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Participation of two different mesenchymes in the developing mouse mammary gland: synthesis of basement membrane components by fat pad precursor cells

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SUMMARY

Two different types of mesenchyme, fat pad precursor cells (FP) and fibroblastic cells (MM) are involved in the morphogenesis of mammary gland epithelium of mouse embryo. Especially, an interaction between FP and the epithelium is necessary for its characteristic shaping of ductal branching structure. To assess the relative participations of the mesenchymes, we have analysed the extracellular matrix products by immunofluorescent staining method using antibodies to laminin, proteoheparan sulphate, and fibronectin. The staining patterns suggested that, after the 16th day of gestation when fatty substances first appeared in FP and the epithelial rudiments started to elongate and branch rapidly, FP initiated synthesis of laminin and proteoheparan sulphate, while MM synthesized fibronectin at all times. Attention was also paid to differences in the epithelial basement membranes (BM) concomitant with ones in the mesenchyme. BM were always stained with antibodies to laminin and proteoheparan sulphate. However, topographical differences in thickness were observed: the one facing FP, often seen at the tip region of the end bud, was thin, while the other surrounded by MM, often at the flank region of the duct, was thick. Specific elaboration of BM-like extracellular matrix products by FP may attribute to observed differences in BM thickness which are related to the characteristic shaping of the mammary gland.

INTRODUCTION

Mammary gland develops by repetitive budding and elongation of the glandular epithelium. This process is known to require a specific interaction between mammary epithelium and mesenchyme (Kratochwil, 1969). When 16-day foetal mammary epithelium is recombined with foetal mammary mesenchyme or 14-day salivary mesenchyme, its subsequent morphogenesis differs according to the source of the mesenchyme (Sakakura, Nishizuka & Dawe, 1976). The studies

Key words: mouse mammary gland, basement membranes, fat pad precursor cells, laminin and proteoheparan sulphate.

show that a specific interaction with mesenchyme is requisite for the morphogenesis of epithelial duct and alveoli systems which are characteristic of mammary gland. Recently, we have provided evidence showing that two distinct types of mammary mesenchyme actually take part in the morphogenesis of mammary gland (Sakakura, Sakagami & Nishizuka, 1982). One component is made up of fibroblastic cells surrounding the epithelial rudiment. The other component consists of the fat pad precursor cells which appear separately, posterior to the mammary rudiment, by the 14th day of gestation. Subrenal transplantation of the epithelium combined with either component suggests that the interaction with the fatty stroma is inevitable for the characteristic shaping of mammary gland.

The basement membrane is a complex morphological entity, consisting of extracellular matrix deposits associated with the basal surface of epithelial cells (Kefalides, 1978). The structure results from supramolecular interactions between macromolecules derived from epithelium and from the adjacent mesenchyme. Several components of basement membranes, such as laminin, type IV collagen, proteoheparan sulphate, and fibronectin, have been identified (Timpl & Martin, 1982). The mammary epithelial cells synthesize laminin, proteoheparan sulphate, type IV collagen, and hyaluronic acid (Gordon & Bernfield, 1980; Liotta *et al.* 1979; Ormerod, Warburton, Hughes & Rudland, 1983; Rapraeger & Bernfield, 1983; Silberstein & Daniel, 1982). Sugrue & Hay (1981) have found that the actin filaments in isolated epithelia reform the basal cortical mat typical of epithelia *in situ* in response to exogenous laminin, collagen, and fibronectin, and suggested that basement membranes influence and organize not only the basal cell surface but also the actin-rich basal cell cortex of epithelial cells. It is likely, therefore, that the molecular composition and structural features of mammary gland basement membrane provide the specific conditions necessary for mammary epithelium to undergo its characteristic monopodial branching during morphogenesis.

In this report, by immunofluorescent staining using antisera to laminin, proteoheparan sulphate, and fibronectin, we examined the precise distribution of these molecules during development of mouse mammary gland. We found that, accompanied by initiation of ductal branching of the epithelium on the 16th day of gestation, the fat pad precursor cells themselves appeared to start synthesizing basement membrane components, laminin and proteoheparan sulphate, while fibroblastic mesenchymal cells continued to synthesize fibronectin. We also noticed apparent thinning of the epithelial basement membranes facing the fat pad precursor cells, although the basement membranes were always stained with antisera to laminin and proteoheparan sulphate. Different extracellular matrix products of the fat pad precursor cells might modify the basement membrane assembly which is related to the branching pattern of the mammary gland.

MATERIALS AND METHODS

Preparation of specimens

BALB/C mice were purchased from the Japan Charles River (Atsugi). Embryos of specific ages were obtained by checking females for vaginal plugs at 9:00 a.m. The morning of detection

of a vaginal plug was designated as day 0. Embryos were removed aseptically. Adult specimens were taken from 5-week-old female mice. Mammary gland rudiments, including mammary stroma and fat pad tissues, were dissected, fixed in cold 95% ethanol, and embedded in polyester wax after dehydration in cold absolute ethanol (Kusakabe *et al.* 1984). Thin sections (5 μm) were prepared at 4 °C.

