Epithelial and mesenchymal cell interactions with extracellular matrix material in vitro

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Epidermal organogenesis (thyroid gland, salivary gland, feather, hair, skin, thymus gland, tooth, etc.) generally follows a basic rule; epithelium exhibits well-documented interdependence with adjacent mesenchyme for a specific path of development (Grobstein, 1967, for review). Koch (1967) demonstrated in rodent embryos that isolates of incisor epithelial and mesenchymal tissue, separated by a millipore filter, continued to develop. When homotypic tissues were placed in juxtaposition to the filter, no evidence of continued differentiation was observed.

Isolated cervical loop tissues of tooth germs from mammalian embryos have been shown to develop into an entire tooth in vitro (Slavkin & Bavetta, 1968a; Kollar & Baird, 1969). Our laboratory recently reported that isolated tissue preparations (Slavkin & Bavetta, 1968a) or cell suspensions (Slavkin, Beierle & Bavetta, 1968) of epithelial and mesenchymal cells from the embryonic cervical loop, in recombination on the chick chorioallantoic membrane (CAM), reconstituted and developed into a tooth germ. Isolated homotypic tissue or cell suspensions did not differentiate in culture.

Epithelio-mesenchymal interactions in embryos characteristically demonstrated a periodic acid-Schiff (PAS)-positive reaction in the interface between heterotypic cells. Several investigators have outlined hypotheses for cellular interactions with extracellular matrices during such embryonic inductions (Moscona, 1962; Kallman & Grobstein, 1965; Slavkin & Bavetta, 1968b). The experiments to be described were designed to evaluate possible biological activity of tooth extracellular matrix material cultivated with either mesenchymal or epithelial cells. Moreover, might this extracellular matrix material induce epithelial or mesenchymal cells from heterotypic sites to differentiate in an odontogenic path? It has been proposed that the mechanisms that control multicellular organization and morphogenesis are intimately related to the surface or extracellular matrix properties of individual cells (Moscona, 1965).

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Ambrose (1967) and co-workers have demonstrated that the establishment of cell contacts between cells and various surface textures (substratum) has profound effects on cell behavior and may control embryonic development. If competent homotypic cells from different tissues adhere preferentially to a specific extracellular matrix and develop histotypic characteristics related to the organ source of the matrix, then one could assume the matrix to possess biological activity. If such activity could be demonstrated, the extracellular matrix isolated from an epithelio-mesenchymal system could be used to isolate the factor(s) involved in the induction of organ-specific cellular differentiation.

![Diagram of microdissection to isolate cervical extracellular matrix](image)

Fig. 1. Diagrammatic scheme of the microdissection to isolate the cervical extracellular matrix from 24-day embryonic rabbit maxillary and mandibular incisors. The cervical or germinative region of the tooth germ was selected in the area where the extracellular matrix is 10 μ thick. The isolated cervical matrix was used as an acellular cylinder.

MATERIALS AND METHODS

Maxillary and mandibular incisor tooth germs were excised from embryonic New Zealand white rabbits during the 24th day of gestation. The tooth germs were immediately placed in a calcium-magnesium-free phosphate-buffered saline solution adjusted to pH 7.2 (CMF–PBS). The dental papilla was separated from the extracellular matrix (Fig. 1). Subsequently, the entire extracellular matrix was dissected free of adherent dental papilla mesenchymal and inner enamel
Cell interaction with extracellular matrix

epithelial cells. The cervical portion of the extracellular matrix was excised and employed in these studies. The isolated cervical matrix was a hollow cylinder, 10 μ thick, 450 μ high, and appeared as a semi-solid fibrous material. During embryonic odontogenesis this material could be considered as a progenitor for subsequent dentinogenesis. Each matrix weighed 0.3 mg (fresh weight); the dry weight was 0.08 mg/matrix. Microdissection was deliberately selected for these experiments, as opposed to proteolytic enzyme treatment, to avoid the possibility of enzymatic degradation of the matrix material. Phase microscopy of the isolated cervical matrices showed no apparent cellular contamination. Freshly dissected cervical matrix material was placed in CMF–PBS at 37.5 °C until needed.

Electron microscopy of the isolated cervical matrices was employed as a method to test for possible cellular contamination. Twelve randomly selected cervical matrices, following microdissection to remove cellular contamination, were processed for electron microscopy. Matrices removed from the CMF–PBS were prefixed for 10 min in 2.5% phosphate-buffered glutaraldehyde (pH 8.4) and postfixed for 20 min in similarly buffered 1% osmium tetroxide (Millonig, 1961). Subsequent to dehydration in a series of graded alcohol, specimens were embedded in Maraglas (Spurlock, Kattine & Freeman, 1963), sectioned on a Porter–Blum MT-2 ultramicrotome, stained with lead hydroxide (Karnovsky, 1961), and then silver-grey sections were examined in an RCA EMU-3 electron microscope. Orientation 1 μ sections were stained with 1% toluidine blue for light microscopy.

