Production of fibronectin and collagen types I and III by chick embryo dermal cells cultured on extracellular matrix substrates

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ABSTRACT. Dermal cells isolated from the back skin of 7-day chick embryos were cultured on homogeneous two-dimensional substrates consisting of one or two extracellular matrix components (type I, III, or IV collagen, fibronectin and several glycosaminoglycans (GAGs) : hyaluronate, chondroitin-4, chondroitin-6, dermatan and heparan sulfates). The effect of these substrates on the production of fibronectin, of types I, III and IV collagen by cells was compared with that of culture dish polystyrene.Using immunofluorescent labeling of cultured cells, it was observed that, on all substrates, in 1-day and 7-day cultures, 85 to 95% of cells contain type I collagen in the perinuclear cytoplasm; label was absent from cell processes. Type I collagen was also detected in extracellular fibers extending between neighboring cells. By contrast, on all substrates, only 5 to 20% of cells produced type III collagen. Otherwise distribution of type III collagen was similar to that of type I collagen. With anti-type IV collagen antibody no staining of either cell content or extracellular spaces was detected. Staining with anti-fibronectin antibody revealed two types of distribution patterns. On polystyrene and on all but type I collagen substrates, labeling revealed clusters of short thick strands and patches of fibronectin-rich material in extracellular spaces. On type I collagen substrate, however, immunostaining revealed a delicate network of regularly spaced parallel fibrils of fibronectin extending between and along cells. Using quantitative radioimmunoassay of the culture media, it was shown that, after 7 days of culture, cells secreted more type I than type III collagen. Type I collagen production increased with time of culture. Substrates consisting of type IV collagen or GAGs (alone or in mixtures with type I collagen) exerted an inhibitory influence on the production of type I collagen. Production of type I collagen was unaffected by fibronectin, by type I or type III collagen substrates.

KEY WORDS : *embryonic dermal cells, extracellular matrix biosynthesis, collagen, fibronectin, glycosaminoglycans*

Introduction

Several studies on the development of various embryonic organs have suggested that the extracellular matrix (ECM) might play an important role in epithelial-mesenchymal interactions (Grobstein, 1967; Slavkin and Greulich, 1975). Dermal-epidermal recombination experiments have shown that the development of skin and cutaneous appendages in the amniote embryo results from precisely timed and precisely located interactions (Sengel, 1976). It is well established that the dermis controls size, shape, distribution pattern, and regional specificity of cutaneous appendages (Sengel et al., 1980; Sengel, 1983). However the mechanism whereby the dermis transmits its morphogenetic messages to the epidermis is still almost completely unknown. Histological studies on embryonic chick (Sengel et al.,1962; Mauger et al.,1982a, b, 1983; Jahoda et al., 1987) or mouse skin (Mauger et al., 1987) using indirect immunofluorescence and other histochemical labelings have revealed that several extracellular matrix constituents (types I and III collagen, fibronectin and GAGs) are distributed in a heterogeneous manner related to major morphogenetic events. Likewise, in other systems, it appeared that the extracellular matrix might play a determinant role in the development of various epithelial-mesenchymal organs, such as cornea (Hay, 1981), kidney (Ekblom et al., 1982), salivary gland (Bernfield, 1981), and tooth (Thesleff et al., 1981; Kollar, 1983; Lau and Ruch, 1983; Lesot et al., 1985).