Antibodies and antigens

Laminin was purified from mouse basement membrane tumour (EHS tumour) by the method of Foidart, Timpl, Furthmayr & Martin (1982). Plasma fibronectin was purified from freshly drawn rat plasma by a series of affinity chromatography on a gelatin column and on a heparin column (Yamada, 1982). Proteoheparan sulphate from EHS tumour was purified by the method of Hassell *et al.* (1980) with a slight modification. Proteoheparan sulphate from EHS tumour has been shown to be separated into two forms of high and low densities (Fugiwara *et al.* 1984). The preparation used for the present study appeared to correspond to the low-density one and the relative molecular mass was over 400 000, judging from the kD value of the Sepharose CL-4B column chromatography in 4 M-guanidine and 1% Triton X-100. The laminin and plasma fibronectin were injected into rabbits to induce antibodies as described previously (Kimata, Barrach, Brown & Pennypacker, 1981). Briefly, each antigen (200 μg in 0·1 ml of saline) mixed with an equal volume of Freund's complete adjuvant was injected into a lymph node in the popliteal region of one leg of each rabbit. The rabbits were boosted twice with 50 μg antigens in an equal volume of incomplete Freund's adjuvant after three weeks and after six weeks. Titres were tested by the enzyme-linked immunosorbent assay (ELISA) (Rennard *et al.* 1980). Each serum had a titre of more than 12 000 against the injected antigen. Specificity was examined by ELISA using heterologous antigen, including EHS tumour proteoheparan sulphate, rat skin type I collagen, rat chondrosarcoma type II collagen, and the EHS tumour type IV collagen, and immunofluorescent labelling of target tissues. Antiserum to laminin showed little activity (titre, less than 50 by ELISA) against the proteoheparan sulphate, plasma fibronectin, and other components tested. Antiserum to fibronectin showed little activity against laminin, proteoheparan sulphate, and other components tested. Anti-EHS tumour proteoheparan sulphate antibodies which were raised in rabbits were the generous gift of Drs G. R. Martin, J. R. Hassell, H.-J. Barrach (National Institute of Dental Research, NIH). After purification by cross immunoadsorption and affinity chromatography, their specificity and lack of cross reactivity were confirmed by ourselves using ELISA and immunofluorescent staining of target tissues.

Immunofluorescent staining

Thin sections (usually 5 μm, but in the case of adult mammary gland, 10–15 μm thick) were freed from wax in ethanol at 4 °C and hydrated in Dulbecco's phosphate-buffered saline. A conventional method to prepare cryostat sections for immunofluorescent staining was not applicable to the mammary glands, since large lipid inclusion in fat pad cells made the structures very fragile. The hydrated sections were treated with diluted antiserum (1:50) and processed for immunofluorescent staining as described previously (Kimata *et al.* 1981 and 1982). Staining of specific components could be blocked by preincubation of the antisera with their respective antigens. As additional controls, some samples were treated with preimmune serum or antibodies to ovalbumin (Cappel Lab. Inc.). In these cases, non-specific staining was negligible, although brownish autofluorescence was sometimes observed on the epithelia. Some sections were treated with testicular hyaluronidase (0·5 mg ml^{-1}, Type V, Sigma) for 15 min before the processes for immunofluorescent staining.

RESULTS

Day-14 embryo

The mammary gland epithelial rudiment at this stage has the appearance of a knob inserted into the mesenchymal tissues. Two different regions become

