Epithelial–mesenchymal interactions in the production of basement membrane components in the gut

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Summary

The production and deposition of extracellular matrix proteins and the cellular origin of type-IV collagen have been analysed immunocytochemically in cocultured or transplanted intestinal epithelial–mesenchymal cell associations.

In the first experimental model, rat intestinal endodermal cells were cultured on top of confluent monolayers of rat intestinal or skin fibroblastic cells. Under these conditions, interstitial matrix and basement membrane proteins were deposited within the fibroblastic layer over the whole culture period; interactions between the epithelial cells and the fibroblastic cell population, whatever their organ of origin, were required for the production of the basement membrane. In addition, its formation was progressive as assessed by the shift of a spot-like labelling to a continuous linear pattern at the epithelial–mesenchymal interface, and paralleled epithelial cell differentiation.

In the second experimental model, chick–rat epithelial–mesenchymal recombinants developed as intracoelomic grafts were used, and the immunocytochemical detection of a basement membrane protein, type-IV collagen, was performed with species-specific antibodies. The major role of the mesenchyme in the deposition of type-IV collagen is supported by the fact that anti-chick but not anti-mammalian antibodies stained this antigen in chick mesenchyme–rat endoderm recombinants.

These observations emphasize the role of tissue interactions in the formation of a basement membrane and show that the mesenchymal compartment is the principal endogenous source of type-IV collagen.

Key words: intestine, ECM production, rat embryo, chick embryo, epithelial–mesenchymal interactions, basement membrane.

Introduction

Differentiation of the intestinal epithelium during development is closely associated with ordered sequence of morphogenetic events (for a review, see Kedinger et al. 1986a). Several experimental studies using *in vivo* transplantation of various tissue recombinants have emphasized that these developmental processes are dependent upon reciprocal epithelial–mesenchymal interactions, in which mesenchyme plays a major role in epithelial differentiation (for reviews, see Kedinger et al. 1986a; Haffen et al. 1987).

Among the factors that could be involved in such tissue interactions during morphogenesis and cytodif-ferentiation, extracellular matrix (ECM) components have been proposed (for reviews, see Hay, 1981; Bissell et al. 1982; Slavkin, 1982; Timpl & Dziadek, 1986). Yet the cellular origin of these components is still controversial. Although the basement membrane is generally considered to be an epithelial product, there are known examples of mesenchymal production of basement membrane components (Kühl et al. 1984; Kimata et al. 1985; for a review, see Timpl & Dziadek, 1986).

In the intestine, some observations suggested a possible role of the ECM molecules in morphogenesis and in the maintenance of epithelial cell polarity. We could show that basement membrane components

(laminin, nidogen, type-IV collagen) are present at the epithelial–mesenchymal interface early in embryonic development and that changes in the spatial distribution of some ECM proteins are associated with morphogenetic processes (Simon-Assmann *et al.* 1986). On the other hand, severe perturbations of proteoglycans, collagens and some glycoproteins have been reported in human colonic tumours in parallel to tissue disorganization including loss of cell polarity and invasion processes (Burtin *et al.* 1982; Iozzo & Wight, 1982; Iozzo *et al.* 1982).

In the present study, we describe attempts to answer two major questions related to ECM production. The first question concerns the relationship between the establishment of the basement membrane and epithelial cell differentiation. For this purpose, we examined immunocytochemically the deposition of ECM components in epithelial–fibroblastic cocultures. Using this model previously, we showed that the fibroblastic population was necessary to support survival, proliferation and morphological as well as functional differentiation of the epithelial cells (Kedinger *et al.* 1987). The second aim of our work is to define the cellular origin of the ECM components by means of interspecies recombinants and species-specific antibodies.

Materials and methods

Animals

Wistar rat fetuses were used at 14, 15 and 20 days of gestation and White Leghorn chick embryos at 3 and 5 days of incubation. The developmental stages were counted as days of gestation for the former (the existence of a vaginal plug being taken as evidence of mating and designated as day 0) and as days of incubation at 38°C for the latter.

