

Influence of various extracellular matrix components on the behavior of cultured chick embryo dermal cells

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ABSTRACT. Dermal cells isolated from the back 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: hyaluronate, chondroitin-4, chondroitin-6, dermatan or heparan sulfate). The effect of these substrates on cell behavior was compared with that of culture dish polystyrene. Three parameters of cell behavior were examined: cell proliferation and patterning, spreading (cell surface) and locomotion (velocity and directionality). Data were collected by sequential microphotography and analyzed by computer assisted morphometry. Types I and III collagen, hyaluronate and heparan sulfate had a slowing down effect on cell proliferation and patterning. The inhibitory effect of type I collagen was also detected in mixtures with glycosaminoglycans. The other components had no effect. While the smallest spreading was observed on fibronectin substrate, the largest was recorded on chondroitin-6 sulfate and heparan sulfate. The slowest velocity of locomotion was measured on fibronectin, types I and IV collagen and a mixture of type I collagen and chondroitin-6 sulfate. The fastest speed was recorded on chondroitin-4 sulfate. These effects are discussed in view of our knowledge of the role of the dermis in the development of skin and cutaneous appendages, and in the light of the morphogenetically related microheterogeneous distribution of collagens, fibronectin and various glycosaminoglycans in the developing skin.

KEY WORDS: *embryonic dermal cells, cell behavior, extracellular matrix, collagen, fibronectin, glycosaminoglycans.*

Introduction

Dermal-epidermal recombination experiments have established that the formation of skin and cutaneous appendages during embryonic development of amniotes results from precisely timed and precisely located dermal-epidermal interactions (Sengel, 1976) leading to cell differentiation and morphogenesis. Several studies have suggested that the extracellular matrix (ECM) might play a determinant role in the development of various epithelial-mesenchymal organs (Bernfield, 1981; Hay, 1981; Ekblom *et al.*, 1982; Kollar, 1983; Lau and Ruch, 1983; Lesot *et al.*, 1985; Ruch *et al.*, 1984).

Histological studies on embryonic chick (Mauger *et al.*, 1982a, b; 1983) or mouse skin (Mauger *et al.*, 1987) using indirect immunofluorescence and other histochemical labelings showed that several ECM components exhibit a heterogeneous distribution during the development of cutaneous appendages. Changes in the composition of the ECM appear to correlate with major morphogenetic events. Thus interstitial collagen (types I and III) is abundant and fibronectin is scarce in zones of low morphogenetic activity, such as interappendage or glabrous skin. Conversely, in morphogenetically active zones of cutaneous append-

ages, the density of interstitial collagen is low, whereas that of fibronectin is high. Likewise the density and distribution of glycosaminoglycans change in relation to the development of cutaneous appendages (Sengel *et al.*, 1962; Jahoda *et al.*, 1987). The abundance or the scarcity of one or several ECM components in the dermis during formation of cutaneous appendages suggests that these components might significantly influence the individual and social behavior of dermal cells.

In order to experimentally test this hypothesis, the effect of several constituents of the ECM was tested on *in vitro* cultured embryonic skin cells. Experiments were carried out on primary cultures of 7-day chick embryo dermal cells seeded on homogeneous two-dimensional substrates of various ECM components: type I, type III, or type IV collagen, fibronectin, and several glycosaminoglycans (GAGs). Parameters of cell behavior which were studied were proliferation and cell patterning, spreading and locomotion. Data were collected by sequential microphotography and analyzed by computer assisted morphometry.

We found that certain ECM components indeed exert significant influence on cell behavior, in agreement with previous preliminary studies (Sengel and Kieny, 1984 and 1986; Sengel, 1985; Sengel *et al.*, 1985).

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Results

Influence of culture substrate on cell proliferation

Certain ECM components exerted a significant influence on cell proliferation (Table 1), expressed as the arbitrary stage of cell density reached after 1-7 days in culture. When cells were cultured on type I collagen, proliferation was significantly slower than on polystyrene (Figs. 2a-h and 5A,5E and 5G). Type III collagen substrate was found to also have a retarding effect, but to a lesser extent than type I collagen, whereas type IV collagen had no significant influence when compared to polystyrene. There was no difference between cells growing on fibronectin and on polystyrene substrates. Heparan sulfate (Fig. 5B), and possibly to a minor extent also hyaluronate (Fig. 5C), exerted an inhibitory influence. Chondroitin-4 sulfate, chondroitin-6 sulfate (Fig. 5D) and dermatan sulfate (Fig. 2i-l) substrates had no significant influence when compared to polystyrene. When cells were cultured on a mixture of type I collagen and one GAG [type I collagen:chondroitin-6 sulfate (Figs. 3e-h and 5E), type I collagen:dermatan sulfate (Fig. 5F), or type I collagen:heparan sulfate (Fig. 5G)], cell proliferation was significantly slower than on polystyrene.

TABLE 1

INFLUENCE OF EXTRACELLULAR MATRIX COMPONENTS ON CELL PROLIFERATION AS COMPARED TO CULTURE DISH POLYSTYRENE

Culture substrate	Total number of culture series	Effect		NS
		+	-	
Fibronectin	7		2	5
Type I collagen	12		10	2
Type II collagen	3		2	1
Type IV collagen	3		2	1
Hyaluronate	3		3	
Chondroitin-4 sulfate	2	1		1
Chondroitin-6 sulfate	2			2
Dermatan sulfate	3			3
Heparan sulfate	4		4	
2:1 mixtures of type I collagen and chondroitin-4 sulfate	3		3	
and chondroitin-6 sulfate	3		3	
and dermatan sulfate	4		4	
and heparan sulfate	5		5	

Mann and Whitney's non parametric ranking test was used to compare means of data. Figures represent the number of culture series, each of which comprised 4 dishes.

+ accelerating effect

- retarding effect

NS no significant difference.