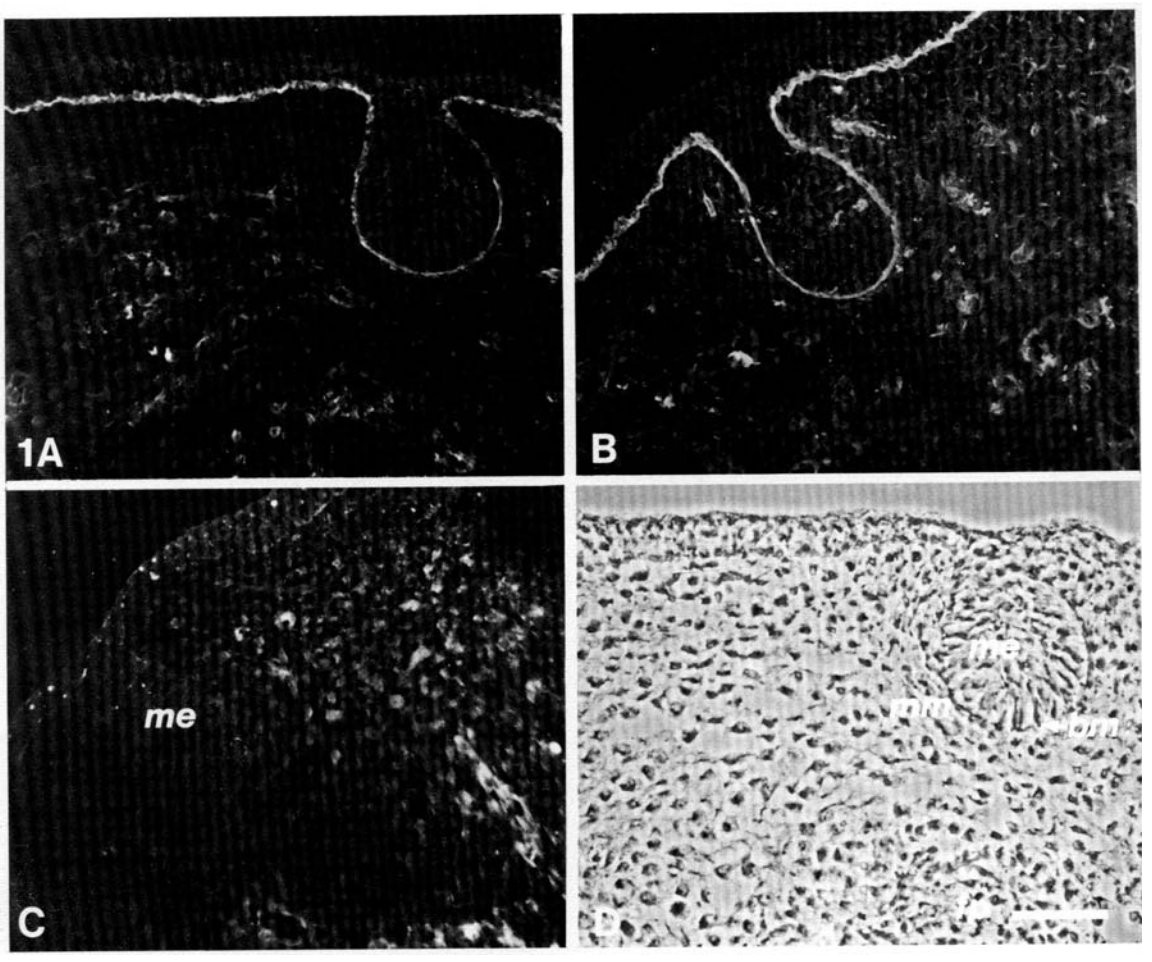


Fig. 1. Immunofluorescent localization of laminin (A), proteoheparan sulphate (B), and fibronectin (C) in 14-day embryo mammary gland and associated tissues. (D), a phase-contrast illumination of (A). Bar, 100 μm. Original magnification, ×200. Staining with antisera to laminin and proteoheparan sulphate delineates basement membranes (*bm*) between mammary epithelial rudiment (*me*) and surrounding mesenchymal tissues (*mm*) and in developing vascular systems. Fluorescence of fibronectin is seen on mesenchymal tissues surrounding the epithelial rudiment. Fat pad precursor cells (*fp*) beneath the mammary rudiment appear as condensed mesenchymal tissue and show little or no fluorescence of laminin and proteoheparan sulphate. The treatment of the sections with bovine testicular hyaluronidase gave no significant changes in the staining patterns.

apparent; a thin layer of fibroblastic cells concentrically surrounding the mammary epithelial rudiment, and fat pad precursor cells slightly distant from the mammary epithelial rudiment (Fig. 1D). The basement membranes around the epithelial knob were visualized by immunofluorescent staining with antisera to laminin (Fig. 1A) and proteoheparan sulphate (Fig. 1B). The vascular system and the basement

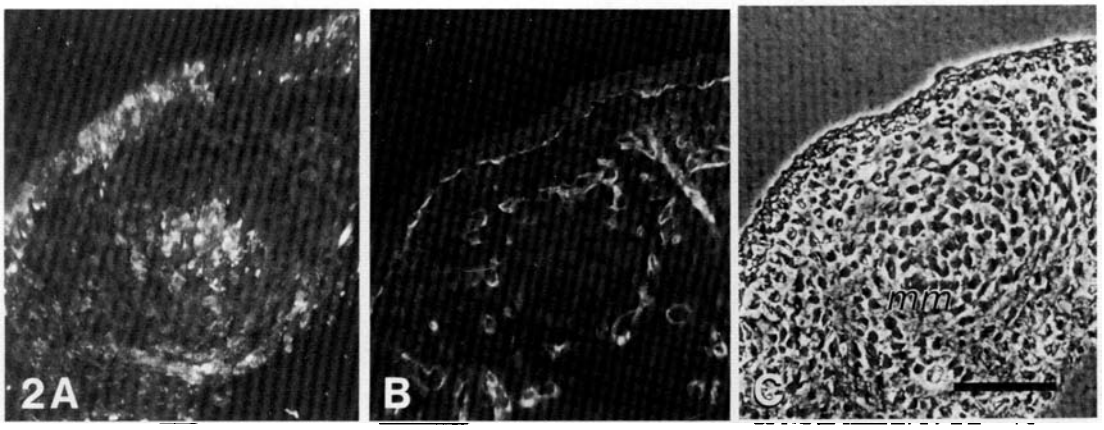


Fig. 2. Immunofluorescent localization of fibronectin (A) and proteoheparan sulphate (B) in 14-day embryo mammary gland mesenchymal cells. (C), a phase-contrast illumination of (B). Bar, 100 μm. Original magnification, ×200. Fibronectin is packed concentrically in the peripheral region of the epithelial knob, while proteoheparan sulphate is not seen in this region.

membranes underlying the epidermis were also stained (Figs 1B, 2B). In contrast to this, fluorescence of fibronectin was distributed throughout the mesenchymal tissues (Figs 1C, 2A). The concentric packing of the mesenchymal cells in the peripheral region of the knob showed strong reaction with antiserum to fibronectin but almost no reaction with antiserum to proteoheparan sulphate (Fig. 2A,B). The fat pad precursor cells were also stained with antiserum to fibronectin but not stained with antisera to laminin and proteoheparan sulphate at this stage.