Cocultures

Cocultures of endodermal cells and fibroblastic cells were performed as previously described (Kedinger *et al.* 1987). Two types of fibroblastic cell populations were used: intestinal mesenchymal and skin fibroblastic cells. The former were derived from intestinal mesenchymal microexplants after separation of the mesenchyme from the endoderm by collagenase treatment (0·03 % collagenase type IV; 147 i.u. mg^{-1}, Worthington) of 14- and 15-day fetal rat intestines. Skin fibroblasts were derived from enzymatically (collagenase 0·01 % and trypsin 0·01 % solution) dissociated 20-day fetal rat dermis (Haffen *et al.* 1983). Both cell types were used at early passages (until passage 3) when they reached confluency, approximately 7 days after plating. Endoderms, separated from their mesenchymes by 0·03 % collagenase treatment of 14- and 15-day fetal intestinal anlagen, were cut into small fragments and seeded on top of confluent monolayers of both types of fibroblastic cells. Each cell population was also cultured in isolation. The culture medium used was composed of a mixture (1:1) of DMEM and Ham's F12 (Gibco Biocult,

Glasgow, Scotland) supplemented with 7·5 % fetal bovine serum (Gibco, containing 2·5 % SerXtend serum substitute, NEN) and with 10^{-7} M-dexamethasone (Sigma). Gentamycin (200 μg ml^{-1}) was added as an antibiotic.

Interspecies recombinants

Associations between rat and chick tissue components have been performed using an experimental procedure described previously (Kedinger *et al.* 1981). Briefly, after collagenase dissociation of 5-day chick embryonic and 14- to 15-day fetal rat intestinal anlagen, chick mesenchymes were recombined with rat endoderms (Cm/Re) and *vice versa* (Rm/Ce). Both types of recombinants were then grafted into the coelomic cavity of 3-day chick embryos. The grafts were recovered 11–15 days later.

Antibodies

Affinity-purified rabbit antibodies against mouse laminin (Rohde *et al.* 1979), the globular domain NC$_1$ of type-IV collagen (von der Mark *et al.* 1985), nidogen (Timpl *et al.* 1983), bovine type-III procollagen aminoterminal propeptide (Nowack *et al.* 1976), and human plasma fibronectin (Fleischmajer & Timpl, 1984) have been kindly provided by Drs R. Timpl and K. von der Mark (Max-Planck Institut für Biochemie, Martinsried, FRG). All antibodies exhibited sufficient cross-reactivity with rat tissues. Rabbit antibodies specific for chick type-IV collagen provided by Dr K. von der Mark were prepared against the pepsin fragment (F1)2F2 of type-IV collagen obtained from chick smooth muscle (Mayne & Zettergren, 1980; Mayne *et al.* 1982) and their species specificity has been shown in immunochemical and immunohistologic analyses (Sariola *et al.* 1984b).

Indirect immunofluorescence

Cell sheets of the cocultures and of isolated endodermal or fibroblastic monolayers were detached mechanically from the bottom of the dish under the dissecting microscope after different culture periods (between 2 days and 2 weeks). They were immediately embedded in Tissue-Tek compound and frozen in freon cooled in liquid nitrogen. Transverse sections (5–6 μm thick) were cut at −25°C and placed on gelatin-coated slides. Interspecies recombinants were processed similarly.