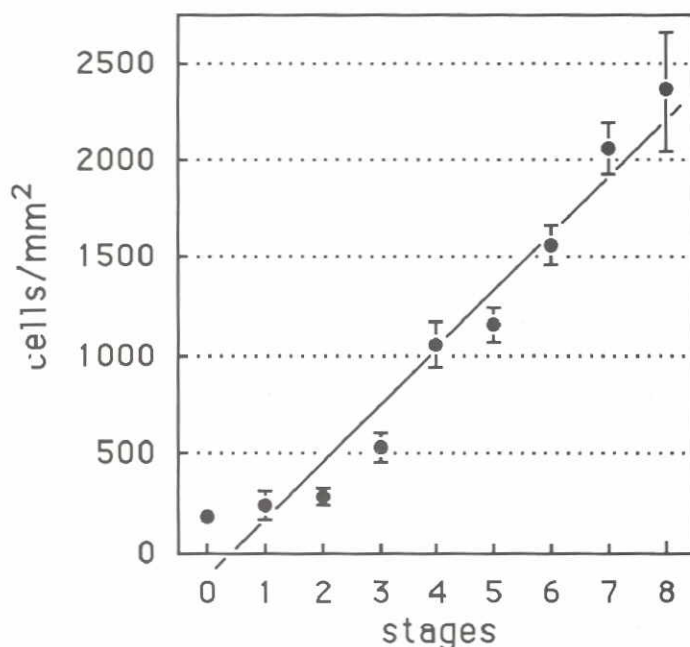


Fig. 1. Correlation between arbitrary stages (0-8) of cell patterning and cell density (cells/mm²). Cell density was measured by counting cells on x235 magnified photographic prints of staged cultures. Each point on the graph represents the mean of at least 10 counts. The straight line is the linear regression ($y = -111.7 + 288.7x$). The correlation coefficient is 0.98.

The mixture of type I collagen:chondroitin-4 sulfate (Figs. 3a-d and 5H) appeared to have no significant influence, in spite of a minor inhibiting effect.

There was no difference in the growth rate between cells grown on type I collagen and on mixtures of type I collagen:chondroitin-4 sulfate or type I collagen:dermatan sulfate (data not shown). On the other hand it was found that the growth rate on mixtures of type I collagen:chondroitin-6 sulfate (Fig. 5E) or type I collagen:heparan sulfate (Fig. 5G) was lower than on type I collagen alone. Thus the inhibitory effect of mixtures of type I collagen:chondroitin-6 sulfate and type I collagen:heparan sulfate was higher than that of type I collagen alone.

Influence of culture substrate on cell spreading (Fig. 3i-k).

In 1-day cultures (Fig. 6A), when cells were cultured on fibronectin, the mean area occupied by the cells was significantly smaller ($388 \pm 43 \mu\text{m}^2$ (SEM)) than on polystyrene ($525 \pm 80 \mu\text{m}^2$). Heparan sulfate ($840 \pm 79 \mu\text{m}^2$) and chondroitin-6 sulfate ($803 \pm 133 \mu\text{m}^2$) significantly increased cell spreading. On the other substrates, cell spreading was intermediate between those extreme values. In 2-day cultures (Figs. 4 and 6A), on type I collagen ($776 \pm 138 \mu\text{m}^2$), on type IV collagen ($681 \pm 150 \mu\text{m}^2$) (Fig. 3i and j) and on dermatan sulfate ($806 \pm 181 \mu\text{m}^2$), the mean area was significantly