Day-17 to -18 embryo

On day 17, the epithelial cells start to show a ductal branching growth pattern. The fat pad becomes distinctive, forming a lobular structure composed of rather densely packed cells separated by loose connective tissue. The basement membranes were clearly visualized by staining with antisera to laminin (Fig. 3A,B) and proteoheparan sulphate (Fig. 3C,D). In most cases, however, the basement membranes facing the fat pad precursor cells showed thinner bands of fluorescence (Fig. 4A,B). Weak but significant staining with antisera to laminin and proteoheparan sulphate was first observed on the cells of the day-17 fat pad precursor tissues (Fig. 3A,C). On day 18, clustering fat pad precursor cells gave a more characteristic fluorescent-staining pattern (Fig. 3B,D). Both laminin and proteoheparan-sulphate-positive materials coated the fat pad cells and appeared to connect the cells to each other. The vascular systems were also stained with these antisera, but their morphology of a few cells encircling a cavity made it easy to distinguish them from the fat pad. On the other hand, thin filamentous structures, stained with antiserum to fibronectin, were observed on the

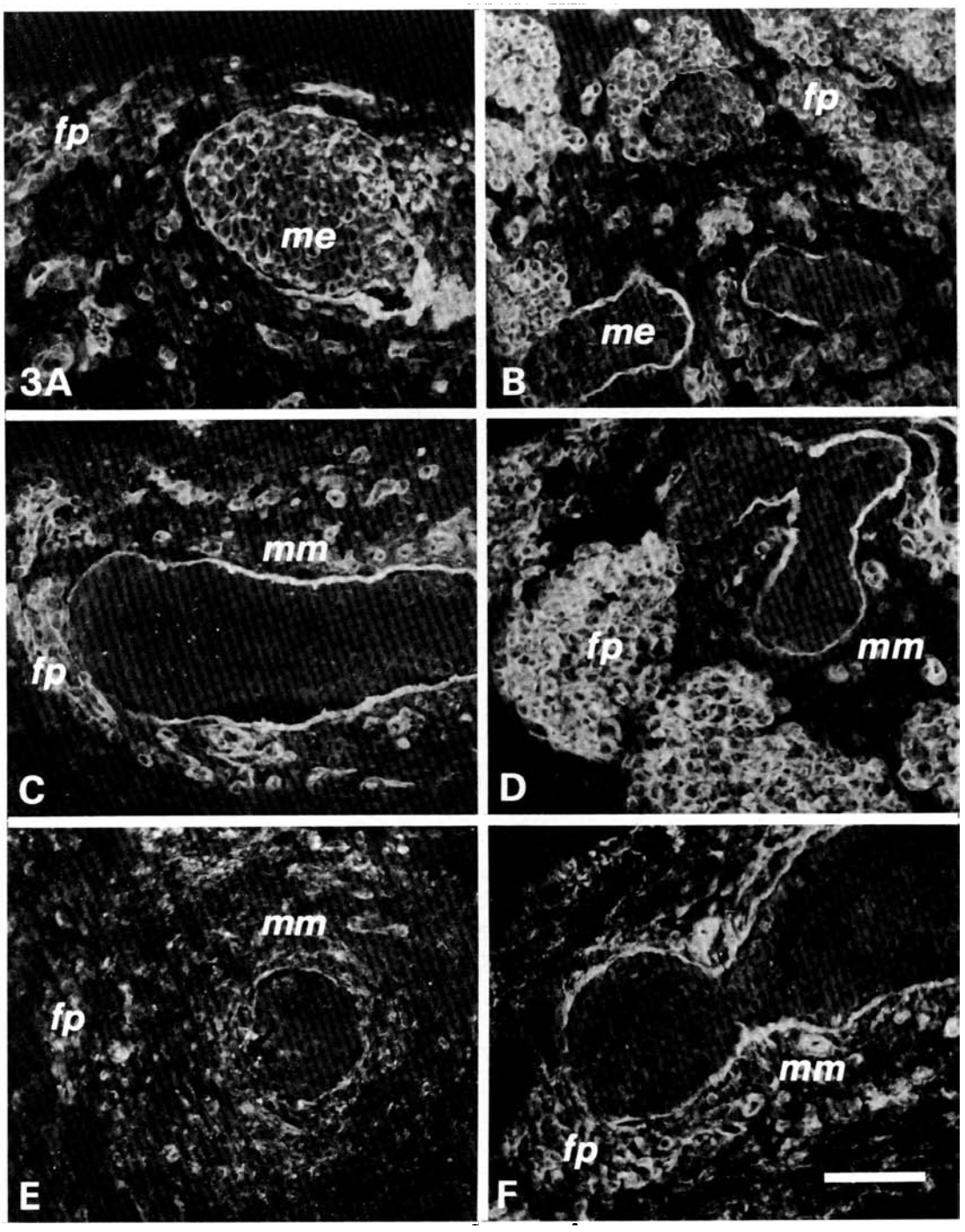


Fig. 3. For legend see p. 250

mesenchymal fibroblasts surrounding the mammary epithelial rudiments, but the fat pad precursor tissues were often stained less with antiserum to fibronectin (Fig. 3E,F).