Other experiments carried out on dissociated cultured chick embryo dermal cells (Sengel and Kieny, 1984, 1986; Sengel, 1985; Sengel et al., 1985; Robert et al., 1989) showed that certain ECM components exert a significant influence on various parameters of cell behavior, such as proliferation and cell patterning, spreading and locomotion. Results have indicated that type I and type III collagen, hyaluronate, heparan sulfate and mixtures of type I collagen and one GAG, inhibit cell proliferation, while fibronectin deposited on top of type I collagen eliminates the inhibiting effect of type I collagen on cell proliferation. The smallest spreading was observed on fibronectin, while the largest was measured on chondroitin-6 sulfate and on heparan sulfate substrates. The slowest cell velocity was
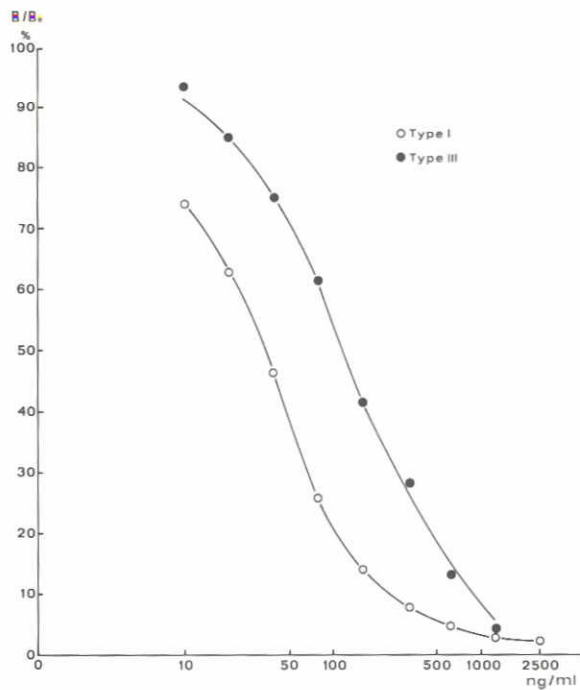
Fig. 1. Radioimmunoassay of chick types I and III collagen.
Abscissa : Concentration of non-labelled type I or type III collagen. Ordinate : Proportion B/B_0 of bound ^{125}I-labeled (a) type I collagen, (b) type III collagen, as percentage of initial binding (B_0) to the antibody.

recorded on fibronectin, on type I and type IV collagen, and on a mixture of type I collagen and chondroitin-6 sulfate, while the fastest speed was recorded on chondroitin-4 sulfate.

It was interesting to test whether extracellular matrix substrates might influence the production of collagen (types I, III and IV) and of fibronectin by 7-day chick embryo dermal cells. Dissociated dermal cells were seeded on homogeneous two-dimensional substrates of various extracellular matrix components : type I, type III, or type IV collagen, fibronectin, and several GAGs. The production of collagen (types I, III and IV) and of fibronectin in the intracellular and extracellular spaces, and in the culture medium was studied using immunohistochemistry and immunoassays. It was found that certain extracellular matrix components exert a significant influence on the production of types I and III collagen, and of fibronectin.

Results

Indirect immunofluorescence

A large majority (85 to 95%) of cells were stained with anti-type I collagen antibody (Figs. 2-7). Labeling was essentially localized within the cells, in the perinuclear cytoplasm. It was stronger and more widespread within the cytoplasm in 7-day cultures (Figs. 4 and 5) than in 1-day cultures (Figs. 2 and 3). Dermal cell processes were devoid of label (Figs. 5 and 6). Anti-type I collagen antibody label

was also detected in extracellular spaces, bound to fibers extending between neighboring cells (Fig. 7).

Using anti-type III collagen antibody, after 1 day of culture, virtually no cell was stained, and, after 7 days of culture, only a minority of cells (5 to 20%) were labeled (Figs. 8 and 9). The distribution of anti-type III label was practically identical to that of anti-type I collagen label.

With both anti-type I and anti-type III collagen antibody, the intensity and distribution of label were not influenced by the nature of the substrate, plastic or ECM-coated dishes.

Cultures treated with anti-type IV collagen antibody were constantly negative.