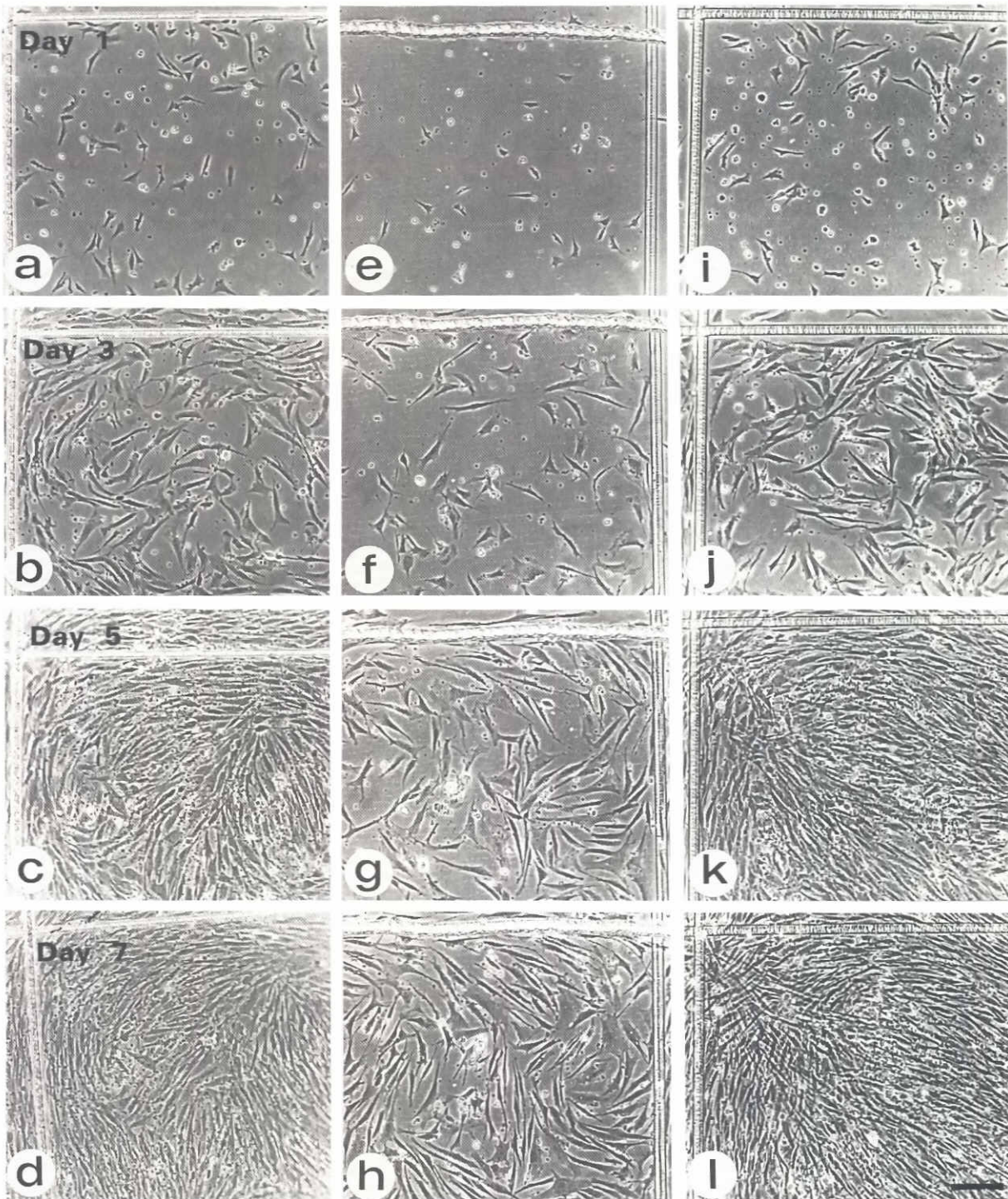


Fig. 2. Comparison of growth rate and cell patterning of dermal cells cultured on culture dish plastic (**a-d**), type I collagen (**e-h**), or dermatan sulfate (**i-l**). The same area of the cultures, identified by landmarks scratched on the bottom of the dish, was photographed on days 1 (**a, e, i**), 3 (**b, f, j**), 5 (**c, g, k**) and 7 (**d, h, l**). Note striking difference of cell density, throughout days 1 to 7, between polystyrene substrate and type I collagen. There was no great difference between polystyrene and dermatan sulfate, except on days 5 and 7 when cell density on dermatan sulfate increased more rapidly than on polystyrene. The areas shown were staged as follows: **a, b, c, d**: stages 1, 3, 6, 7 respectively; **e, f, g, h**: stages 0, 2, 3, 4 respectively; **i, j, k, l**: stages 1, 3, 7, 8 respectively. Scale bar: 100 μm .

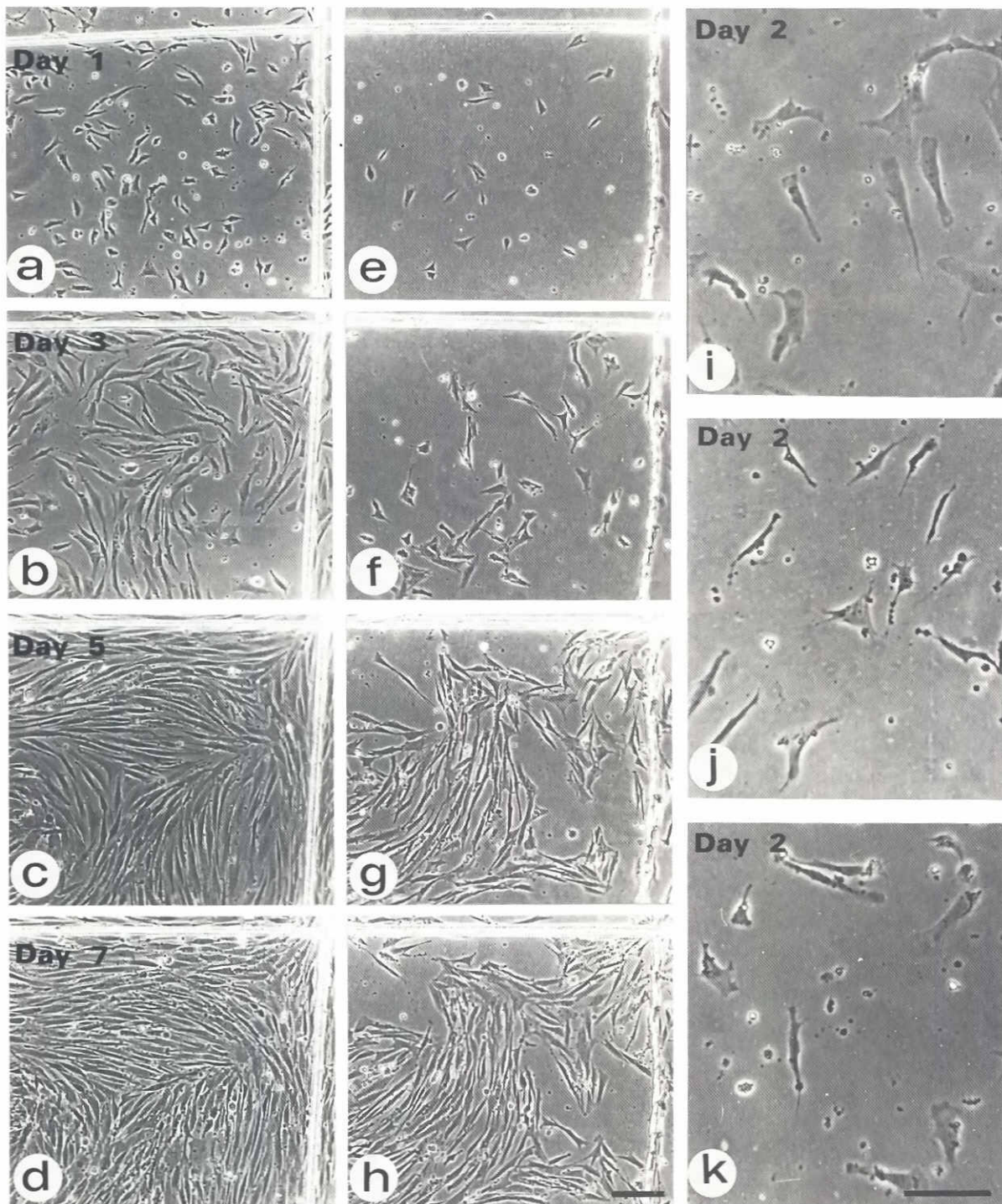


Fig 3. a-h. Comparison of growth rate and cell patterning of dermal cells cultured on 2:1 mixtures of type I collagen and chondroitin-4 sulfate (a-d), or type I collagen and chondroitin-6 sulfate (e-h). The same area of the cultures, identified by landmarks scratched on the bottom of the dish, was photographed on days 1 (a,e), 3 (b, f), 5 (c, g) and 7 (d, h). Note striking difference of cell density, throughout days 1 to 7, between the two substrates. The shown areas were staged as follows: a, b, c, d: stages 1, 3, 5, 6 respectively; e, f, g, h: stages 0, 1, 3, 4 respectively. i-k. Typical aspects of cells cultured on polystyrene (i), type IV collagen (j), or a 2:1 mixture of type I collagen and dermatan sulfate (k), and photographed on day 2 (see also Figure 4). Scale bar: 100 μ m.

smaller than on polystyrene ($1137 \pm 171 \mu\text{m}^2$). On the mixtures of type I collagen:heparan sulfate ($1487 \pm 377 \mu\text{m}^2$), or type I collagen:dermatan sulfate ($1221 \pm 177 \mu\text{m}^2$), cell spreading was increased but not significantly with respect to polystyrene; it was however significantly more pronounced than on type I or type IV collagen substrates (Fig. 3j and k). On the other substrates cells occupied an area intermediate between those extremes. Irrespective of the types of substrate, the mean area of cells increased by a mean factor of 1.79 between the end of the first and the end of the second day of culture, indicating that cells tended to spread out progressively with time.