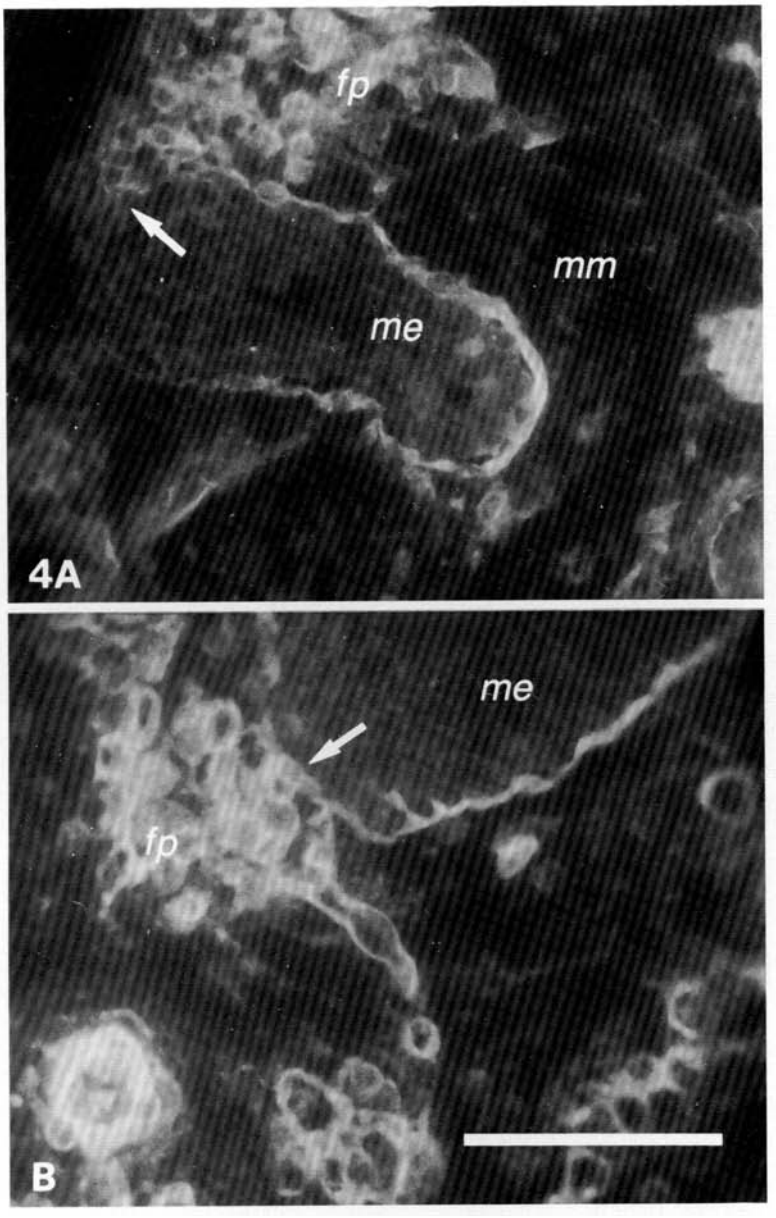


Fig. 4. For legend see p. 250

Neonatal embryo

The fat pad precursor cells accumulate large amounts of fatty substances and become typical adipose tissue. The gland epithelium appears to penetrate the fat pad tissue with ductal elongation and branching. The antisera to laminin and proteoheparan sulphate stained the basement membranes of the glandular epithelial rudiments intensely. The basement membranes at the sites where epithelial cells faced the fat pad precursor cells were often thin (Fig. 5A,B). Staining of the fat pad tissues with antisera to laminin and proteoheparan sulphate clearly outlined each cell in the fat pad tissues, but the lipid inclusions remained unstained. Therefore, each fat pad tissue looked like a honeycomb under the fluorescent microscope (Fig. 5A,B). Fibroblastic mesenchymal cells and associated extracellular matrices were preferentially stained with antiserum to fibronectin with a fibrillar pattern (Fig. 5C).