Using anti-fibronectin antibody, label was located preferentially in extracellular spaces, with but little stain inside the cell cytoplasm (Figs. 10-13). In 1-day cultures, as well as in 7-day cultures, extracellular label was organized in fibrillar arrays in close proximity to the cell plasma membrane or extending between cells as a complex meshwork of branched fibrils. Coarsely and finely structured labelled material was also found attached to the bottom of the culture dishes at spots where no living cells could be seen (Figs. 16, 17).
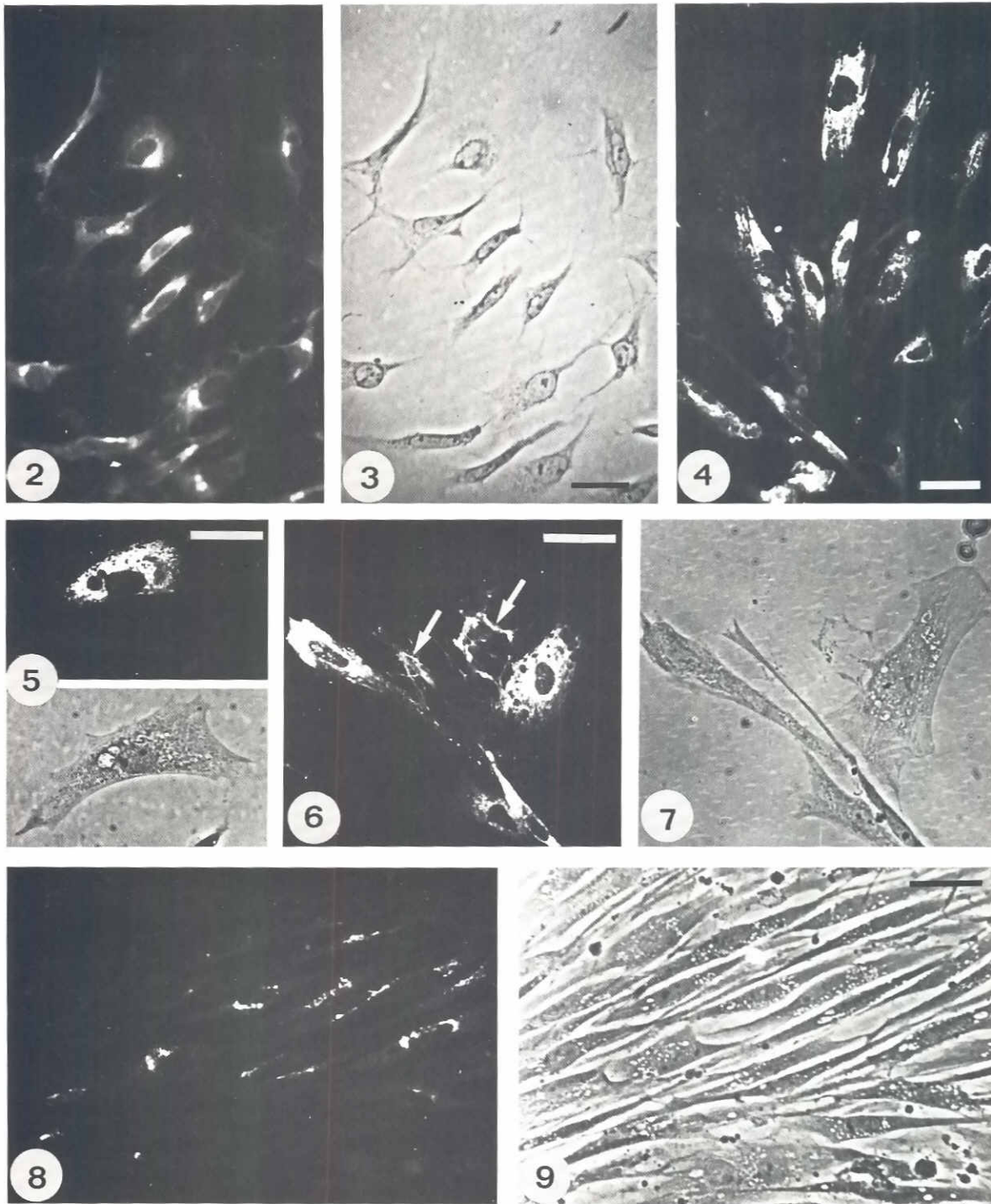
The distribution pattern and fibrillar organization of anti-fibronectin label was found to vary according to the nature of the substrate. On polystyrene (Figs. 10-13) and on all ECM substrates, except on type I collagen substrate, it consisted of clusters of short thick strands and patches of material bound to the substrate. By contrast, when grown on type I collagen substrate (Figs. 14, 15), cells were surrounded by or juxtaposed to a fine and delicate network of thin, often regularly spaced parallel fluorescent fibrils.