Cryostat sections were incubated for 30 min at room temperature with the antibodies diluted in phosphate-buffered saline (PBS) at concentrations of 20–70 μg ml^{-1} (Simon-Assmann *et al.* 1986). The sections were then washed with PBS and stained for 30 min with fluorescein-isothiocyanate-labelled goat anti-rabbit γ-globulin (Nordic) diluted (1:20) in PBS. The preparations were mounted under coverslips in glycerol–PBS–phenylenediamine and observed under an Orthoplan microscope (Leitz). Control sections were processed as above but affinity-purified antibodies were omitted or replaced by normal rabbit serum. None of these controls showed any specific fluorescence.
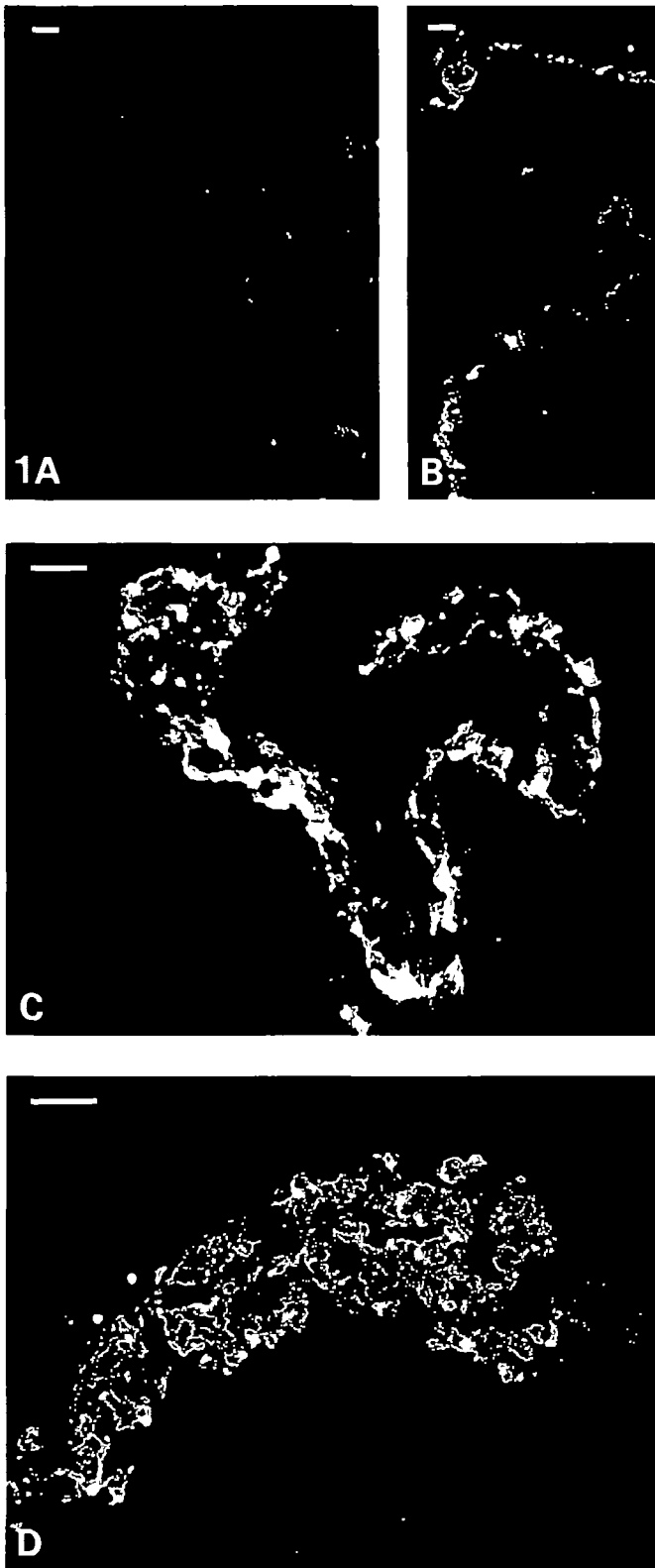
Fig. 1. Representative indirect immunofluorescence micrographs of ECM components in endodermal (A,B) and in fibroblastic (C, D) cell populations cultured in isolation. (A) Type-IV collagen; (B) laminin; (C) fibronectin; (D) type-IV collagen. Bar, 20 μm.
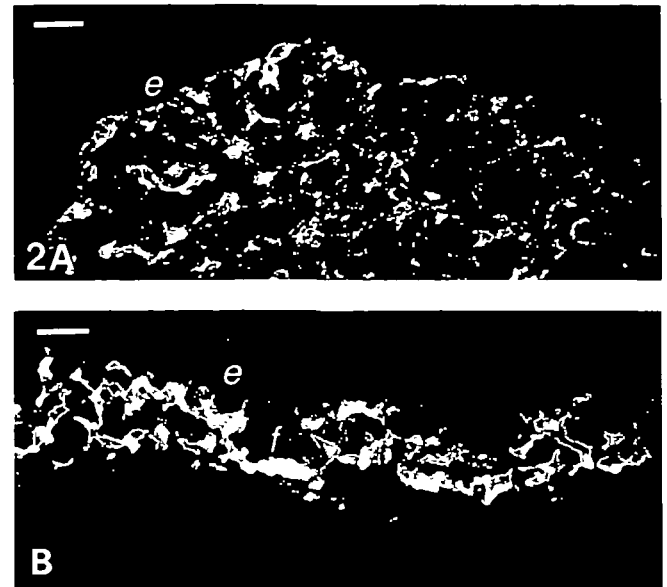

Fig. 2. Patterns of deposition of interstitial matrix proteins in cocultures of intestinal endodermal cells and skin fibroblasts. Deposition of fibronectin (A) at 8 days and of type-III procollagen (B) at 9 days within the fibroblastic layer. Epithelial (e) and fibroblastic (f) cell layers; Bar, 20 μm (A) or 40 μm (B).

