreign stromal environment on the isolated epithelial components we have xenografted such purified epithelial structures. In this preliminary study we have examined a) the maintenance of specific differentiated phenotypes using immunocytochemical markers of both myoepithelial and luminal differentiation and b) whether such xenografts can be induced to proliferate and to secrete milk proteins in response to the stimulus of pregnancy in the host.

Materials and methods

Human breast "organoids" (Fig. 1) from three individual reduction mammoplasties prepared by collagenase digestion as previously described (Easty et al. 1980) were used in this study. No pathological abnormality was evident in the material. The donors were aged 19–25 years. Approximately 10,000 freshly isolated organoids which previous studies have shown are essentially free of stromal components were suspended in 0.2 ml of medium 199 and injected subcutaneously into the right inguinal mammary fat pad of each of 65 female Balb/C (nu/nu) mice. Twenty-five animals were mated with Balb/C males, 17 became pregnant and littered, 8 did not become pregnant. Unmated control animals were killed by cervical dislocation at 4 weeks (12 animals), 8 weeks (8 animals), and 12 weeks (20 animals). The pregnant mice were terminated on the day of littering; the mated but not pregnant mice were killed 21 days after mating. Mating was used in preference to hormone pellet as the latter has been shown by other workers to be an unsatisfactory lactogenic stimulus (McManus and Welsh 1981).

All tissues from the mammary fat pad region were fixed in methacarn (Puchtler and Leblond 1958) (60% methanol, 30% chloroform and 10% glacial acetic acid) and processed in the usual way with final embedding in paraffin wax. 4 μ sections were cut and stained with hematoxylin and eosin.

Details of anti-keratin and anti-myosin antisera have been described previously (Gusterson et al. 1982). The antiserum to α-lactalbumin was a gift from Dr. M. Ormerod of the Institute of Cancer Research, London, and the characteristics of the antibody and the staining pattern in human breast tissue have been described (Bailey et al. 1982). Mouse monoclonal antibodies to the milk fat globule membrane (M8 and M18) were provided by Drs. C. Foster and P. Edwards and their reactivity in resting and lactating breast has been described in detail elsewhere (Foster et al. 1982). The binding of specific rabbit antisera to histological sections was visualised by an indirect procedure using alkaline phosphatase conjugated goat anti-rabbit second antibodies. Using mouse monoclonals, the second antibodies were rabbit anti-mouse reagents similarly conjugated (Avrameas 1969). The method used for immunocytochemical staining was similar to that described in detail elsewhere (Mitchell and Gusterson 1982). Briefly sections were dewaxed and rehydrated firstly in water and subsequently in calcium: magnesium free PBS. All sections were treated with 15% acetic acid (v/v) for 5 min at room temperature to block endogenous alkaline phosphatase. In addition to the use of normal resting and lactating human breast control tissues, antisera to keratin, myosin and α-lactalbumin were also absorbed with 1 mg/ml of the appropriate antigen at room temperature for $1\frac{1}{2}$ h prior to staining. Incubation of histological sections with the absorbed antisera resulted in complete abolition of the alkaline phosphatase staining. Human tissue was distinguished from mouse glandular elements by both of the morphological differences and the M8 positivity.

Xenografts of human breast tissue in 3 pregnant and 6 non-pregnant mice were used to assess the thymidine labelling of the tissues. At the time of littering in the pregnant and three weeks after mating in the case of the mated but non-pregnant animals, the mice were injected intra-peritoneally with 5 μCi/g [^{3}H] thymidine. Animals were sacrificed 1 h after injection and the tissue processed as previously described. The slides were dewaxed by two changes of 10 min each in xylene followed by a final wash in absolute alcohol. They were dipped in Ilford Nuclear Research Emulsion K5, dried and exposed for 14 days at 4° C. The slides were developed in D19 (Kodak) for 3 min, washed in distilled water and fixed in Kodafix for $2\frac{1}{2}$ min. Sections were washed in distilled water and lightly counterstained with Harris's Hematoxylin. Five sections were examined from each mammary fat pad. Only cells with >10 silver grains (nuclear or cytoplasmic or both) were counted and all slides were coded prior to counting to avoid bias. The labelling index (L.I.) was calculated as the number of labelled nuclei/unit area (0.1 mm^2) of epithelium as previously described by McManus & Welsch (McManus and Welsch 1981). Using a drawing tube attachment on a Dialux microscope (Leitz) and a Kontron MOP-Videoplan to measure the area of epithelium examined – a total area of approximately 4.5 mm^2 of xenografted human epithelium was assessed for L.I. in the pregnant and non-pregnant mice.