Immunolabeling of control skin sections of 7- to 10-day chick embryos with anti-type I, anti-type III, and anti-fibronectin antibody was constantly positive and revealed a microheterogeneous distribution pattern similar to that previously described (Mauger et al., 1982a, b, 1983).
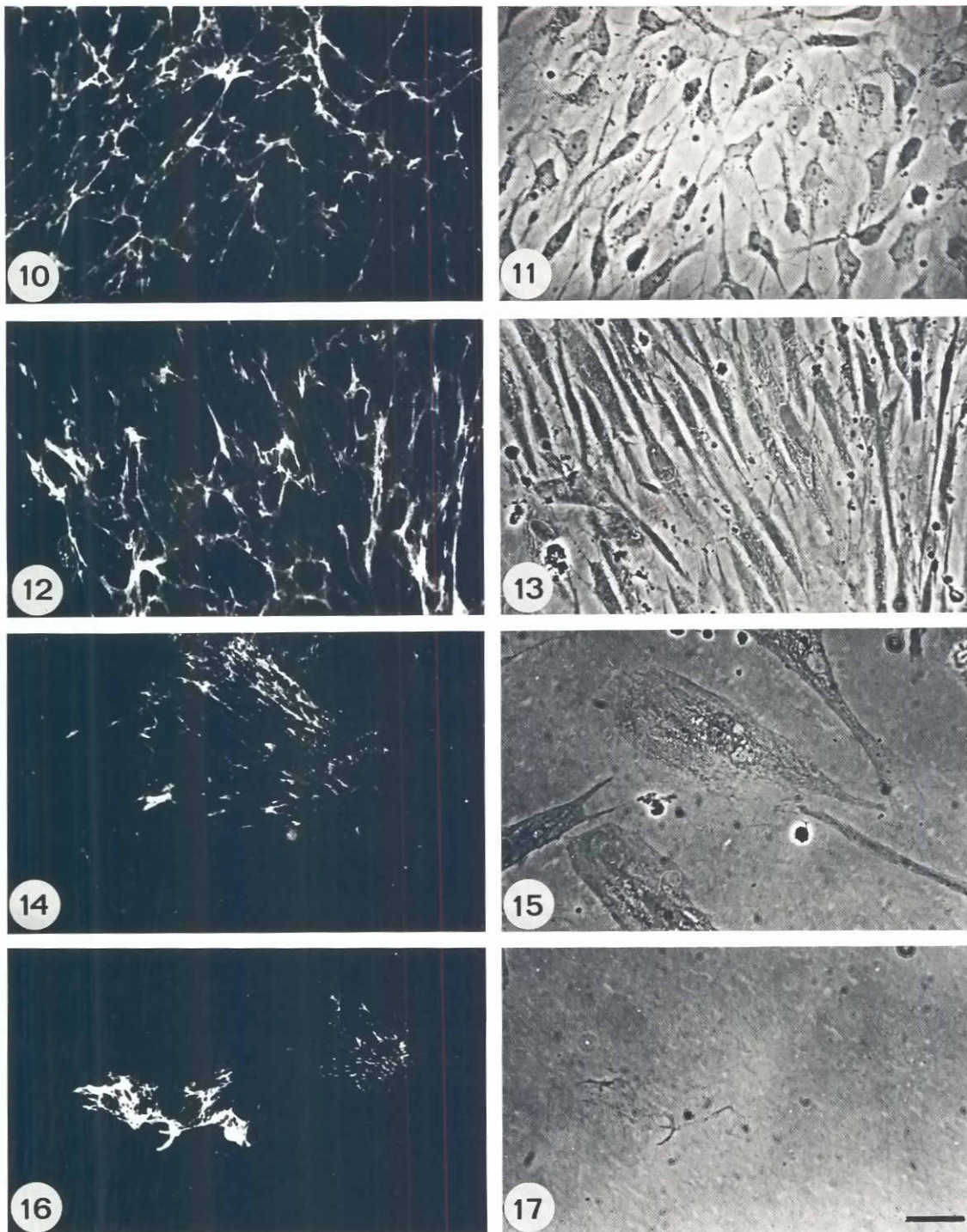
Radioimmunoassays

Radioimmunoassays of culture media revealed that dermal cells secrete types I and III collagen (Fig. 18). In 1-day cultures, only low amounts of type I collagen were measured (between 17 and 25 ng/ml) and no differences with the nature of the substrate could be noted (Fig.18). By contrast, in 7-day cultures, high amounts of type I collagen were secreted by dermal cells and the amounts produced varied from 80 ng/ml to 174 ng/ml according to the nature of the substrate (Fig. 18).

When cells were cultured on fibronectin, the production of type I collagen was not significantly different (174 ng/ml) from what it was on polystyrene (167 ng/ml). Type I collagen and type III collagen substrates likewise had no signif-

Figs. 2-7. Localization, by immunofluorescence and phase contrast microscopy, of type I collagen *in cultures of chick embryo dermal cells on a polystyrene substrate (85 - 95% of the cells are labeled by anti-type I collagen antibody). After 1 day of culture (Fig. 2), immunofluorescent label is localized inside the cells, predominantly as a clump of fluorescent material located near the nucleus (see phase contrast image in Fig. 3). After 7 days of culture (Fig. 4), labeling is still mostly intracellular, more widespread and seemingly stronger than at 1 day (in Fig. 5 immunofluorescence and phase contrast microscopy of an isolated cell show that dermal cell processes are not labeled); at places, extracellular material (arrows) also bind anti-type I collagen antibody, (see Figs. 6 and 7)*

