Type-specific Morphogenesis of Cartilages developed from Dissociated Limb and Scleral Mesenchyme in vitro

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WITH ONE PLATE

The emergence of definite organ and tissue structure during development implies that the component units—cells and intercellular materials—assume patterned space relations. These reveal themselves in geometric features of position, proportions, orientation, grouping, alignment, and so on. The ordering processes involved are variously referred to as 'organization', 'differentiation', 'morphogenesis', 'field action', and the like. Such terms, while useful as identifying labels for the respective problems, are largely lacking in concrete operational meaning. To realize this, one need only consider how little is known about the practical devices and tools by which any given product of development is achieved. The study of the 'biotechnology' of developmental mechanisms has indeed been lagging behind the preoccupation with general principles.

Development is a complex network of interlocking chains of processes. For an illustration of its intricacy we may refer to a diagram (P. Weiss, 1955, fig. 144) summarizing the results of efforts at resolving 'the development of the nervous system' into a series of more elementary events. To single out from such complex networks of relations simpler component threads thus becomes a prime prerequisite to a more penetrating factual analysis of developmental mechanisms. The following report offers a small contribution to such a programme, as applied to the problem of 'tissue architecture'; specifically, the architecture of cartilage as a prototype of a structurally simple tissue.

The objective of resolving histogenesis into component cell activities has recently been furthered by the introduction of procedures for the dissociation of tissues and of embryonic organ rudiments into their constituent cells and for

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the recombination of such cells into reconstituted tissues (Moscona, 1952, 1956). For demonstrations of the analytical possibilities of this approach see Moscona, 1957a, b; Moscona & Moscona, 1952; Weiss & James, 1955; Trinkaus & Groves, 1955. To recapitulate the essential points: embryonic tissues can be dissociated by enzymatic action (trypsin) in such a manner as to free the individual cells in viable condition, whereupon these isolated cells, when allowed to aggregate in vitro, can reestablish tissue-like fabrics and, under appropriate conditions, give rise to typical differentiations in accordance with their original character. Evidently this method lent itself readily for the investigation of the problem of whether the architecture of a given skeletal unit was a composite result of contributions of the constituent cells, or something inherent to the organ rudiment as a whole.

Cartilages differ characteristically by their shapes, which in turn are but indices of the differential patterns of their antecedent growth and differentiation. Growth patterns, in turn, are resolvable into more elementary component features: the configuration of the original cell population of a blastema; secondary regroupings; differential rates of proliferation, resulting in unequal expansion and crowding of different regions; different rates of production, deposition, and swelling of intercellular ground substances; confinement, creating internal strains; and passive deformation by external stresses. External factors of the latter sort definitely cannot account for the basic differences of growth pattern that distinguish different skeletal elements. For in tissue culture, under otherwise identical conditions, various precartilaginous blastemas continue to grow into different shapes corresponding essentially to their sites of origin. Thus, explanted precartilaginous mesenchyme from the limb-bud of a chick embryo turns into parts of a typical limb skeleton (Fell & Canti, 1934; Niven, 1933); while explanted mesenchyme from the ventral midline forms a sternum with its diagnostic median keel (Fell, 1939). Similarly, precartilaginous mesenchyme of the coat of the eye in culture gives rise to a flat plate characteristic of scleral cartilage (Weiss & Amprino, 1940). Although the eventual shapes to which these different growth patterns give rise vary in morphological details according to the nutrient conditions (Fell & Mellanby, 1952) and mechanical stresses (Glücks- mann, 1941; Weiss & Dorris, 1936; Weiss & Amprino, 1940), which have prevailed during their gradual elaboration, their basic architectural features are already pre-established in the mesenchymal blastemas at the time of their explantation.

Here, then, are tissue fragments which, in a common medium, take conspicuously divergent developmental courses and which, because of the relative simplicity and geometric definition of their eventual distinctions, seemed to be singularly suited for the next step of analysis—the identification of critical differentials among the different chondrogenic blastemata. Gross microscopic inspection has furnished no clue. Since the most obvious distinctions are those of shape—that is, of the particular patterns in which the cells are arranged—and
since the positions of the cells in the group are relatively fixed by the cementing action of the common ground substance, however tenuous at first, it seemed most plausible to look to the intercellular matrix as the seat of the critical structural differences. In all the experiments referred to above, the embryonic fragment had been removed and transferred into culture in one piece, thus preserving whatever internal architecture might have existed in it at the time. But whether or not this preservation of group integrity, as a patterned entity, was actually relevant for the subsequent pattern of development, had remained undecided. For a crucial test of this question, one had to go further and disrupt the inner organization of the blastemas thoroughly prior to explantation, as had become feasible by the trypsin dissociation method. It had been established that disassociated and reaggregated mesenchyme cells from the embryonic chick limb-buds formed compact nodules of cartilage bearing the typical cytological and histological characteristics of chondrified tissue (Moscona, 1952, 1956; Trinkaus & Groves, 1955). But whether such pieces, beyond becoming just generalized cartilage, also assumed definite morphological features, indicative of their origins, could only be decided by further experiments, comparing the differentiation in vitro of cells from dissociated blastemas of two different cartilage types of grossly divergent architecture. For this we chose scleral cartilage with its sheet-like growth to be contrasted with limb cartilage with its massive expansion.