Results

ECM molecule production by the endodermal and the fibroblastic cells cultured in isolation

When isolated intestinal endoderms were cultured directly on plastic culture dishes, they did not exhibit any labelling with the various antibodies (type-IV collagen, nidogen, fibronectin, type-III procollagen) either within the cell layer or at the basal surface of the cells (illustrated for type-IV collagen in Fig. 1A). One exception concerned the occurrence of some fluorescent spots scattered within the epithelial cell sheet with anti-laminin antibodies (Fig. 1B). By contrast, in intestinal and skin fibroblastic cell cultures, a peri- or inter-cellular deposition of interstitial molecules (fibronectin: Fig. 1C, and type-III procollagen), as well as of basement membrane components (type-IV collagen: Fig. 1D, laminin, nidogen), was obvious.

Temporal expression of ECM components in the cocultures

A general feature was that, at any time, none of the antigens were detected immunocytochemically within the epithelial cell layer either intracellularly or in the extracellular spaces (Figs 2, 3). By contrast, positive extracellular sheaths surrounding the cellular elements were detected within the intestinal and skin
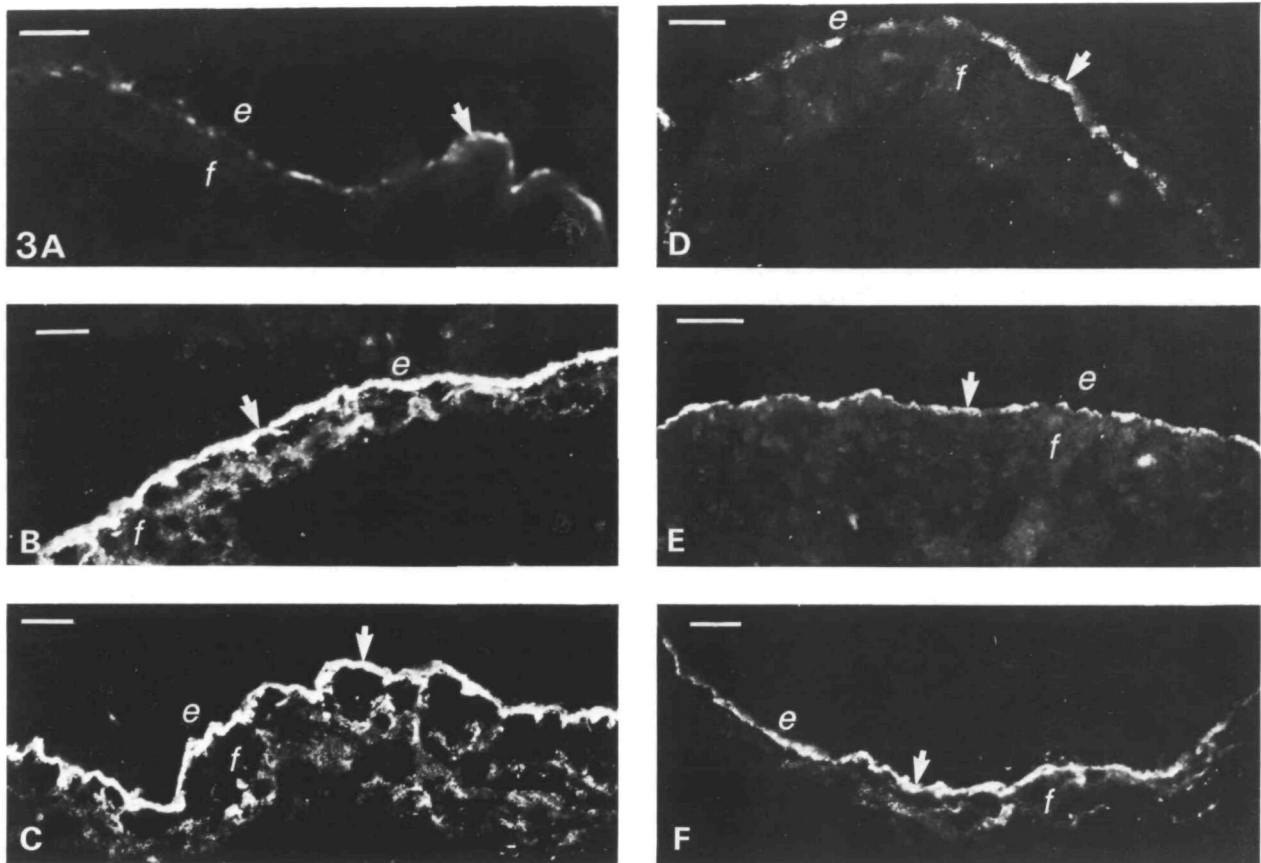
Fig. 3. Temporal expression of basement membrane molecules in intestinal endodermal and fibroblastic cell cocultures after 2 (A,D), 4 (B,E), 6 (F) and 8 (C) days, exemplified for laminin (A–C) and type-IV collagen (D–F). Epithelial (*e*) and fibroblastic (*f*) cell layers; epithelial–mesenchymal interface (arrow). Bar, $20\,\mu m$ (B,C,D,F); $40\,\mu m$ (E); $80\,\mu m$ (A).

fibroblastic cell layer with all antibodies tested, the staining of basement membrane components being less intense, however (Fig. 3 *versus* Fig. 2).