Results

When breast organoids consisting of portions of ducts and lobulo-alveolar units digested free of both interlobular and intralobular stroma (Fig. 1) were injected into the mammary fat pad they formed small nodules. When examined histologically at 4 weeks the nodules were seen to be composed of ducts and acini in a connective tissue stroma. A similar appearance was seen at eight (Fig. 2) and twelve weeks after implantation. These ductal structures formed an inner epithelial layer which stained strongly with antibodies to keratin (Fig. 3) and an outer myoepithelial layer which stained for myosin (Fig. 4). This staining distribution is similar to that previously described in the normal resting breast with the same antisera (Gusterson et al. 1982). The monoclonal antibodies to the milk fat globule membrane

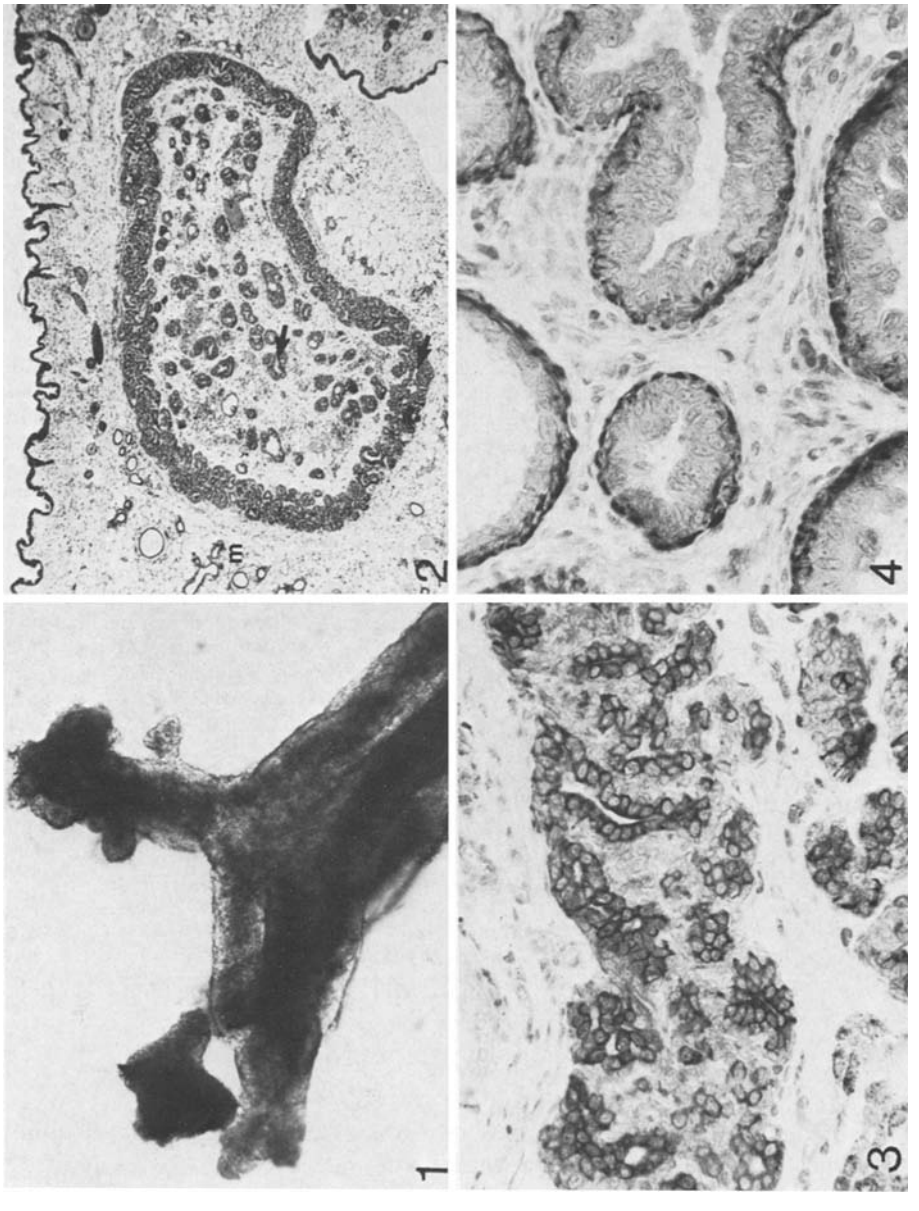

Fig. 1. Phase contrast photomicrograph of a human 'organoid' composed of a duct and lobuloalveolar units. Magnification ×96

Fig. 2. Human tissue at 8 weeks in the subcutaneous fat and mouse mammary fat pad. Lumina are present (*arrow*) and there is resting mouse mammary tissue (*m*). Magnification ×16

Fig. 3. Breast organoids at 8 weeks stained for keratin. Cells adjacent to the lumina are strongly stained and surrounding cells are weakly stained. Magnification ×340

Fig. 4. Breast organoids at 8 weeks stained with anti-myosin antibody. Cells at the periphery of the ducts are strongly stained (myoepithelial cells). Magnification ×340