MATERIAL AND METHODS

The experiments were done with cells from chick embryos. Since appendicular cartilage develops earlier than scleral cartilage, test mesenchyme of limb-buds was taken from 3½- to 4-day embryos, that of eye coat from 6- to 7-day ones. If explanted at these stages in toto, the former would give rise to limb-like cartilages (Fell & Robison, 1929; Fell & Canti, 1934), while the latter would form scleral plates (Weiss & Amprino, 1940). The former is characterized by essentially three-dimensional growth centres, with the cells assuming the configuration of concentric whorls; while the latter grows as a plate of uniform thickness, normally about 3–4 cell layers in width, bounded by smooth parallel surfaces and showing in its interior heavy fibrous tracts in planes parallel to the surface, which give it an appearance of abortive lamination.

The rudiments were removed under the dissecting microscope and dissociated into the component cells by the trypsin procedure, as previously described (Moscona, 1952, 1956). Plate, figs. A, B, show fixed and stained smears of cell suspensions from the two types of chondrogenic rudiments. There were no perceptible microscopic differences between the individual cells of the two types after isolation. The suspended cells were then allowed to settle and to aggregate in the concavity of a Maximow slide containing culture medium consisting of equal quantities of chick serum (Difco), fresh chick embryo extract (10–12 days), and Earle’s balanced salt solution. The aggregating cells formed cohesive
clusters or sheets. These formations were removed after 24 to 48 hours from the liquid medium and transferred to the surface of clots of chicken plasma and embryo extract mixed in a ratio of 3:1. Each culture was set up to contain only one of the two types of mesenchyme tested and no mixtures have been tried in these experiments.

The cultures were fixed after 2 to 5 days in vitro, sectioned perpendicularly to the clot surface, and stained with hematoxylin-eosin or with the periodic acid-Schiff reagent.

RESULTS

Already during the phase of aggregation in the liquid medium, a marked difference in the behaviour of the two types of cells became noticeable: while the limb-bud cells, as described previously (Moscona & Moscona, 1952), aggregated into numerous separate clusters, each of which formed a rounded nodule, the prescleral cells usually settled in a single continuous sheet coating the bottom of the slide. These configurations thus seemed to anticipate the basic morphologies of their later products. During further cultivation on plasma clot, both cell types developed into histologically typical cartilage. Morphologically and structurally, however, these cartilages were distinguished from each other by the possession of features referrable to their original prospective architecture. Thus, cartilage derived from limb mesenchyme appeared as scattered nodules, irregularly shaped, with cells arranged in concentric whorls around numerous foci (Plate, figs. C, G). By contrast, cartilage derived from prescleral cells had formed smoothly bounded plates in which the cells were predominantly arranged in planes parallel to the surface, interspersed with fibre systems in the ground substance of corresponding parallel orientation (Plate, figs. D, F). The results thus point to the conclusion that the individual cells of the two types of blastemas had already been constitutionally different at the time of their isolation and had each contained properties which would enable them to elaborate their own appropriate tissue architecture when reassembled in a group.

Before accepting this conclusion, however, an alternative explanation had to be ruled out. As mentioned above, the limb-cell aggregates had already rounded up, and the prescleral cell cultures had assumed the shape of a sheet, during the aggregation phase. This initial difference in arrangement was essentially preserved as they were transferred on to the clots for cultivation. Now, it is known that external shape determines internal stress patterns which, in turn, affect the disposition of cells and ground substance (Weiss & Dorris, 1936; Weiss & Amprino, 1940); and since in these tests we had to rely on cellular configurations as major criteria, differences in the constellation in which the aggregated cells were initially placed on the clot had to be taken into account. Conceivably, chondrogenic mesenchyme, from whatever source, might give rise to either massive cartilage of the limb type or to flat plates resembling sclera, depending
on whether the aggregated cells be cultured in a solid, rounded clump or in a flat sheet.