At the epithelial–mesenchymal interface, changes in the macromolecular composition of the basement membrane occurred as a function of the culture period. At the time of seeding, just after its separation from the mesenchyme by collagenase, the immunostaining of the endoderm revealed that type-IV collagen had disappeared (Fig. 4A) while nidogen and laminin persisted as a punctuate ring at the periphery of the endoderm (Fig. 4B). After 2 days of coculture, laminin (Fig. 3A), nidogen and type-IV collagen (Fig. 3D) were deposited as a discontinuous sheet at the epithelial–mesenchymal interface. This irregular pattern of labelling shifted towards a continuous bright staining visible as early as 4 days for laminin (Fig. 3B,C) or nidogen, but only at 6 days for type-IV collagen (Fig. 3F *versus* 3E). Fibronectin and type-III procollagen never delineated the basal surface of the epithelial cell layer at any culture time (Fig. 2).

The chronological appearance of the basement membrane components was similar whether endodermal cells were cultured with intestinal mesenchymal cells or with skin fibroblasts.

Immunofluorescence pattern of the ECM components in interspecies recombinants

Attempts to define the cellular origin of the ECM components that contribute to the formation of the basement membrane have been made by means of interspecies tissue recombinations and species-specific antibodies. Of the available antibodies raised against ECM components of mammalian origin, those directed against fibronectin, type-III procollagen, laminin and nidogen also reacted with chick intestinal connective tissue (named lamina propria in the intestine) and/or basement membrane (Fig. 5). On the contrary, the antibody raised against mouse type-IV collagen stained rat tissue (Fig. 6C) but did not cross-react with chick tissue (Fig. 6A). *Vice versa*, the antibodies produced against chick type-IV collagen labelled the epithelial–mesenchymal interface as well as the muscular layers of the chick intestine (Fig. 6B), but did not stain sections of rat tissue
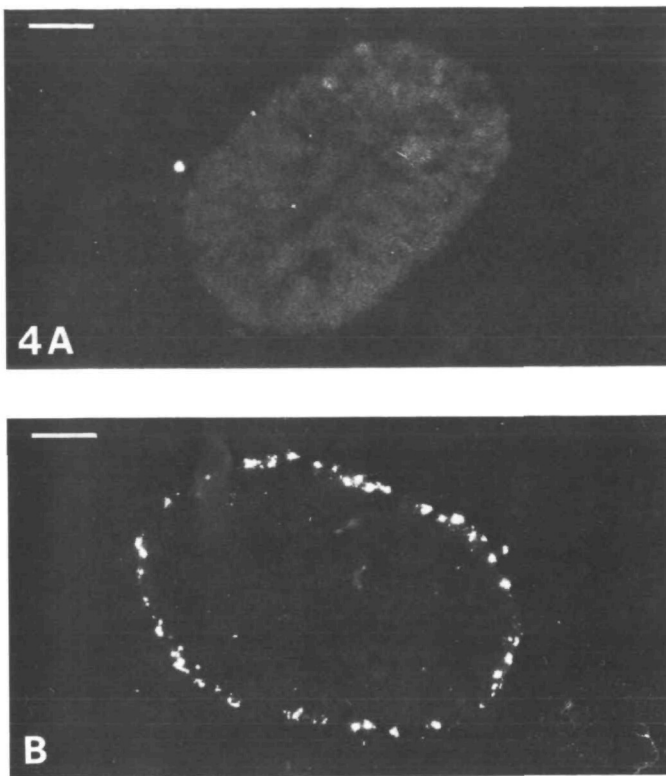
Fig. 4. Immunocytochemical reaction on isolated 14-day fetal intestinal endoderms with (A) type-IV collagen and (B) nidogen antibodies. Bar, 20 μm.

(Fig. 6D). Therefore, type-IV collagen was used as a marker for the study of the cellular origin of the basement membrane in two kinds of chimaeric intestines composed of chick mesenchyme and rat endoderm (Cm/Re) or inversely of rat mesenchyme and chick endoderm (Rm/Ce).