The isolation of the cervical loop tissues and preparation of epithelial and mesenchymal cell suspensions were accomplished by methods previously reported (Slavkin et al. 1968). Sheets of skin were dissected from the dorsal aspect of the 24-day rabbit embryos. These tissues were cut into 1 mm fragments and incubated with 0.5% trypsin (crystallized and lyophilized, Worthington Biochemical Corp.) dissolved in CMF–PBS for 12 min at 37.5 °C. The epidermis and dermis were separated and, following an additional 18 min of enzyme treatment, each tissue dissociated into homotypic cell suspensions. A volume of 0.2 ml of cell suspension per graft was used in these experiments (ca. 2.5 × 10⁶ cells). The experiments are summarized in Table 1. Isolated cervical matrix, intact cervical loop tissues and sham-operated host eggs were used as controls. Previous observations indicated that homotypic cells did not differentiate in vitro. Chick chorioallantoic membrane grafting methods were modified from those previously reported by this laboratory (Slavkin & Bavetta, 1968a). After exposing the site for the graft, the matrix was placed upon the CAM and the homotypic cells then inoculated into the hollow of the matrix (Fig. 2). Each group was incubated for periods up to 10 days in a forced-draft incubator at 37.5 °C; it was assumed that this cultivation period would be adequate to test if extracellular matrix influenced adjacent homotypic cell populations to migrate, align and differentiate, on the basis of previous observations.
The term selective migration was used to describe the migration of homotypic cells towards the surface of the cervical matrix. Differentiation described the cytological transitions of explanted cells, epithelial or mesenchymal, to become tall columnar protein-secretory cells; a process which normally occurred in the differentiation of odontogenic cells. During these studies grafts and adjacent CAM were excised, placed in Bouin–Holland’s solution, embedded in nitrocellulose-paraffin, sectioned at 5 \( \mu \) and stained with PAS. Observations were based upon 105 surviving experimental cultures over a ten day period.

Table 1. Summary of experimental design and number of combinations used

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Degenerated</th>
<th>Survived</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical loop intact</td>
<td>4</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Cervical extracellular matrix (CM)</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>CM plus tooth cervical epithelial cells</td>
<td>8</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>CM plus tooth cervical mesenchymal cells</td>
<td>7</td>
<td>23</td>
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<tr>
<td>CM plus skin dorsal epidermal cells</td>
<td>11</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>CM plus skin dorsal dermal cells</td>
<td>5</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Sham-operated CAM grafting</td>
<td>1</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 2. Procedure for chick chorioallantoic membrane grafting. The homotypic cell suspensions (0.2 mm solution containing \( 2.5 \times 10^5 \) cells) were inoculated within the cervical matrix cylinder after the matrix was grafted to the CAM. AE = allantoic epithelium. MES = mesenchyme of the host membrane. CE = chorionic epithelium. CAM = chorioallantoic membrane. SHM = shell membrane. SH = shell.

RESULTS

Electron and light photomicrographs showed no cellular contamination associated with the cervical matrices (Fig. 3, A, B). Although not readily apparent under the light microscope, several membrane-bound dense bodies were observed in the electron micrographs. Serial sections indicated that these bodies represented cytoplasmic extensions within the cervical matrix. The cytoplasmic extensions, microvilli, or filopodia appeared in association with those regions rich in large collagen fibrils and in regions containing fibrils of a much smaller diameter.
Intact cervical loop tissues, grafted to the CAM for 10 days, developed resembling *in situ* odontogenesis. The cultured cervical loop tissues demonstrated the competence to differentiate into tooth germs in the avian environment. The morphogenesis associated with the cervical loop cultures often showed aberrant forms of odontogenesis, although the cellular differentiation patterns appeared similar to *in situ* development.