To check this possibility, the experiments were repeated with a deliberate effort to make the geometrical conditions during the culture phase more nearly alike for both types of aggregates. This was done as follows. In one set, dissociated limb-cells in a very thick suspension were poured on to the plasma clot surface as a dense, continuous layer, thus imitating the spontaneous layering typical of prescleral cell aggregates formed in liquid culture medium. In the other set, newly formed sheets of prescleral cells, instead of being spread on the clot, as in the preceding experiment, were bunched so as to be given the lumpy shape and dimensions of a limb-cell nodule.

The two types of cartilage developing under these more strictly comparable conditions still exhibited the constitutional differences of their respective architectures. Limb-cells plated out as a sheet gave rise to cartilage that had externally the dimensions of a plate, but internally the characteristic whorl-shaped texture of typical limb cartilage aggregates (Plate, figs. E, G), contrasting with the laminar texture of scleral cartilage developed under the same conditions (Plate, figs. D, F). Reciprocally, prescleral cells, cultured as massive lumps, still produced plate-shaped cartilage, albeit bent and folded inside the lump because of the spatial confinement of the building material (Plate, fig. H). In other words, regardless of whether the initial distribution of the explanted material on the clot favoured essentially planar expanse or more massive clustering, the way in which the cells filled the allotted space, grouped themselves, proliferated, and deposited intercellular ground substance, was determined by the properties of the particular cell type; evidently, the individual cells of either type must have been already endowed with those properties at the stage when they were separated. These structural differences are perhaps more obvious when limb cartilage is allowed to develop *en masse*, and scleral cartilage as a sheet, each type being thus free to assume a shape conforming to its intrinsic growth pattern. Yet, even in the case of an artificially created inconsistency between the growth and grouping tendency of these cells and the external configuration of the growing mass, the intrinsic growth and group patterns still prevail.

**DISCUSSION**

The foregoing results lead to the following conclusions. Different chondrogenic blastemas, destined to form specific parts of the skeleton, consist of cells which, at the stages tested, are endowed not only with a general faculty to turn into cartilage cells, but furthermore with distinctive morphogenetic properties determining the particular patterns of cell grouping, proliferation, and deposition of ground substance which, in due course, lead to the development of a cartilage of a distinctive and typical shape. This implies that the crucial morphogenetic properties in question reside in the individual cells, for whether or not their assembly—the blastema—retains its original structural integrity has
proved to be quite immaterial for the subsequent morphogenesis. Even after the complete disruption of the chondrogenic blastema and the random reassociation of the separated cells, the latter still know how to build that same typical cartilage.

Whatever this remarkable property be, it cannot manifest itself, of course, in single cells and is evidently a group phenomenon. A single cell can form neither a plate nor a whorl. The property in question, therefore, must be of a sort that would enable the individual cells, when they join together with others equally endowed, to execute collectively a group operation of a higher degree of orderliness. Supercellular self-ordering processes of this kind conform to the original definition of ‘field’ effects (P. Weiss, 1923; summarized in P. Weiss, 1939, 1953). It is a great step forward to have come to the realization, from experiments such as here reported and similar previous ones (Moscona & Moscona, 1952; Grobstein, 1952, 1953; Andres, 1953; Weiss & James, 1955), that ‘fields’ may originate by active integration of formerly independent cells, or cell groupings, of given types congregating at random. At the same time, one must not lose sight of the fact that the different cell types endowed with these differential faculties for specific group performances have contracted them during earlier phases of their embryonic history while they themselves resided within integral field districts. In its general features, the phenomenon of field reconstitution from random dispersed cells resembles closely the morphogenesis of the fruiting bodies of slime moulds achieved by a collective action of populations of free amoebae (cf. Bonner, 1951); it also validates, in a purely formal sense, some of the principles of morphogenesis envisaged by Child (1941), though not necessarily his particular assumptions as to the mechanisms involved.

Which brings us just to the crucial question of mechanism. The fact that dispersed and reaggregated epidermal chick-cells can constitute a feather field de novo, even in tissue culture (Weiss & James, 1955), though theoretically of interest, still tells us nothing of just how the reassembled cells go about accomplishing that feat. Perhaps the reproducibility of the process under the simplified conditions in vitro will help elucidate it; but this is still a matter for the future. The same is true of our present cartilage case. Besides telling us that some cells know how to build a plate, and others a nodule, the experiments have yielded no safe clue as to how they do it. However, since cartilage structure is considerably simpler than feather structure, this kind of tissue may provide a more favourable object for a deeper penetration into the mechanisms of structure formation, referred to in the introduction as ‘biotechnology’. The next step should be to detect more elementary differences in behaviour of cells which preferentially form plates versus cells which tend to form lumps: differences in aggregation, in mutual orientation, in proliferative pattern, and perhaps in the fine-structural characteristics of their secreted ground substances. In view of the fact that some extracellular fabrics have recently been intimated to possess ‘pseudo-crystalline’ organization (Weiss & Ferris, 1956), it is not implausible to
conjecture that the ground substances of the cartilage may likewise play a unifying and structure-determining role, the cells thus generating an ordered matrix, to the ordering influences of which they themselves would then reciprocally submit (P. Weiss, 1933).