Influence of culture substrate on cell locomotion

Two parameters of locomotion, defined in Materials and Methods, were studied: mean instantaneous velocity, expressed in micrometers per hour (Fig. 6B), and directionality, expressed as a ratio (Fig. 6C).

In 1-day cultures, when cells were cultured on fibronectin ($12.7 \pm 1.7 \mu\text{m/h}$), on type I collagen ($15.4 \pm 1.5 \mu\text{m/h}$), on type IV collagen ($14.7 \pm 2.4 \mu\text{m/h}$), and on the mixture of type I collagen:chondroitin-6 sulfate ($14.7 \pm 2.3 \mu\text{m/h}$), the velocity was significantly lower than on polystyrene ($20.5 \pm 4.1 \mu\text{m/h}$). Chondroitin-4 sulfate ($28.5 \pm 4.0 \mu\text{m/h}$) significantly increased the velocity. On the other substrates, velocity was not significantly different from what it was on polystyrene. In 2-day cultures, on type IV collagen ($16.7 \pm 2.1 \mu\text{m/h}$), and possibly to a minor extent also on mixtures of type I collagen:chondroitin-4 sulfate ($19.0 \pm 4.6 \mu\text{m/h}$) and of type collagen:dermatan sulfate ($20.7 \pm 2.0 \mu\text{m/h}$), the velocity was lower than on polystyrene ($27.4 \pm 7.0 \mu\text{m/h}$). The other substrates had no significant influence when compared to polystyrene. The global mean velocity (on all substrates) increased from $19.3 \pm 2.6 \mu\text{m/h}$ in 1-day cultures to $25.3 \pm 5.5 \mu\text{m/h}$ in 2-day cultures, which indicated that, on the whole, cells move more quickly after 2 days than after 1 day of culture.

The second parameter, namely directionality, was not significantly different on any kind of substrate (Fig. 6C). Cells appeared to move about at random, which was also illustrated by the fact that, on any type of substrate, the mean angulation of two successive segments of their path was comprised between $84^\circ \pm 14^\circ$ and $120^\circ \pm 10^\circ$, and was thus not significantly different from 90° (data not shown). On the whole, directionality increased between 1 and 2 days of culture from 0.260 ± 0.028 to 0.351 ± 0.052 . This indicated that the path followed by cells became straighter as their velocity increased.

Discussion

In this study we analyzed the effect of various ECM components on the behavior of cultured 7-day chick embryo dermal cells. Data show that, among the components tested, namely type I collagen, type III collagen, type IV collagen, fibronectin, GAGs (chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate, heparan sulfate and

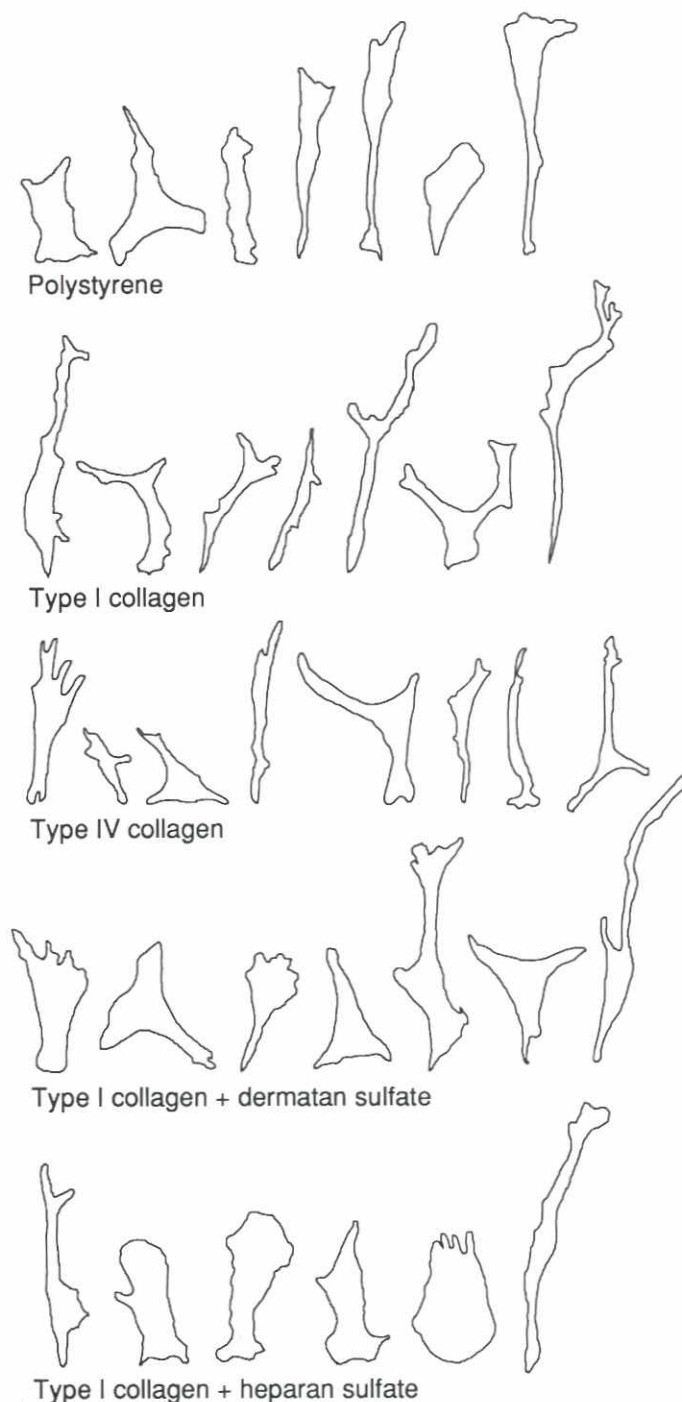


Fig. 4. Representative samples of outlines drawn from photographs of randomly chosen cells in 2-day cultures on various substrates (polystyrene, type I collagen, type IV collagen, type I collagen + dermatan sulfate (2:1), type I collagen + heparan sulfate (2:1)). The cells shown here are a subset of those that were used for evaluation of cell spreading, as determined by the cells' surfaces (see also Figure 6A). Scale bar: 50 μm .