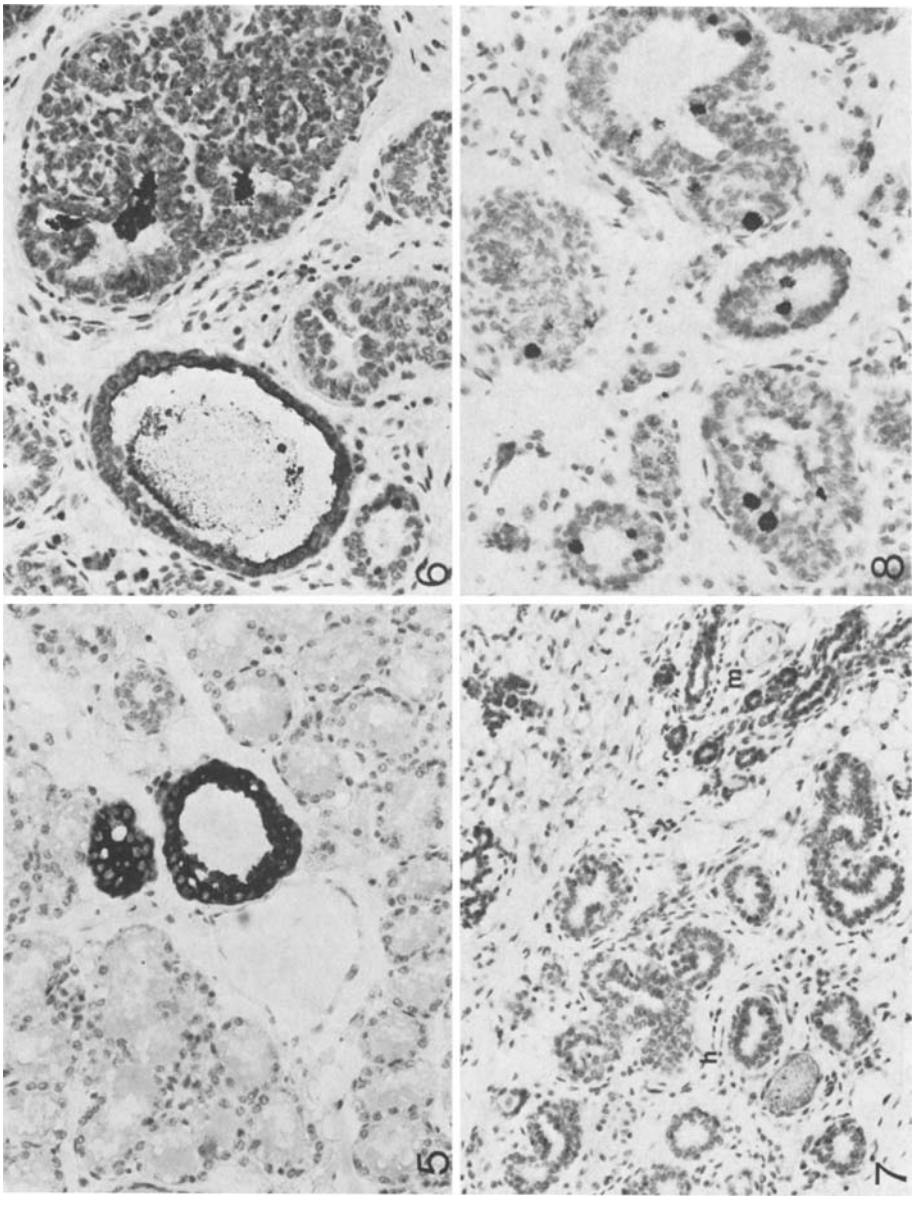

Fig. 5. Human breast organoid stained with M8 in a lactating mouse breast. Note the vacuolation of the cytoplasm of the luminal epithelial cells in the organoid. Magnification $\times 250$

Fig. 6. Human breast organoid stained for α-lactalbumin. Note the staining of the luminal membrane of the dilated duct and strong staining of the luminal contents. Magnification $\times 250$

Fig. 7. Non-lactating organoids at 8 weeks stained for α-lactalbumin. This section demonstrates the difference in morphology between the human breast tissue (*h*) and the mouse tissue (*m*). There are no stained secretions in the lumina or vacuolation of the cytoplasm. Magnification $\times 160$

Fig. 8. Autoradiography of human organoids in a pregnant mouse at term demonstrating an area with particularly high labelling. Magnification $\times 275$

M8 and M18 also gave a similar distribution of staining to that previously reported (Foster et al. 1982) in the intact human breast. Thus there was strong luminal membrane staining with both antibodies. M18 stained heterogeneously with some acini positive and others completely negative. M8 is human specific and was used for the identification of the human breast tissue. This was especially useful in the case of the pregnant animals (Fig. 5) where the proliferation of the endogenous rodent tissue in the vicinity of the xenograft made clear-cut identification of the grafted tissue difficult using solely histological criteria.