The cultures of cervical matrix grafted onto the CAM showed no evidence of matrix interaction with host tissues. The matrix material did not lose its

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**Fig. 3** (A) Phase microscopy of the cervical matrix employed in these studies. The arrow points to the mesenchymal surface (surface of the matrix which faced the dental papilla mesenchyme *in situ*) which was selected for fine structure observations. Note the lack of apparent cellular contamination. (Oil immersion, ×1800). (B) Numerous collagen fibrils of various diameters appeared as constituents of the isolated cervical matrix. Note the dense bodies which are membrane-bound. These inclusions were found to be cell extensions within the matrix after serial sectioning. Such inclusions were associated with both epithelial and mesenchymal surfaces of the cervical matrix. (Lead hydroxide, ×30000.)
Fig. 4 (A) Embryonic cervical epithelial cells cultured with the cervical matrix (CM) for 10 days on the CAM. (PAS, $\times$ 1200.) During 10 days of culture, the explants on the CAM, these homotypic cells migrated to the surface of the matrix, aligned themselves in juxtaposition to the fibrous material, and differentiated as tall columnar mesenchymal cells. Note the material, presumably synthesized within the homotypic cells during culture and exported to the interface between cell and cervical matrix (arrow). (PAS, $\times$ 1200.) (B) Embryonic rabbit dermal fibro-blasts recombined with cervical matrix (CM).
initial PAS-positive staining reaction nor was degeneration of the integrity of the matrix observed during the ten days of cultivation.

In this culture system 48 h were required to observe selective cellular migration and orientation of homotypic cells to the extracellular matrix. Cervical epithelial and mesenchymal cells from the embryonic tooth, when isolated as homotypic cell suspensions, demonstrated differentiation adjacent to the matrix (Fig. 4A). The skin dermal fibroblasts, normally spindle-shaped in vivo, appeared spherical during trypsinization when monitored by phase microscopy. After cultivation on the CAM with matrix, dermal cells selectively migrated, contacted the matrix and differentiated as columnar mesenchymal cells suggestive of pre-odontoblasts (Fig. 4B). The epidermal cells did not differentiate as a recombinant with the cervical matrix.

Not all cells cultured with cervical matrix migrated to the surface of the matrix material; this phenomenon was observed only at the peripheral margin of the cell suspension inoculated within the perimeter of the matrix cylinder. Homotypic cells within the center of the cell mass did not differentiate into columnar cells, nor was an extracellular ‘secretion’ observed in association with these cells. The central cell populations were viable on the basis of mitotic figures and cytoplasmic basophilia. These cells were primitive and might best be described as polymorphic undifferentiated cells of epithelial or mesenchymal origin.

Not all surface areas of the matrix material showed uniform morphogenetic potential; specifically, the basal surfaces of the cervical matrix (10 μ thick) were repeatedly observed in association with cells in various stages of cellular differentiation. Cells in contact with this surface of the matrix illustrated a reversal of nuclear polarity and increased cytoplasmic basophilia (Fig. 4A). By the tenth day of cultivation, cells in contact with the matrix were columnar and indicated an accumulation of extracellular material(s) interposed (arrow) between matrix and cells analogous to those found in normal odontogenesis (Fig. 4A).

DISCUSSION

The elegant experiments of Weiss & Garber (1952) and Weiss (1961) demonstrated that cells are able to align themselves along linear parallel fibers, grooves, or other molecular discontinuities in their substratum. Therefore, it was a highly suggestive observation that embryonic rabbit dermal fibroblasts or dental papilla mesenchymal cells, which at the 24th day of gestation consisted of irregularly shaped mesenchymal cells, converted into a single layer of elongated non-dividing cells when in contact with tooth cervical extracellular matrix. Further, it was curious that only cells in contact with the cervical matrix showed this behavior. This led to the suggestion that the selective migration of cells to the matrix substrate, and subsequent cellular differentiation to a columnar protein-secretory-type cell, were mediated by properties intrinsic to the cervical matrix,
surface properties of the cells, or both. Since the classic works of Holtfreter (1948) and Weiss (1950), it has become increasingly apparent that the mechanism(s) of regulation and control of cell movements are to be sought in the surface properties of the cells and their substratum (Weiss, 1967; Davis & Warren, 1967, for recent reviews.)

*In vivo,* the cervical matrix represents an increasingly heterogeneous interface between mesodermally derived odontoblasts and ectodermally derived ameloblasts; both cell types contribute to the formation of the extracellular matrix (Gaunt & Miles, 1967). Therefore, the metachromatic interface interposed between epithelial and mesenchymal secretory-type cells possesses two functional surfaces; an epithelial surface and a mesenchymal surface. The observations reported in these experiments of specific cellular aggregation and differentiation suggested that the properties of the matrix enhanced these phenomena. The surface of the matrix material which was in contact with the cells demonstrating differentiation was of mesenchymal origin: the surface facing the odontoblast progenitor cells *in situ.* This factor may be considered crucial although additional information appears necessary. It may be assumed that the matrix constituents were representative of both epithelial and mesenchymal synthetic exports. A variety of evidence has led to the interpretation that epithelial cells exhibit a pattern of differential adhesiveness of their basement lamina reflecting the glycosaminoglycans composition (Okazaki, Fukushi & Dan, 1962; Kallman & Grobstein, 1966; Slavkin & Bavetta, 1968b). The mesenchymal cells with their extended filopodia ‘wander’ over this substratum until selectively ‘trapped’ by an area of high specific adhesiveness. It is suggested that the surface properties of the basement lamina, or extracellular matrix materials interposed between interacting dissimilar cell populations, may act as a template for the positioning and differentiation of cells (Dan, 1960; Gustafson & Wolpert, 1963).