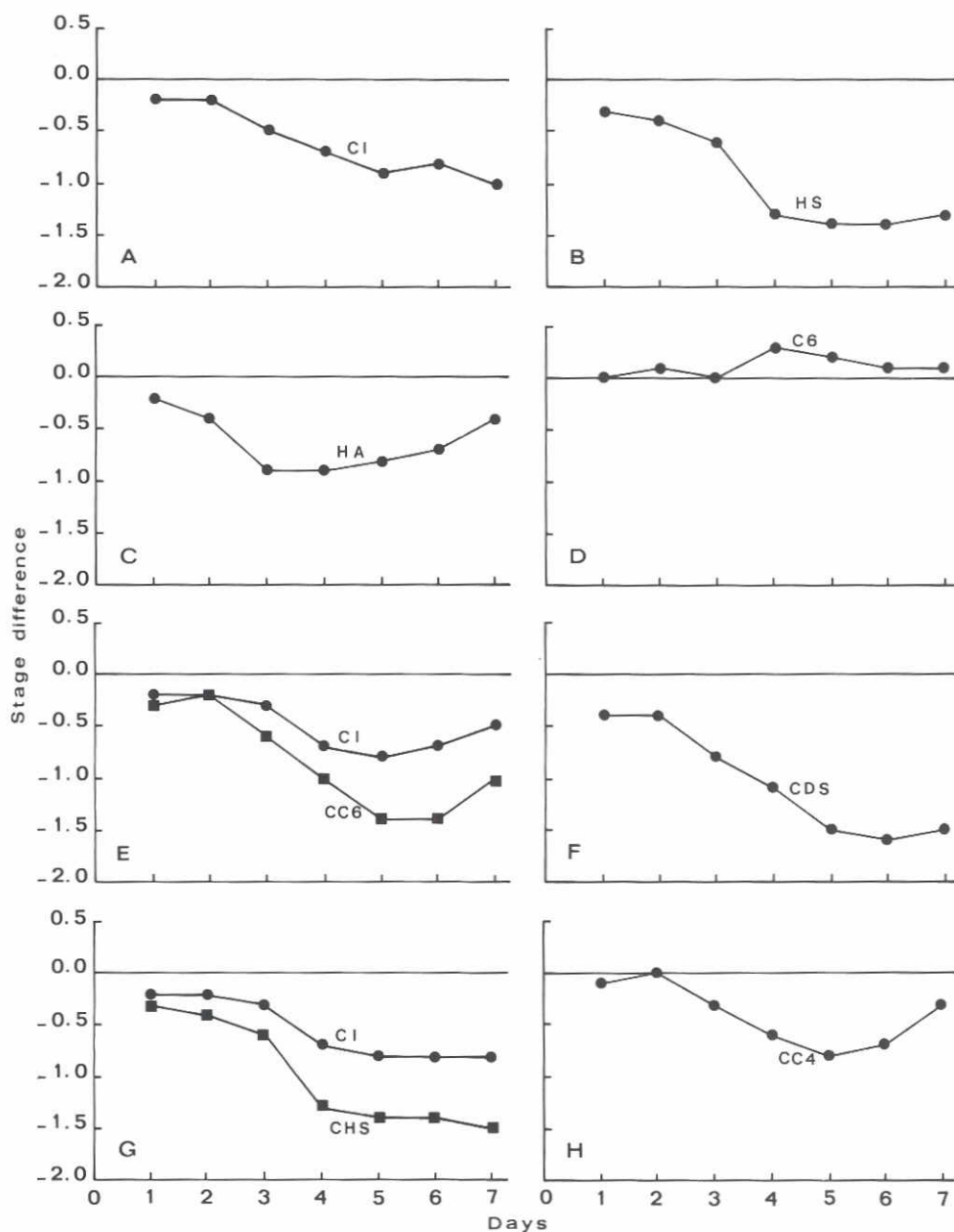


Fig. 5. Effect of various extracellular matrix components on cell proliferation, compared to polystyrene. The ordinate shows stage difference (in cell density) as a function of time between culture dish plastic (ordinate 0.0) and dishes coated with extracellular matrix components. A stage difference of 1 corresponds to approximately 289 cells/mm². CI, type I collagen; C6, chondroitin-6 sulfate; HA, hyaluronate; HS, heparan sulfate; CC4, CC6, CDS, CHS, 2:1 mixtures of type I collagen and chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate and heparan sulfate, respectively.

hyaluronate) and also 2:1 mixtures of type I collagen and one GAG, some indeed have a notable influence on various parameters of cell behavior.

Regarding cell proliferation, recorded as the progression through 10 arbitrary stages of cell patterning (Sengel *et al.*, 1985), the present study revealed that, when compared to culture dish polystyrene, type I collagen, possibly also type III collagen, heparan sulfate, and 2:1 mixtures of type I collagen and either heparan sulfate, chondroitin-6 sulfate or dermatan sulfate, exert an inhibitory effect; the other substrates tested, notably fibronectin, type IV collagen, GAGs other than heparan sulfate, and the mixture of type I collagen and chondroitin-4 sulfate have no significant influence.

Thus the global result is that the substrates tested either have no effect or exert an inhibitory influence on the rate of cell proliferation as compared to culture dish polystyrene. No substrate was found to have an accelerating effect on cell proliferation.

It thus appears that embryonic dermal cells are more strongly stimulated to proliferate on culture dish polystyrene than on substrates comprising natural macromolecules, with which they are presumably already acquainted. Explanted isolated cells would tend to reconstitute their natural environment as quickly as possible by rapidly increasing their numbers. Preliminary data indeed indicate that cells on polystyrene produce more type I collagen than they do on reconstituted ECM substrates (Robert *et al.*, 1989).