The xenografted human tissue in all pregnant mice stained positively for α-lactalbumin (Fig. 6), in contrast to the human tissue in non-pregnant mice which did not stain (Fig. 7). As can be seen in Fig. 7 there are obvious morphological differences between the human and the rodent cells. The human cells are larger with more abundant cytoplasm in the resting state. Also the rodent cells are relatively more haematoxyphilic. There was also some ductal dilatation and staining of the luminal epithelial cells in the pregnant animals (Fig. 6), but the strongest staining was in the luminal contents and the luminal membranes. Although occasional cells showed vacuolation of the cytoplasm the morphological changes in the luminal cells of the human tissue were not as markedly secretory as those seen in the surrounding mouse tissue (Fig. 5).

There was also a highly significant increase in the labelling index (L.I.) of human epithelium in pregnant versus non-pregnant mice (Fig. 8). The relevant labelling indices were $27.56 \pm 8.15/0.1$ mm^2 (SD) in the lactating animals compared with $7.25 \pm 6.68/0.1$ mm^2 (SD) in the control group ($P < 0.001$). The labelling activity in the human tissue in both the 'resting' and the pregnant mice did, however, tend to be focal in nature and this is reflected in the variance noted. There was no obvious increase in the volume of the human epithelial tissues over the three week period, but this was difficult to assess owing to the problem of localising the graft in the extensively enlarged mouse fat pad.