The fine structure of the cervical matrix must be evaluated in terms of the inclusions noted. Although cells *per se* were not observed within the microdissected cervical matrix, membrane-bound cell extensions were noted. Such cell processes could be directly responsible for the inductive behavior of the matrix. Other electron microscopic investigations of the progenitor extracellular matrix associated with dentinogenesis (Takuma, 1967) have reported mesenchymal cell processes within this material. During embryonic odontogenesis Pannese (1962) and Reith (1967) found that the basal lamina which separated the epithelial cells from the connective tissue elements (extracellular matrix) is disrupted and penetrated by epithelial microvilli. The inference was drawn that the microvilli may be structural devices engaged in the exchange of developmental information. Evidence is not yet available to eliminate or implicate cell extensions responsible for secondary embryonic inductions. The fractionation of the matrix material and subsequent assay of the various fractions for biological activity appear essential prior to definitive conclusions.
The experiments described in these studies of induction of homotypic cells *in vitro* are not adequate criteria to implicate either of the tooth cell types responsible, prior to this stage of development of the tooth primordia, for the synthesis and introduction of the morphogenetic property into this matrix. Rather our observations are the first to demonstrate histologically *in vitro* differentiation of an epithelial or mesenchymal cell population in contact with extracellular matrix in the absence of a heterotypic cell population. We assume that physico-chemical properties at the surface or within the matrix are responsible for the induction phenomena.

**SUMMARY**

1. The morphogenetic properties of isolated tooth extracellular matrix material were studied by culturing either epithelial or mesenchymal cells within the matrix as explants on the chick chorioallantoic membrane (CAM).
2. In addition to observations of 24-day embryonic rabbit incisor epithelial and mesenchymal cell suspensions cultured with microdissected cervical extracellular matrix, dorsal skin epidermal and dermal cell suspensions were also employed.
3. During the 10-day period of experimentation, cervical loop epithelial and mesenchymal cells and dermal fibroblasts migrated in contact with the extracellular matrix material, aligned and subsequently differentiated as columnar protein-secretory-type cells. Skin epidermal cells did not differentiate when combined with the matrix.
4. It was concluded that the cervical matrix, a semi-solid fibrous extracellular material, possesses a morphogenetic property capable of inducing epithelial or mesenchymal cells to differentiate analogous to embryonic odontogenesis *in situ*. Although devoid of cell contamination *per se*, membrane-bound cell extensions were observed in electron micrographs as inclusions within the matrix material. A distinction between whether these inclusions or the other matrix components possessed the morphogenetic potential is not possible at this time.

**RÉSUMÉ**

*Intéractions entre cellules épithéliales et mésenchymateuses et matériel de la matrice extracellulaire* in vitro

1. Les propriétés morphogénétiques du matériau de la matrice extracellulaire de dent isolée, ont été étudiées en cultivant des cellules épithéliales ou mésenchymateuses dans la matrice, sur la membrane chorio-allantoidienne (CAM).
2. On a observé des suspensions de cellules épithéliales et mésenchymateuses d'incisive de Lapin de 24 jours de gestation, cultivées avec la matrice extracellulaire cervicale finement disséquée. On a également utilisé des suspensions de cellules épidermiques et dermiques de la peau dorsale.
3. Pendant la durée de l’expérience (10 jours), les cellules épithéliales et mésenchymateuses et les fibroblastes du derme migrent au contact du matériel de la matrice extracellulaire, s’alignent puis se différencient en cellules hautes, de type sécrétrices de protéines. Les cellules épidermiques de la peau ne se différencient pas quand elles sont associées à la matrice.

4. On peut conclure que la matrice cervicale, matériel extracellulaire, fibreux semi-solide, possède des propriétés morphogénétiques capables d’induire les cellules épithéliales et mésenchymateuses à se différencier comme au cours de l’odontogénèse embryonnaire in situ. Bien que la matrice soit elle-même acellulaire, le microscope électronique révèle la présence d’inclusions qui sont des prolongements cellulaires limités par une membrane. Dans l’état actuel des recherches, on ne peut dire si le potentiel morphogénétique appartient à ces inclusions, ou aux autres composants de la matrice.

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