When 2:1 mixtures of type I collagen and one GAG were used, the inhibitory effect of type I collagen was always maintained. Moreover, with one type of mixture, namely type I collagen:heparan sulfate, the inhibitory effects of the two components were additive, as the depression of proliferation was stronger with the mixture than with any one of the two components used alone. With another type of mixture (type I collagen:chondroitin-6 sulfate), the inhibitory effect of collagen appeared to be enhanced by the presence of the GAG, which by itself had no influence.

Qualitative and quantitative changes of GAGs during the development of various organs have been reported (Toole, 1981). A close correlation has been established between the presence of hyaluronate and the migration and proliferation of mesenchymal cells within the hydrated primary stroma of the cornea (Hay, 1983). Hyaluronate is the major GAG in the matrix surrounding the sclerotomy cells migrating from the chick embryo somite. Ectodermal cells at gastrulation (Solursh and Morris, 1977), endocardial cushion cells (Markward *et al.*, 1978) and neural crest cells (Derby, 1978; Pintar, 1978) all move and proliferate in a hyaluronate-rich matrix. In feather development, Jahoda *et al.* (1987) described variations of the localization and density of GAGs which correlate with morphogenetic events of skin development. Notably, hyaluronate was seen to accumulate underneath the epidermal feather

placode, when dermal cells conglomerate to form the dermal condensation.

Regarding cell spreading our observations showed that, when compared to culture dish polystyrene, in 1-day cultures, fibronectin significantly decreases the mean area occupied by the cells; chondroitin-6 sulfate and heparan sulfate enhance cell spreading. In 2-day cultures, type I collagen, type IV collagen, and dermatan sulfate have an inhibitory effect; a mixture of type I collagen and heparan sulfate stimulates cell spreading. The other substrates tested in 1- and 2-day cultures have no significant influence. On all substrates, spreading increases with time at least between 1 and 2 days of culture.

Observations *in vitro* (Davis and Trinkaus, 1981) indicate that the shape of neural crest cells within a hydrated collagen lattice is dependent on the concentration of collagen. Several GAGs have been shown to influence cell behavior. Bernanke and Markwald (1979) observed that hyaluronate tends to increase the spreading of cardiac cushion cells. Turley (1984) noted that involvement of hyaluronate appears to be dependent on the cell, as well as on time. Many cell types can attach to hyaluronate, but few cells can actually spread on it. Turley (1984) noted also that chondroitin sulfate inhibits the spreading of fibroblasts, yolk sac, tumor and neural crest cells. These two inhibitory components (hyaluronate and chondroitin sulfate) were not found to be localized at the site of specialized contacts. In contrast heparan sulfate is found in specialized contacts and is involved in the spreading of cells.

Regarding cell locomotion (speed and directionality), our observations revealed that, on any one substrate, the directionality is not significantly different from what it is on polystyrene, indicating that cells fidget about completely at random and that the substrates have no influence on this behavioral parameter. Regarding speed, the present study revealed that, when compared to polystyrene, in 1-day cultures, fibronectin, type I collagen, type IV collagen, and a mixture of type I collagen and chondroitin-6 sulfate decrease cell velocity; chondroitin-4 sulfate exerts a stimulatory effect. In 2-day cultures, type IV collagen, mixtures of type I collagen and either chondroitin-4 sulfate or dermatan sulfate slow down locomotion. The other substrates tested in 1- and 2-day cultures have no significant effect. On all substrates tested in the present study, mean cell velocity increases with time between 1 day and 2 days of culture.

In other systems, it was observed that the motile rates of heart fibroblasts cultured on glass coverslips decrease with time in culture (Turley and Torrance, 1984). In agreement with our observations, Armstrong and Armstrong (1980) noted that the collagen content of aggregates of chick embryo skin fibroblasts has an inhibitory effect on cell motility. On the other hand, neural crest cells migrate more quickly on collagen than on tissue culture plastic (Maxwell, 1976). Our results show that the speed varies from 0.2 $\mu\text{m}/\text{min}$ to 0.65 $\mu\text{m}/\text{min}$. David and Trinkaus (1981)

observed that the velocity of neural crest cells within a hydrated collagen lattice is dependent on the concentration of collagen: cells move more rapidly ($1 \mu\text{m}/\text{min}$) when collagen concentration is low, and more slowly ($0.5 \mu\text{m}/\text{min}$) when it is high. Chick embryo dermal cells cultured on two-dimensional substrates seem to have a smaller speed than neural crest cells within a hydrated collagen lattice. In opposition with our observations, several studies revealed that fibronectin stimulates cell migration (Nishida *et al.*, 1983; Rovasio *et al.*, 1983; Katow and Hayashi, 1985). Regarding GAGs, our studies showed that chondroitin-4 sulfate increases cell velocity, while chondroitin-6 sulfate appears to have no significant influence. On the other hand, Turley (1984) showed that chondroitin sulfate increases cell motility. While cultured cardiac cushion cells (Bernanke and Markwald, 1979) and many other types of cells (Turley, 1984) have their motility increased by hyaluronate, we observed that hyaluronate has no effect on cell velocity. This might be due to the fact that hyaluronate was presented to the cells in a two-dimensional manner rather than in a more natural three-dimensional environment. Regarding sulfated GAGs, Sengel *et al.* (1962) showed by histo-autoradiography that their density is particularly high at the cranial base of the outgrowing feather bud. The probable stabilizing role that these GAGs might exert during morphogenesis of the reclining feather bud is thus corroborated by the observation that sulfated GAGs indeed inhibit cell proliferation. This was found notably for heparan sulfate alone, or chondroitin-6 sulfate, dermatan sulfate, or heparan sulfate in conjunction with type I collagen, the latter compound having also been shown to asymmetrically accumulate under the cranial slope of the feather bud (Mauger *et al.*, 1982b).