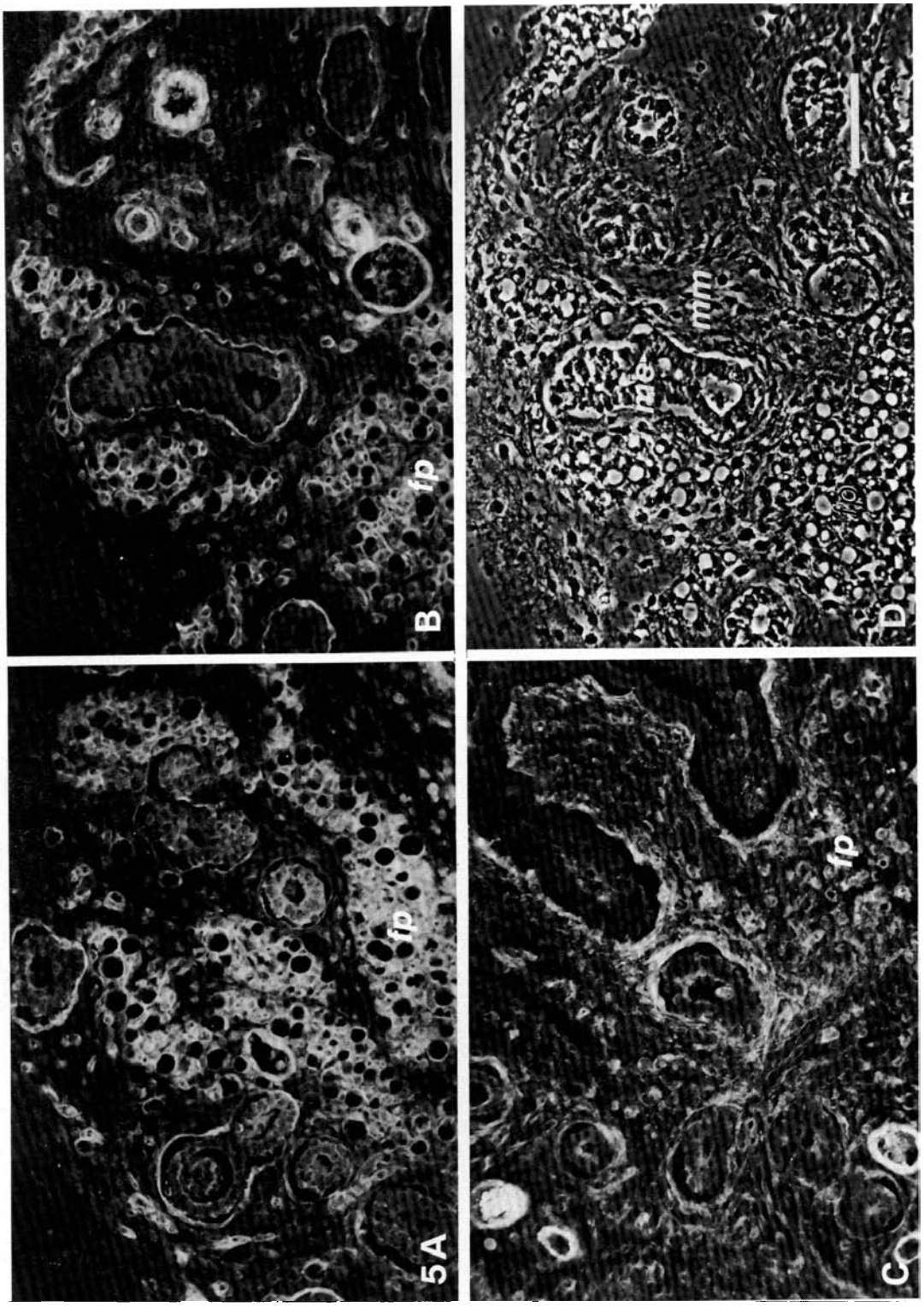
Virgin mouse (five-week-old)

The epithelial structures have a tree-like pattern of ducts surrounded by the fatty stroma. The fat cells characterized by large lipid inclusions were separated from each other by septal structures which were stained intensely with antisera to laminin, proteoheparan sulphate, and fibronectin (Fig. 6). When stained with antisera to laminin and proteoheparan sulphate, the basement membranes associated with the alveolar and ductal forms of the epithelial glands were clearly visible, but the ones at the tip regions usually showed thinner layers (Fig. 6A,B). Antiserum to fibronectin thickly stained fibroblastic cells and fibrillar matrix between the basement membranes and fatty stromal structures (Fig. 6C). The septal structures in the fatty stroma were always stained with antisera to laminin and proteoheparan sulphate, whatever fixation methods were applied.

Fig. 3. Immunofluorescent localization of laminin (A,B) proteoheparan sulphate (C, D), and fibronectin (E,F) in 17-day (left column) and 18-day (right column) embryo mammary glands and associated tissues. Bar, 100 μm. Original magnification, ×200. Fluorescent labelling of laminin and proteoheparan sulphate reveals that the fat pad precursor cells (*fp*) are stained while mesenchymal fibroblastic cells are not. Vascular systems are also visualized as single small circles scattered throughout mesenchymal tissues. (E,F) shows thin filaments of fibronectin localized on the mesenchymal tissues surrounding the epithelial rudiments.

Fig. 4. Immunofluorescent localization of laminin (A) and proteoheparan sulphate (B) on the basement membranes on 18-day embryo mammary gland and associated tissues. Bar, 50 μm. Original magnification, ×400. The tip regions of the buds are often found to closely associate with fat pad precursor cells. Comparison of fluorescence distribution in the basement membranes indicates weaker staining in the ones facing on the fat pad precursor tissues (*fe*) than to the mesenchymal tissues (*mm*).

Fig. 5. Immunofluorescent localization of laminin (A), proteoheparan sulphate (B), and fibronectin (C) in new born mouse mammary gland and associated tissues. (D), a phase-contrast illumination of (B). Bar, 100 μm. Original magnification, ×200. Fluorescence of laminin and proteoheparan sulphate reveals a honeycomb-like structure of the fat pad tissue (*fp*) which have started to accumulate large amounts of fatty substances. Fluorescence of fibronectin scatters throughout mesenchymal tissues.