Figs. 8-9. Immunofluorescent detection of type III collagen *in 7-day cultures of chick embryo dermal cells on a polystyrene substrate (5 to 20% of the cells are labelled with anti-type III collagen antibody). Note that staining is essentially, if not exclusively, intracellular. A phase contrast image of the same area (Fig. 9) shows the fact that only a relatively small proportion of cells are labeled with anti-type III collagen antibody. Scale bars : 25 μm.*

Figs. 10-17. Immunofluorescent detection of fibronectin produced by chick embryo dermal cells cultured on polystyrene (Figs. 10-13, 16 and 17) or on collagen type I substrates (Figs. 14-15). *On polystyrene substrate, in 1-day cultures (Fig. 10), anti-fibronectin antibody binds to an essentially extracellular network of rather coarse fibers and patches; in 7-day cultures (Fig. 12), the extracellular anti-fibronectin-positive network comprises alternatingly thick strands and thin translucent veil-like meshes. Phase contrast views (Figs. 11 and 13) of areas shown in figures 10 and 12, respectively, reveal that there is no close morphological relationship between cells and pattern of labelled fibers. On type I collagen substrate (Fig. 14) in 7-day cultures, anti-fibronectin antibody labeling decorates a delicate extracellular network of more or less regularly spaced parallel fibril. A phase contrast image of the same area (Fig. 15) clearly shows that most fluorescent fibrils lie between two cells, oriented parallel to the cells' long axes. On polystyrene substrate, in a 7-day culture, comparison of immunofluorescent (Fig. 16) and phase contrast views (Fig. 17) of same area reveals the existence of two types of anti-fibronectin-positive structures deposited on the bottom of the culture dish: (left) thick coarse patches of labeled material presumably adherent to the plasma membrane remnants of a partially detached cell, and (right) array of regularly distributed fine dots and strokes of fluorescent material probably representing the attachment points of a cell having temporarily adhered on that spot and later migrated elsewhere. Scale bar : 25 μm.*

icant influence on the secretion of type I collagen (respectively 162 ng/ml and 155 ng/ml), whereas type IV collagen substrates exerted a minor inhibiting effect (148 ng/ml), when compared to polystyrene. Cells cultured on hyaluronate and on chondroitin-4 sulfate exhibited a moderate decrease in the amounts of secreted type I collagen (respectively 151 ng/ml and 156 ng/ml) when compared to polystyrene. When cells were grown on mixtures of type I collagen and chondroitin-4 sulfate (139 ng/ml), or chondroitin-6 sulfate (80 ng/ml), or dermatan sulfate (80 ng/ml), or heparan sulfate (121 ng/ml), on chondroitin-6 sulfate (145 ng/ml), on dermatan sulfate (139 ng/ml) or on heparan sulfate (120 ng/ml), the production of type I collagen by dermal cells was significantly lower than on polystyrene (167 ng/ml). It was noted that on mixtures of type I collagen and chondroitin-6 sulfate or dermatan sulfate the level of secreted type I collagen was approximately half that obtained on polystyrene. Furthermore, it was found that the amount of type I collagen produced on mixtures of type I collagen and chondroitin-4 sulfate or chondroitin-6 sulfate or dermatan sulfate was lower than on chondroitin-4 sulfate, on chondroitin-6 sulfate or on dermatan sulfate, respectively. Thus the inhibitory effect of mixtures of type I collagen and chondroitin-4 sulfate or chondroitin-6 sulfate or dermatan sulfate was stronger than that of the GAG alone. However, when cells were cultured on a mixture of type I collagen and heparan sulfate, the production of type I collagen was not significantly different from what it was on heparan sulfate alone. Irrespective of the types of substrate, the amount of secreted type I collagen increased by a mean factor of 7.26 between the first and the seventh day of culture.

Regarding the secretion of type III collagen, no significant differences in the amounts measured (between 5 and 10 ng/ml) could be detected at 1 and at 7 days of culture or on any kind of substrate.