The finding that type I collagen and possibly also type III collagen have a retarding effect on cell patterning and cell proliferation *in vitro* is in agreement with the observation that these two interstitial collagens accumulate during skin development in morphogenetically inactive zones of cutaneous appendages (Mauger *et al.*, 1982a, b; 1983), and also with the idea that interstitial collagens (Bernfield, 1981; Hay, 1983) play a stabilizing role in morphogenesis. Type IV collagen was found in the present study to have no effect on dermal cell proliferation, which again agrees with the observation that this type of collagen is evenly distributed along the dermal-epidermal junction without regional or temporal variation throughout the development of cutaneous appendages (Mauger *et al.*, 1982a; 1983). Fibronectin likewise was seen to have no effect on the rate of cell patterning, although it is known that this glycoprotein accumulates preferentially in morphogenetically active zones of cutaneous appendages (Mauger *et al.*, 1982a, b; 1983). Explanted cells possibly need to reconstitute their own fibronectin environment before they can use it as an efficient substrate, the more so as the fibronectin used in the present study was of human origin. However, human fibronectin deposited on top of bovine type I collagen was previously found to counterbalance the inhibitory effect of collagen on the proliferation of cultured chick dermal cells (Sengel and Kieny, 1984).

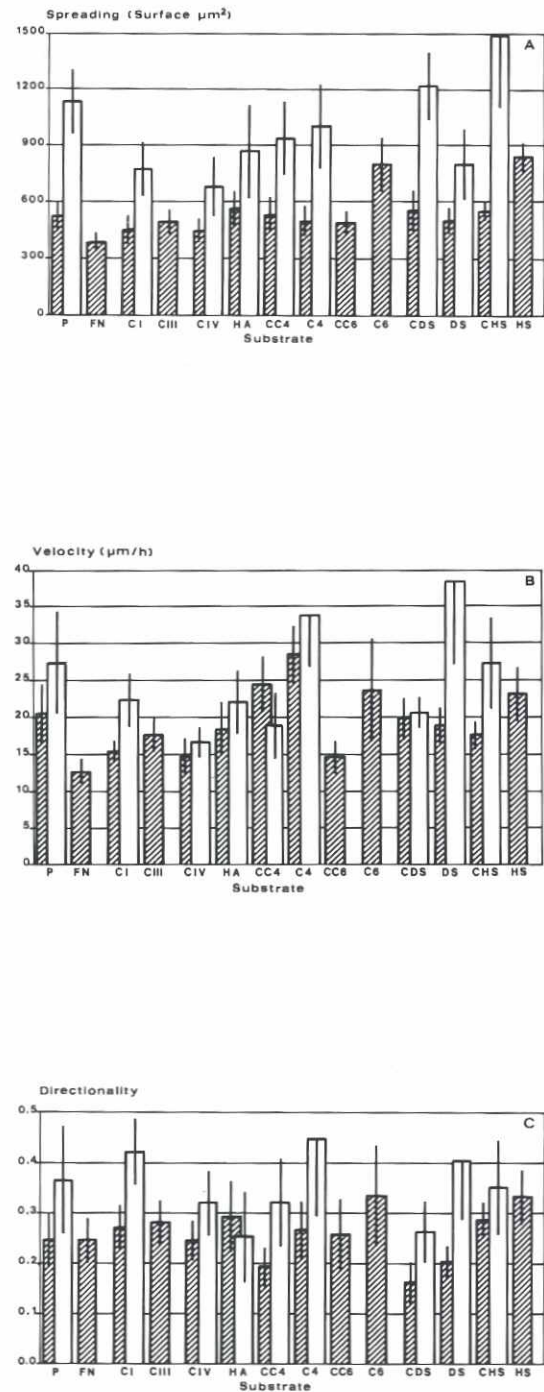


Fig. 6. Quantitative analysis of cell spreading and locomotion. **A**, spreading; **B**, velocity; **C**, directionality. Observations were performed on 1-day cultures (striped columns) and 2-day cultures (open columns). CI, CIII, CIV, types I, III, and IV collagen, respectively; C4, chondroitin-4 sulfate; 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. Vertical bars represent confidence limits at the level of 95%.

Despite several points of divergence between our observations and those of previous studies, it is clear that several ECM components influence the behavior of dermal cells *in vitro*. The discrepancies between our findings and those of others probably reflect variations in the culture methods used (origin of cells, medium with or without fetal calf serum, two- or three-dimensional cultures). Homogeneous two-dimensional substrates are but a crude oversimplification of *in vivo* conditions. Further studies, using heterogeneous two-dimensional substrates and three-dimensional environments, now under way in our laboratory, will probably lead to a better understanding of how ECM macromolecules influence cell behavior and what precise role they play in morphogenesis.

Materials and Methods

Cell culture

Dermal cells were obtained from the back skin of 7-day chick embryos, at a stage when the first mid-dorsal feather rudiments begin to appear. Pieces of skin were immersed for 20 min at 4°C in calcium- and magnesium-free Earle's saline containing 1% trypsin (lyophilized, Choay). Epidermis was mechanically separated from the dermis and discarded. The dermis was chopped up into small pieces with iridotome scissors, further treated with the same trypsin solution for 20 min at room temperature and then dissociated into single cells by repeated pipetting. The dispersed cells were diluted by the addition of two volumes of Tyrode's solution to one volume of cell suspension. The resulting diluted suspension was centrifuged (1000 rpm, 10 min) and the resulting pellet was resuspended in Eagle's Minimum Essential Medium (MEM, Gibco) with 20% fetal calf serum (FCS, Gibco) for trypsin inactivation. The suspension was again centrifuged (1000 rpm, 10 min), and the pellet resuspended in MEM with 5% FCS. The suspension was sieved through a double layer of sterile nylon cloth to eliminate most of the undissociated cell clusters. Visual examination revealed that 2- to 3-cell clusters were rare, and larger ones absent. Cell density was determined with a haemocytometer, and cell mortality (<1%) was determined by the Trypan blue exclusion test.

Dermal cells were seeded at a density of 3.5×10^5 cells per 35 mm plastic culture dish (Falcon) in 1.8 ml of culture medium, which consisted of MEM supplemented with 5% FCS and penicillin (50 IU/ml, Spécia). Cultures were maintained at 38.5°C in a gas phase of 5% CO₂ in air and saturating humidity, for a period of 7 days, without change of medium, except on the day following initial seeding.