These results demonstrated that both ^{3}H thymidine labelling and function were significantly enhanced during the 3 weeks of pregnancy in the mouse under quasi-physiological conditions.

Discussion

The present study indicates that the structural epithelial components of human breast tissue i.e. (the luminal epithelial cells and the myoepithelial cells) are retained when implanted as xenografts in nude mice and that they respond to the 'normal' stimuli of pregnancy in the host with both a proliferative and lactogenic response. It is of interest that the time scale of this response (i.e. 3 weeks) is compatable with the typical monolayer tissue culture experiment in which the human breast cells are essentially refractory to added mammotrophic/lactogenic hormones (Gaffney et al. 1976; Hallowes et al. 1980; Kirkland et al. 1979), thus demonstrating that a failure to respond under the latter conditions is not simply a consequence

of an inadequate length of exposure in relation to the duration of normal human pregnancy. The nude mouse model is, therefore, a useful test system to assist in defining what improvements in in vitro culture systems are necessary in order to elicit a full spectrum of phenotypic differentiation and hormonal responsiveness.

Specialised human epithelia such as bronchus (Barrett et al. 1976), endometrium (Sananes et al. 1978) and breast (Easty et al. 1980; Foster et al. 1983; Hillman et al. 1983) tend to lose many of their definitive characteristics after a short time in culture and often exhibit evidence of squamous metaplasia with increase in tonofilaments and reversion to a common morphological phenotype. It has also been demonstrated that stratified squamous epithelia such as cornea, oesophagus, conjunctiva and skin which exhibit individual distinctive features in vivo convert to a similar biochemical phenotype with expression of a common keratin profile in culture (Doran et al. 1980; Sun T-T and Green 1977). When, however, the same cells are xenografted they re-express the keratins of the parent tissue (Doran et al. 1980; Sun T-T and Green 1977). These observations indicate that the typical culture environment in vitro does not provide the necessary extrinsic factors for the expression of the normal differentiation of these cells. It has, therefore been concluded that while both intrinsic and extrinsic factors control overt differentiation; extrinsic factors in the form of the local environment play a major role. In the presence of a 'pseudo-stromal' environments such as a repolymerized collagen gel (Foster et al. 1983) or the insoluble tissue biomatrices (Wicha et al. 1982) it appears that both squamous and breast epithelial cells can express a more differentiated phenotype than is observed in the typical monolayer or explant culture systems. Architecture, however, is retained to a limited degree by the use of organ cultures, exemplifying the effects of a correct stromal support (Hillman et al. 1983). These data would support the view that if we understood more about these extrinsic factors we could develop improved in vitro systems to look at isolated cell populations.

The experiments described here provide further evidence that the nude mouse is, in several respects, an ideal culture system for specialised human epithelial tissues and is, furthermore, a useful system for examining the response of the human breast to 'physiological' stimuli. Responses to a variety of hormones including oestrogens, progestogens, insulin and growth hormone have been observed in this system (McManus and Welsch 1981), but the implantation of exogenous oestrogens (or progestogens) and the grafting of hormone secreting tumours (McManus and Welsch 1981) cannot be expected to accurately simulate the complex hormone changes occurring during pregnancy. Using the pregnant xenograft model in conjunction with other tissue culture methods, it should be possible to dissect the intrinsic factors controlling phenotypic expression from those modulated by the environment. By the use of appropriate agents to block or enhance the secretion of steroid and/or pituitary and placental polypeptide hormones it may be possible to define more precisely the role of individual hormones in the vaious stages of growth and differentiation of human breast cells during

pregnancy. Current investigations are also underway to asses the relevance of the rodent breast stroma for this response by injection of organoids into other sites.

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