In Cm/Re recombinants, no staining with anti-mouse type-IV collagen antibodies was found

(Fig. 6E), while a bright staining was produced with the anti-chick antibodies at the basement membrane region as well as within the lamina propria and the muscular layers (Fig. 6F). In the reciprocal recombinants Rm/Ce, mouse antibodies labelled the muscular layers, some cellular elements within the connective tissue and the epithelial–mesenchymal interface of the developed grafts (Fig. 6G). It is noteworthy that the anti-chick antibody stained some structures within the lamina propria and underlying the basement membrane region or scattered within the muscular layers (Fig. 6H). This reaction is presumably due to the presence of blood vessels originating from the chick embryonic host which have invaded the grafts. This assumption is supported by a similar labelling pattern in 14-day fetal rat intestines grafted under the same conditions (not illustrated). On the other hand, in these two kinds of chimaeric intestines, nidogen, laminin, fibronectin and type-III procollagen were produced and exhibited their characteristic distribution pattern.

Discussion

In the present study, an *in vitro* coculture model system simulating the *in vivo* 'epithelial–mesenchymal entity' was used to study immunocytochemically the temporal aspects of expression and the distribution of extracellular matrix molecules during epithelial differentiation. Furthermore, the tissue source of type-IV collagen was analysed by means of interspecies tissue combinations. The data obtained emphasize, first, that ECM components are produced in cocultures and that basement-membrane-specific molecules are deposited at the epithelial–mesenchymal interface. This deposition does not seem to be
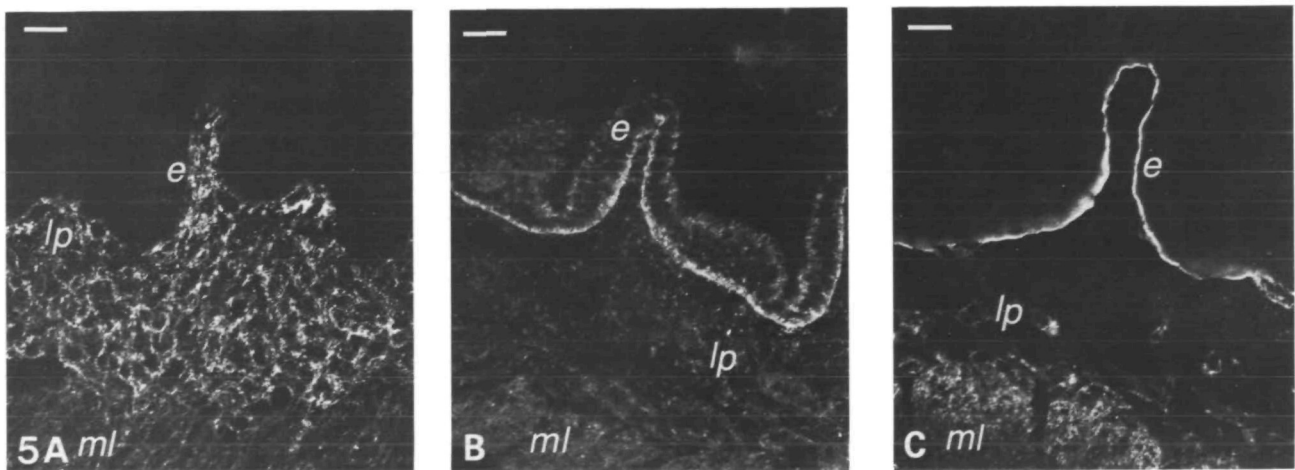