B
fp
D
mm
5A
fp
fp
C

Evaluation of the immunofluorescent staining

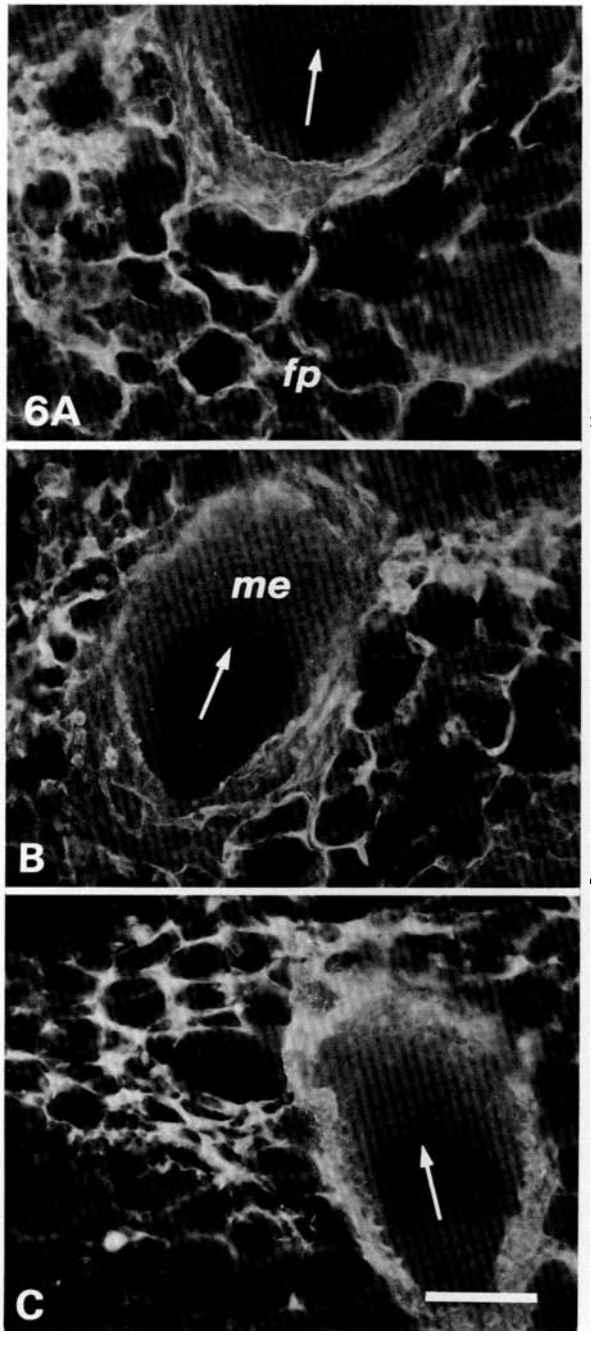
No significant fluorescence was observed on the sections when stained with preimmune serum (Fig. 7) or antibodies to ovalbumin (data not shown). In addition, some sections were treated with testicular hyaluronidase to expose antigens possibly masked with proteoglycans and hyaluronic acid. No effect on the staining patterns was observed (data not shown), suggesting that the antigens themselves showed changes during development.

DISCUSSION

The present study provides evidence that one of two types of mammary mesenchymal tissue, the fat pad precursor cells start to synthesize basement membrane components, laminin and proteoheparan sulphate on day 16 when the mammary epithelium undergoes its characteristic morphogenesis, while the other, fibroblastic mesenchymal cells synthesize fibronectin from the time the mammary gland first appears as a small spherical knob of epithelium. Adipocytes in the adult mouse mammary gland also appear to synthesize basement membrane components. Metabolic labelling of the isolated tissues with [^{35}S]methionine and subsequent immunoprecipitation of the labelled tissue extracts have given direct evidence for the synthesis of laminin by the fat pad precursor cells and adipocytes (Kimata *et al.*, manuscript in preparation). As early as 1960, Wasserman & MacDonald (1960) noted that the fat cell was enveloped by a structure similar to, and probably identical with, the basement membrane. Other investigators have also observed the existence of the external lamina beyond the plasma membrane of adipocytes (Cushman, 1970; Napolitano, 1963; Slavin, 1972; Suter, 1969). However, the molecular characterization had not been done. Our present results have, for the first time, given molecular bases for their morphological observations.

Laminin is usually synthesized by epithelial cells (Timpl & Martin, 1982; Foidart *et al.* 1982). However, several exceptions have been found recently. Kühl, Timpl & von der Mark (1982) have shown that not only muscle fibroblasts and myoblasts from embryonic mouse and quail, but also L6 myoblasts, a cell line derived from the rat skeletal muscle, produced laminin and type IV collagen. In this case, individual myofibres in skeletal muscle were also known to be ensheathed by a basement membrane. Furthermore, cultures of Schwann cells (Cornbrooks *et al.* 1983) and astrocytes (Liesi, Dagl & Vaheri, 1983), mouse embryos at preimplantation stage (Cooper & MacQueen, 1983; Leivo, Vaheri, Timpl & Wartiovaara, 1980; Wu, Wan, Chung & Damjanov, 1983), and dermal

Fig. 6. Immunofluorescent localization of laminin (A), proteoheparan sulphate (B), and fibronectin (C) in 5-week-old virgin mouse mammary gland and associated tissues. Bar, 100 μm. Original magnification, ×200. Arrows indicate the direction of the tip region of the end bud. The mammary epithelium penetrates the fat pad tissues in which the septal structures show intense fluorescence of laminin, proteoheparan sulphate, and fibronectin. Fibroblastic cells surrounding the flank region of the end bud are stained thickly with anti-fibronectin antiserum.


6A
fp
B
me
C

components of the rat skin immediately before and after birth (Hogan, Taylor, Kurkinen & Couchman, 1982) produce laminin.