Discussion

The present study analyzes the effect of several extracellular matrix components (type I, type III, or type IV collagen, fibronectin, hyaluronate, chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate, heparan sulfate and also 2:1 mixtures of type I collagen and one GAG) on the production of types I and III collagen, and of fibronectin, by 7-day chick embryo dermal cells cultured *in vitro*. Results show that certain GAG substrates decrease the amount of secreted type I collagen, and that type I collagen as a substrate influences the extracellular organization of secreted fibronectin-rich material.

Using anti-type I, anti-type III, or anti-type IV collagen antibody, immunofluorescent labeling of cultures revealed that 1) type I collagen was present in cultured cells from the first day of culture onwards; 2) after 7 days of culture, 85% to 95% of the cells contained type I collagen, which remained essentially intracellular in the perinuclear cytoplasm; 3) dermal cell processes were devoid of label; 4) extracellular anti-type I collagen label was scarce and
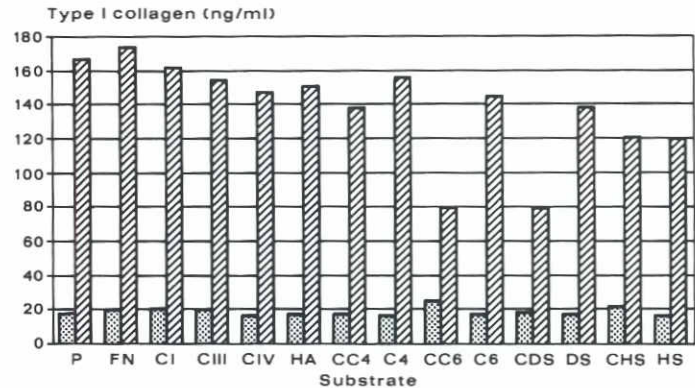

Type I collagen (ng/ml)

Fig. 18. Effect of substrate of various extracellular matrix components on the secretion into the medium of type I collagen by chick embryo dermal cells. *Radioimmunoassays were performed on 1-day (dotted columns) and 7-day cultures (striped columns). Substrates: CI, CIII, CIV, types I, III, and IV collagen, respectively; C4, chondroitin-4 sulfate; C6, chondroitin-6 sulfate; DS, dermatan sulfate; FN, fibronectin; HA, hyaluronate; HS, heparan sulfate; P, culture dish plastic (polystyrene); CC4, CC6, CDS, CHS, 2:1 mixtures of type I collagen and chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate, and heparan sulfate, respectively.*

located on fibrous structures extending from one cell to another; 5) type III collagen was not detected in 1-day cultures and only 5% to 20% of cells contained type III collagen after 7 days of culture; the distribution of anti-type III collagen label was identical to that of anti-type I collagen label, except that no extracellular staining was seen; 6) intensity and distribution of label with both anti-collagen antibodies were not affected by the nature of the substrate; 7) anti-type IV collagen antibody did not bind to the cultured cells nor to the substrate at any time and on any substrate.

Radioimmunoassays of interstitial collagen types I and III in the culture medium revealed that 1) in 1-day cultures, the level of secreted type I collagen was low, near the assay detection limit (17 to 25 ng/ml); 2) in 7-day cultures, high amounts of type I collagen were secreted (80 to 174 ng/ml); 3) chondroitin-6 sulfate, dermatan sulfate, heparan sulfate, mixtures of type I collagen and chondroitin-4 sulfate, or chondroitin-6 sulfate, or dermatan sulfate, or heparan sulfate and also to a minor extent type IV collagen, chondroitin-4 sulfate and hyaluronate exerted an inhibitory effect on the secretion of type I collagen, when compared to the amount secreted on culture dish polystyrene; 4) type I collagen, type III collagen, and fibronectin substrates had no significant influence on the secretion of type I collagen; 5) very low amounts of type III collagen could be detected (5 to 10 ng/ml), even after 7 days of culture; so that it was impossible to assess whether this production was or was not influenced by the substrate.

Quite a number of previous studies have shown that cultured fibroblast-like cells synthesize and secrete collagen into the medium, although intracellular accumulation of anti-collagen positive material is generally observed (Furcht et al., 1980; Tajima and Pinnell, 1981; Kleinman et al., 1982; Thesleff, 1986; Katsuoka et al., 1988). Some authors have pointed to the depressing effect of culture conditions, either on primary cultures or suspension cultures, on the amount of type I collagen synthesized : chick embryo tendon cells produce less collagen *in vitro* than they do *in situ* (Quinones et al., 1986). However, the addition of extracellular matrix components to the culture medium has been shown to promote collagen production. Thus, synthesis of short-chain (Mr = 60,000) collagen by vascular smooth muscle cells was enhanced 3-15 times in the presence of heparin and related glycosaminoglycans (Majack and Bornstein, 1985). Similarly, collagen, fibronectin, and other glycoproteins in the environment of cultured cells have been reported to stimulate collagen production by chick chondrocytes grown in collagen gels (Gibson et al., 1982).