Fig. 5. Immunofluorescence pattern of extracellular matrix proteins in 16-day chick embryonic intestine stained with a rabbit anti-human fibronectin (A), rabbit anti-mouse laminin (B), and rabbit anti-mouse nidogen (C) antibodies. Epithelium (*e*); lamina propria (*lp*); muscular layers (*ml*). Bar, 20 μm.
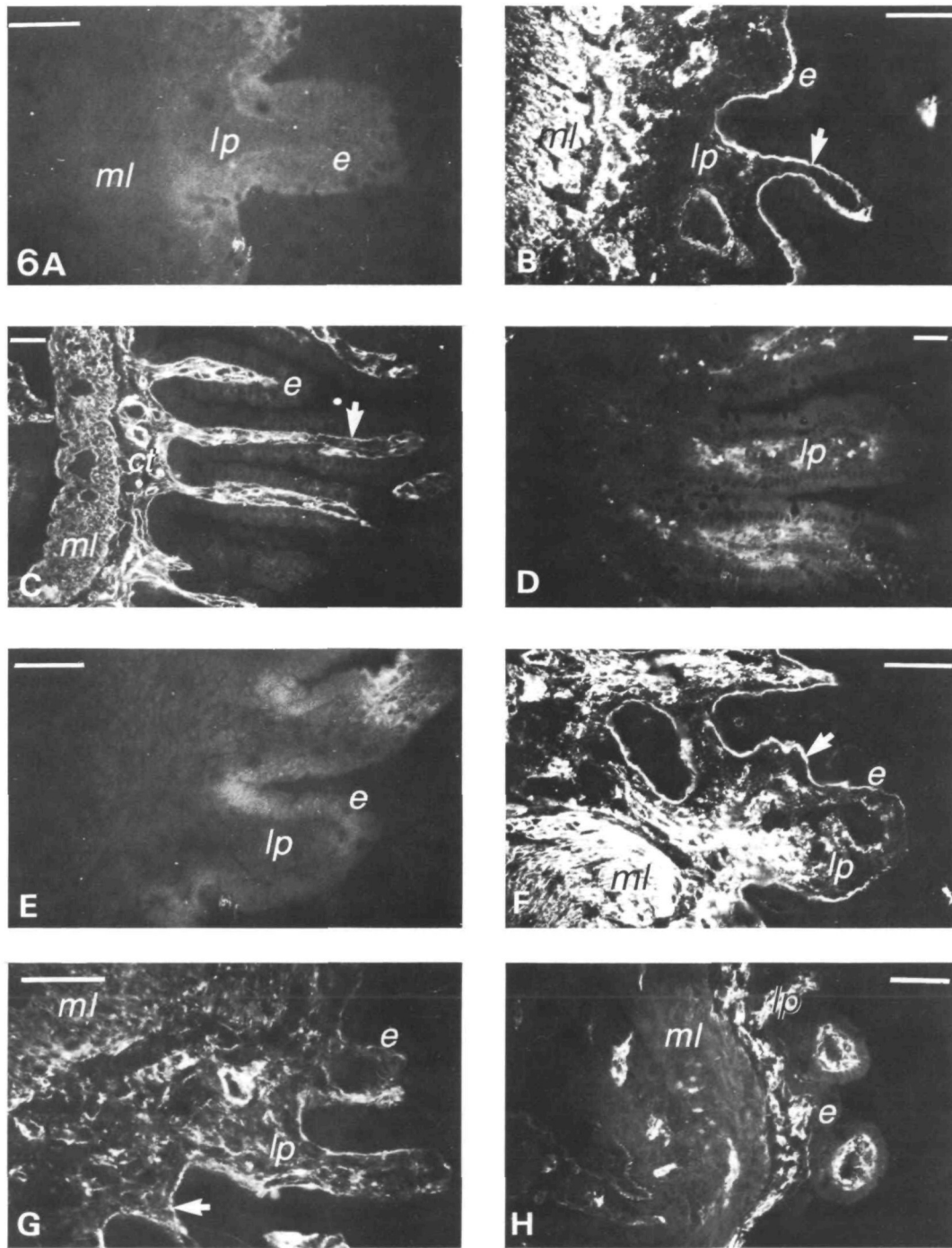
Fig. 6. Immunodetection of type-IV collagen with anti-mouse (A,C,E,G) and anti-chick (B,D,F,H) antibodies on chick (A,B), rat (C,D) intestines, Cm/Re (E,F) and Rm/Ce (G,H) recombinants. Some unspecific yellowish fluorescence is found within the lamina propria of the rat intestine in D. When anti-mouse type-IV collagen antibody was absorbed with mouse type-IV collagen, no residual antibody activity was detectable on sections of rat intestine, showing that the anti-mouse antibody cross reacts specifically with rat type-IV collagen (not illustrated). Epithelium (*e*); muscular layers (*ml*); lamina propria (*lp*); epithelial–mesenchyme interface (arrow). Bar, 40 μm (A–G); 80 μm (H).

organ specific as assessed by the substitution of skin fibroblasts for intestinal mesenchymal cells. The second main result concerns the role of the fibroblastic cells in the production and deposition of type-IV collagen in the basement membrane.