Interaction with the fat pad precursor tissue is necessary to support the normal development and shaping of characteristic structures of the mammary gland, i.e. the combination of 17-day mammary epithelium with 14- or 17-day fat pad precursor tissue produced the typical mammary gland structures (ductal branching pattern), but, when combined with 12- to 17-day fibroblastic mesenchyme, an abnormal structure with ductal hyperplasia was formed by frequent branching and without the 'stretching out' of these ducts (Sakakura *et al.* 1982). The fatty tissue

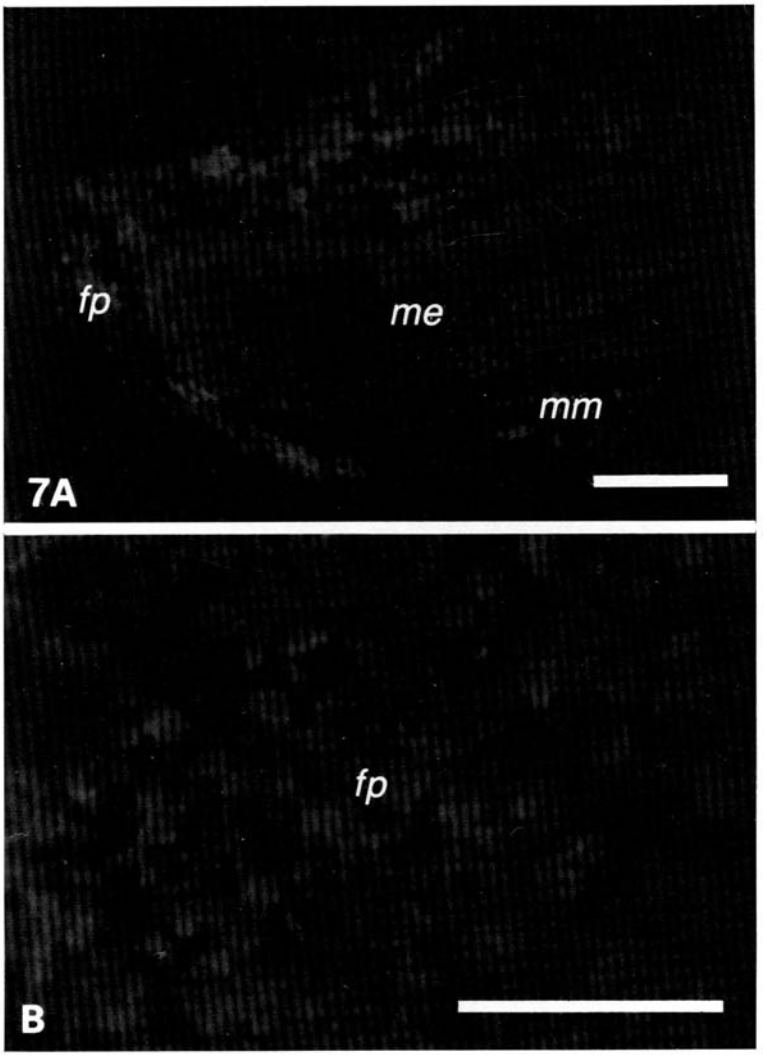


Fig. 7. Controls for immunofluorescent staining. 17-day embryo mammary gland and associated tissues (A) and 18-day embryo mammary fat pad precursor cells (B) were stained with pre-immune serum. Bars, 100 μm.

also acts inductively in the adult stage, associating with the mammary gland to maintain its characteristic morphology. For example, Hoshino (1978) observed that various sites of fatty tissue supported regeneration and milk secretion of transplanted small duct segments taken from adult and neonatal mammary glands. Precise histological studies on elongating mammary ducts in the immature mouse by Williams & Daniel (1983) have shown that cap cells, epithelial cells at the anterior region of the end bud penetrating the adipose tissues abut closely against adipocytes. These results are all suggestive of the participation of the fatty tissue in mammary gland morphogenesis.

At present we do not know how the fat pad functions or how its functions are related to our finding that the fat pad precursor tissue and its prospective mammary fat pad have an extracellular matrix consisting of basement membrane components. However, we have noted that the basement membranes are different in thickness topographically, the one adjacent to the fat pad precursor cells being thinner and often corresponding to the growing tip region of the end bud. There are several reports describing the heterogeneity in component and structure of the basement membrane underlying the mammary epithelium (Bernfield, 1981; Silberstein & Daniel, 1982; Williams & Daniel, 1983). Since the epithelial and mesenchymal interactions have to occur through the basement membrane, such differences in thickness of the basement membrane may be of significance when we consider the role of fatty tissues in the ductal morphogenesis of mammary gland.

The question then arises as to how the fat pad tissues are involved in producing such differences in the basement membrane. Basement membrane components have been found to form a supramolecular complex in a highly specific manner, quantitatively as well as qualitatively (Kleinman, McGarvey, Hassell & Martin, 1983). In relation to this observation, the findings by Grover, Andrews & Adamson (1982) are noted who investigated the effect of exogenous laminin on the retinoic acid-induced formation and maturation of the epithelial layer by F9 embryonal carcinoma cells. Exogenous laminin prevented the accumulation of a basement membrane and resulted in the disruption of the organization of the epithelium. Therefore, one role of fatty tissues may be a factor to modify the organization of the epithelial basement membrane and, in turn, influence the growth, locomotion, and cytodifferentiation of the epithelial cells.

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