Culture substrates

Isolated dermal cells were cultured on homogeneous two-dimensional substrates consisting of one or two matrix components (type I collagen, type III collagen, type IV collagen, fibronectin and several GAGs: chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate, heparan sulfate and hyaluronate). The effect of these substrates on cell behavior was compared with that of culture dish plastic (treated polystyrene).

Human plasma fibronectin was obtained from the Institut Pasteur Lyon (Dr. D.J.Hartmann). Culture dishes were coated with fibronectin using a solution of fibronectin (40 µg/ml) in MEM, which was left in place for 30 min at room temperature. Dishes were then rinsed twice with MEM and filled with 1 ml of culture medium.

Dishes coated with collagen (either bovine type I, human type III, or human type IV collagen), with heparan sulfate, or with a mixture of bovine type I collagen and one of the following GAGs: chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate or heparan sulfate, were obtained from Bioética (Lyon). The coating of culture dishes by this supplier is performed as follows. Purified acid-soluble type I collagen is extracted from calf skin, and sprayed on the culture dishes as a methanol solution (0.4 mg/ml). As methanol evaporates, about 100 µg per dish native collagen is uniformly deposited. For type III or type IV collagen 0.5 ml of a collagen solution in 0.5 M acetic acid (0.1 mg/ml) is poured into culture dishes and allowed to evaporate in a ventilated incubator at 30°C, resulting in 50 µg collagen deposited per culture dish.

Coating with 2:1 (w:w) mixtures of type I collagen and one of the above-mentioned GAGs is done in the following manner. Purified acid-soluble type I collagen is sprayed as a methanol solution as before. After evaporation of the methanol, 0.125 ml of an aqueous solution of one GAG (0.2 mg/ml) is added, and left to dry out completely at 37°C, resulting in the deposition of 50 µg type I collagen and 25 µg GAG per culture dish. Coating of culture dishes with heparan sulfate is prepared using 0.25 ml of a solution of heparan sulfate (0.2 mg/ml) in water, which is also left to dry out completely, resulting in 50 µg heparan sulfate being deposited per culture dish.

Lyophilized GAGs (chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate or hyaluronate) were also supplied by Bioética. Culture dishes were coated with these GAGs, using 1 ml of an aqueous solution of the GAG (0.04 mg/ml) in water, which was left to dry out completely at 37°C, resulting in the deposition of 40 µg GAG per culture dish. To evaluate release of GAGs into the culture medium during cultivation, culture dishes coated with one of these GAGs were incubated with culture medium at 38.5°C. After 4 days, the medium was removed and its GAG content was determined by the method of Elson and Morgan (1933) modified by Cessi and Piliago (1960). GAGs were found in the culture medium at concentrations lower than the sensitivity of the method (4 µg/ml). We concluded that the major part of the GAGs remained adherent to the culture dish during cultivation.

Observation and recording of cell behavior

In order to allow sequential daily observation and photographic recording of selected culture areas, landmarks were scratched on the bottom of culture dishes prior to seeding of the cells. Landmarks consisted of two orthogonally oriented sets of three parallel lines, the latter being approximately 3 mm apart.

Cell proliferation was quantified by the definition of an arbitrary scale of cell patterning consisting of 10 arbitrary stages (Sengel and Kieny, 1984), progressing from isolated cells (stage 0), through the establishment of first cell contacts (stage 1), initial alignment and coordinated grouping of neighboring cells (stage 2), formation of a loose network where cells cover less than half the available surface (stage 3), formation of a dense network where cells cover more than half the available surface of substratum (stage 4), formation of a subconfluent network where cells occupy almost all the available surface (stage 5), initial confluence where cells occupy all the available surface, but still maintain their initial width (stage 6), crowded confluence where cells become narrow and crammed together on the available surface (stage 7), formation of a criss-cross pattern where cells pile up on top of each other in several layers (stage 8), to the eventual conglomeration of cells into bulging clusters (stage 9). Cell counts performed in previous experiments (Sengel *et al.*, 1985) showed that there was a strong correlation between arbitrary stages 0-8 of cell patterning and cell density, a difference of one stage corresponding to approximately 289 cells/mm² (Fig. 1).

Time-lapse analysis

Sequential microphotography (magnification x31.5) was used to analyze cell behavior. Selected areas of the cultures were observed with a Leitz Diavert inverted microscope and photographed at 15 min intervals for a period of 9 to 19 h during days 1 and/or 2 of culture. Areas were chosen among those where a majority of cells were isolated and presumably not yet directly influenced by their neighbors. For each substrate, a minimum of 9 and a maximum of 66 cells were analyzed.

Negative film was projected at x9.25 magnification (total magnification x291), frame by frame, on an x-y coordinate digitalizing tablet (Hipad) connected to an Apple III microcomputer. The coordinates of successive positions of the dermal cells' geometric center of gravity were recorded with the aid of the tablet's electronic pen, and stored in the computer. These data were then processed to evaluate locomotion and spreading of the cells.

Two parameters were used to describe cell locomotion. The *mean instantaneous velocity* is defined as the average of velocities recorded between each two consecutive positions of the cell. In all, 10 consecutive positions of cells were recorded between 20 and 34 hours of culture (so-called 1-day cultures), and between 44 and 58 hours of culture (so-called 2-day cultures). The *directionality* is the ratio of the distance between starting and end positions to the total length of the track.

Spreading of the cells was quantitated by measuring the *surface* occupied on the bottom of the culture dish by the orthogonal projection of the same randomly chosen cells as those used for the study of locomotion.

Statistics

Series of cultures consisted of 2 to 5 sets of 4 culture dishes. One set in each series comprised uncoated plastic dishes and served as control. The other sets used dishes coated with any one of the 13 ECM substrates tested.

Staging of the cultures, i.e. determination of cell density, was evaluated as follows. First, at least 16 defined areas were staged by visual examination on days 1-7 in each culture dish. From these 16 or more data, the mean stage reached by each dish was then calculated and the mean stage for each day and each substrate was computed for the series from these four means. In addition, for the presentation of the graphs (Fig. 5), sets from different series were used to calculate an overall mean for each day and each substrate. This overall mean thus represented from 12 to 44 culture dishes.

Statistical significance of data was determined by both Student's t test and Mann and Whitney's non parametric ranking test. Both tests yielded identical significance levels.

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