All ECM antigens studied were detected within the fibroblastic cell layer, whether these cells were cultured in isolation or in contact with endodermal cells. By contrast, the deposition of the major basement membrane components (nidogen, laminin and type-

IV collagen) in a polar fashion was found only when both epithelial and mesenchymal cell types were associated. Such a cellular cooperation for basement membrane formation has already been demonstrated in various organs (Lipton, 1977; Sariola *et al.* 1984*a* and for a review, see Timpl & Dziadek, 1986). Moreover, from the present study it appears that the re-establishment of such a complex interface is gradual and parallels the epithelial cell differentiation described previously (Kedinger *et al.* 1987). Indeed, as early as 2 days after coculture, a spot-like distribution of these proteins at the basement membrane region occurred. At this stage, endodermal areas had spread; the cells are linked together by junctions, some apical microvilli become obvious but they do not express any digestive enzymes. With increasing coculture time, a gradual assembly of the antigens occurred, leading to a continuously labelled epithelial–mesenchymal interface after one week; morphologically, the epithelial cells resemble the characteristic absorptive cells at this stage with their elongated shape and their apical brush borders. Furthermore, they synthesize microvillar digestive enzymes and are able to respond to glucocorticoids *via* the mesenchyme (Kedinger *et al.* 1987). The present data can also be correlated to the previously described ultrastructural observations at the epithelial–mesenchymal junction during morphogenesis (Mathan *et al.* 1972) and in coculture (Kedinger *et al.* 1987), which emphasized that contacts between the epithelial cytoplasmic processes and the mesenchymal cells precede the formation of a continuous basement membrane.

Taken together these data point to a parallel development of epithelial differentiation and basement membrane formation, provided that both epithelial and fibroblastic cell layers are present. However, it still remains impossible to determine whether one of these two events acts primarily as trigger. Such a correlation has been investigated by different authors and led to somewhat controversial conclusions. In the kidney, a complete basement membrane seems to be required for terminal differentiation of epithelial cells (Ekblom *et al.* 1980, 1982). On the other hand, during skin development, it has been suggested that the basement membrane is not a prerequisite but rather an additional criterion of epithelial differentiation (Lavker & Sun, 1983; Bohnert *et al.* 1986).

Concerning the cellular source of the basement membrane components, we employed as an experimental approach, immunological methods (use of species-specific anti-type-IV collagen antibodies) and embryonic tissue recombinations. The model of *in ovo* grafted interspecies associations was preferred to the cocultures since it ensured a three-dimensional

structure and allowed the differentiation of all epithelial cell types of the mature intestine (Kedinger *et al.* 1981, 1986*b*). We could show first, using chick mesenchyme/rat endoderm recombinants, that the epithelial cells did not produce any detectable type-IV collagen and second, in the inverse associations, that the basement membrane was labelled with the antibodies directed against the species from which the mesenchyme was taken. This provided evidence that collagen IV, a component of the basement membrane, is produced by the mesenchymal compartment. In relation to this conclusion, there are known instances of mesenchymal basal lamina formation. Kühl *et al.* (1984) have emphasized the role of the muscle fibroblasts in the deposition of type-IV collagen in the basal lamina of myotubes. In the developing mammary gland, mesenchymal fat pad precursor cells have been shown to synthesize laminin and proteoheparan sulphate; these components are deposited around individual fat cells in basement membrane-like structures (Kimata *et al.* 1985). Yet, the finding that mesenchymal cells produce and deposit a basement membrane component at the epithelial––mesenchymal interface is novel. However, from our observations, one cannot exclude an additional participation of the invading host capillaries in the formation of the basement membrane. Such a possibility is supported by the demonstration of a cooperation between interstitial and vascular cells for the formation of embryonic kidney basement membrane (Sariola *et al.* 1984*a*; Sariola, 1985).

Finally, the fact that intestinal endodermal cells cultured alone did not reveal any staining with the various ECM antibodies tested is not consistent with data reported for other cultured epithelial cell types that have been shown to produce ECM molecules (for a review, see Timpl & Dziadek, 1986). In particular this was the case for intestinal crypt cell lines (IEC cells), which synthesize and secrete collagens and fibronectin, the latter being detected in regions of cell–cell contacts (Quaroni *et al.* 1978; Quaroni & Trelstad, 1980). Although it cannot be excluded that low levels of immunocytochemically undetectable ECM molecules are synthesized by the cultured endodermal cells, this discrepancy could be due to the fact that such a production by established cell lines does not necessarily reflect the *in vivo* situation.

In conclusion, our model systems may be instrumental in further investigations of the cellular origin of extracellular products by *in situ* hybridization using cDNA probes and also the biosynthetic activity of both cell populations.

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