It appears from the present study that none of the tested ECM substrates exerts a stimulating influence on collagen production. On culture dish polystyrene, on type I or type III collagen, or on fibronectin, cells produce more type I collagen than they do on several GAGs, notably chondroitin, heparan or dermatan sulfate. Of interest is the fact that mixtures of type I collagen and one GAG (notably sulfated GAGs) significantly depress the biosynthetic activity of embryonic dermal cells, indicating that exogenous GAGs - either alone or in mixtures with type I collagen - probably provide the cells with an environment which is closer to their natural environment and thus less stimulatory for the production of collagens than pure plastic, collagen or fibronectin, stressing the important role that GAGs play in the physiology of mesenchymal cells.

Regarding the production and deposition of fibronectin, our study showed that 1) anti-fibronectin labeling was conspicuous in 1-day and in 7-day cultures, but was stronger and denser at 7 than at 1 day; 2) labeling was essentially extracellular; 3) the pattern of the anti-fibronectin positive meshwork appeared to have but little relationship to the distribution and morphology of the cells, indicating that the fibronectin-rich network was attached to the culture dish rather than to the cell plasma membrane; this was obvious in cell-less regions of the bottom of the culture dish where anti-fibronectin-positive «foot-steps» were detected; 4) on polystyrene and on all matricial substrates tested, except on type I collagen, extracellular labeling with anti-fibronectin antibody decorated thick strands and patches of fibrous material bound to the substrate; 5) by contrast, on type I collagen substrate, cells produced a fine and delicate array of regularly spaced parallel anti-fibronectin binding fibrils extending between and beneath cells.

A variety of cultured cell types have been reported to produce fibronectin as a pericellular matrix protein (Yamada and Weston, 1974; Hedman et al., 1978; Choi and Hynes,

1979). Using indirect immunofluorescence, Baum and Wright (1980) demonstrated the presence of fibronectin in a complex network of branched fibrils on and between human gingival fibroblasts cultured on glass cover slips. Similarly, cultured human skin (Fromme et al., 1982) or embryonic mouse fibroblasts (Geuskens et al., 1986) were surrounded by a fibrous network of pericellular fibronectin extending in the interspaces between neighboring cells. Cultured rat embryo and human diploid fibroblasts produced fibronectin fibrils which were deposited between cell and substrate (Hynes et al., 1982). In cultured human skin and chick heart fibroblasts, fibronectin was essentially found to be localized in areas of close contact (Fox et al., 1980; Fromme et al., 1982), but was absent from intimate focal contacts between cells and substrate (Fox et al., 1980). Our observations demonstrate that the microscopic organization of the fibronectin network produced by cultured embryonic dermal cells is dependent on the nature of the substrate. While on polystyrene and GAG substrates cells deposit a coarse matrix of thick fibronectin rich fibers and clusters, on type I collagen substrate they organize the extracellular fibronectin into a delicate network of fine and regularly spaced fibrils. Similar parallel arrays of fibronectin-rich fibrous structures were also observed in cultures of fibroblasts from normal human skin (Fyrand, 1983). This type of distribution pattern suggests that fibronectin organized in this way is produced and/or used by motile cells, involved in morphogenetic movements. Indeed it resembles the way in which the fibronectin network can be seen to be organized in situ in morphogenetically active zones of developing skin (Mauger et al., 1982a and b, 1983, 1987), and presumably corresponds to a state more closely related to normal physiological conditions than do the coarse deposits obtained on polystyrene or other non collagenous compounds.

In conclusion, the present findings demonstrate that several extracellular matrix components may influence the biosynthetic activity of embryonic dermal cells. In this respect, the composition of the matricial environment encountered by the cells during embryonic development might constitute part of the signals that they perceive as morphogenetic messages. These in turn induce the local deposition of newly synthesized extracellular matrix, leading to an alteration of other cells' environment. Previous culture experiments with chick embryo dermal cells have shown that the matricial environment also influences cell proliferation, adherence and motile activity (Robert et al., 1989). Thanks to these results obtained from cultures of isolated dermal cells, and despite the fact that two-dimensional culture conditions do not reflect natural *in situ* conditions, the micro-heterogeneous distribution of interstitial collagens and of fibronectin in the dermis of developing skin as reported earlier (Mauger et al., 1982a and b, 1983, 1987) can, with a certain degree of confidence, be interpreted as having a morphogenetic significance. Further studies using three-dimensional collagen lattices as culture environment are expected to provide more pertinent data on the role of extracellular matrix components in organogenesis.

Materials and Methods

Cell cultures

Dermal cells isolated from the back skin of 7-day chick embryos were cultured on homogeneous two-dimensional substrates consisting of one or two matrix components (bovine type I, human type III, or human type IV collagen, human plasma fibronectin and/or several GAGs : hyaluronate, chondroitin-4, chondroitin-6, dermatan or heparan sulfate) as described previously (Sengel and Kieny, 1984; Robert *et al.*, 1989). Cultures were grown in Eagle's Minimum Essential Medium (MEM, Gibco) supplemented with 5% fetal calf serum (FCS, Gibco) and penicillin (50 IU/ml, Spécia). They were maintained at 385° C in a gas phase of 5% CO_2 in air and saturating humidity, for a period of 1 to 7 days, without change of medium.

Immunodetection

Antibodies

Antibodies directed against chick type I or chick type III collagen or against human serum fibronectin were produced in rabbits. They were prepared, purified by immunoabsorption, and tested for cross-reactivity as described previously (Mauger et al., 1982b). Antibodies to bovine type IV collagen were raised in rabbits and purified by affinity chromatography as described elsewhere (Bride *et al.*, 1982). The anti-chick types I and III collagen antibodies recognized chick antigens, but neither human nor bovine ones.

Immunofluorescence

Cells grown on tissue culture dishes were immunolabeled at room temperature by the indirect immunofluorescence method. Dishes were rinsed three times with phosphate-buffered saline (PBS) at pH 7.4. Cells adhering to the bottom of the dish were fixed and permeabilized with a 75% ethanol solution for 10 min. After washing twice in PBS, cells were immersed in the antibody solution (1:20 dilution in PBS for anti-type I collagen and anti-fibronectin antibodies; 1:30 dilution for anti-type III collagen antibody and 1:4 dilution for anti-type IV collagen antibody). After 30 min, dishes were rinsed three times in PBS and cells were incubated for another 30 min in fluoroisothiocyanate-labeled goat anti-rabbit IgG globulin (Institut Pasteur, Paris) solution (1:80 dilution) containing 70 mg/ml of Evans blue as a background counterstain.

The bottom of tissue culture dishes were mounted in buffered glycerin and observed with a Leitz Ortholux II fluorescence microscope equipped with epi-illumination.

Controls consisted of frozen transversal sections of skin from the back of chick embryos. Embryos were frozen by immersion in liquid nitrogen-cooled liquid dichlorofluoromethane (CCl_2F_2). Six-micrometer-thick cryostat sections were cut at -20°C, air-dried, and processed for indirect immunofluorescence according to the procedure previously described (Mauger *et al.*, 1982b). Previous studies using indirect immunofluorescence (Mauger *et al.*, 1982a, b, 1983, 1987) have shown that types I, III and IV collagen, and fibronectin are present in the dermis of embryonic skin from 5 through to 16 days of incubation.

Photographs were taken on Kodak Ektachrome 200 professional color film. Black and white prints were obtained from the color transparencies via an internegative on Kodak professional negative orthochromatic film.

Radioimmunoassays

The experimental protocol was as follows: to 100 μl of culture medium, or of standard type I or type III collagen in PBS, 100 μl of PBS and 100 μl of antibody, diluted in order to give between 30% and 50% binding with the corresponding [125]-labelled antigen, were added. Following incubation overnight at 4°C, 100 μl of iodinated collagen were added. Tubes were shaken, then incubated at 4°C for 24 h, after which 100 μl of an antiserum to rabbit gamma globulins and 1 ml of 1.5% (w/v) PEG 6000 solution were added. After 45 min incubation at room temperature, the precipitate was collected by centrifugation in the cold at 4000 g for 20 min and the radioactivity of the precipitate was measured in a gamma scintillation counter (LKB 1260 Multigamma). All experiments were performed in duplicate. Non specific binding was measured by replacing anti-collagen antibodies by normal rabbit serum. Results were expressed in nanogram collagen per milliliter of culture medium (Fig.1).

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