Although these experiments have been the first to establish explicitly that morphogenetic distinctions between cartilages are based on properties of their individual constituent cells, rather than on a property of the blastema 'as a whole', it may be worthwhile to point out that this outcome might have been actually suspected long ago from observations on the development of branchial cartilage in amphibians, which takes its origin from the distant neural crest. Crest material contributes circumscribed portions to the head skeleton, mosaic-fashion (Stone, 1929; Hörstadius, 1950). The cells concerned migrate in streams from their dorsal positions in the neural crest to their peripheral destinations and, to all appearances, they are not firmly assembled during this trek. How, then, do they manage to build a particular skeletal element, e.g. a palatoquadrum, once they have aggregated again at their final site? One might have invoked a moulding role of 'organizing' factors of the local environment were it not for the fact that in xenoplastic combinations (e.g. frog hosts provided with toad neural crest) the foreign cells, even though under general guidance from the host, form the appropriate skeletal pieces strictly according to the rule of their own species-specific pattern (Wagner, 1949). Since the swarming from the neural crest can be legitimately compared to experimental dissociation, it would seem equally valid to consider the orderly group performances after recombination and aggregation as essentially of the same nature—and equally remarkable—in both cases. The origin of the limb-bud from freely migrating mesenchyme of the somatopleura in amphibians (Balinsky, 1929; Taylor, 1943) may also have to be reconsidered in this light.

**SUMMARY**

1. Precartilaginous blastemas of chick limb-buds (3½–4 days) and chick sclera (6–7 days) were dissociated into their constituent cells; the cells from each type were allowed to settle and to reassociate in a liquid tissue culture medium, and the newly established groups were cultivated on plasma clots *in vitro*. Both types of cells produced cartilages, yet each according to the specific pattern characteristic of its origin: limb mesenchyme formed lumpy nodules with whorl-shaped cell arrangements, which eventually gave rise to masses of cartilage similar to that found in normal appendicular skeleton; whereas prescleral mesenchyme formed flat plates with pseudostratified texture, much as normal scleral cartilage.

2. Either cell type maintained its intrinsic structural characteristics even when the explanted cell masses were experimentally given external shapes corresponding to the other type (limb-bud cells by layering on plasma clot and prescleral cells by compacting, respectively).
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3. The individual cells of each cartilage type were thus shown to have possessed at the time of their dissociation distinctive properties, including a type-specific rule of assembly and subsequent growth which enabled them eventually to elaborate collectively a supercellular architecture of the same type as that which they would have formed if they had been left undisturbed.

4. The phenomenon described may be regarded as a simple instance of a ‘field’ effect and as such lends itself readily to further experimental analysis.

REFERENCES


EXPLANATION OF PLATE

Fig. A. Suspension of disassociated chondrogenic cells from the limb-buds of a 4-day chick embryo. (Ehrlich’s hematoxylin, Biebrich’s scarlet. × 760.)

Fig. B. Suspension of disassociated chondrogenic cells from the prescleral mesenchyme of the eye coat of 64-day chick embryos. (E.h., B.s. × 760.)

Fig. C. Clusters of aggregated and differentiated limb cartilage cells, grown in vitro for 5 days. Notice the concentric or whorl-like arrangement of cells in the differentiated and chondrified masses. (E.h., PAS. × 200.)

Fig. D. Scleral cartilage formed by aggregated prescleral cells. Following aggregation in a liquid culture medium, the cellular sheet was transferred to a plasma clot and cultured for 5 days. (E.h., B.s. × 80.)

Fig. E. Limb cartilage formed by disassociated limb-bud cells spread on a plasma clot to encourage sheet-like development. Five-day culture. Notice the whorl-like clusters and compare the structural appearance of this tissue with the scleral sheet in fig. D. (E.h., B.s. × 80.)

Fig. F. Reconstituted scleral sheet in culture, similar to that in fig. D, magnified to show the architecture of the tissue. (E.h., B.s. × 800.)

Fig. G. Limb-bud cartilage, reconstituted in sheet-like form, similar to fig. E, magnified to show the architecture of the tissue. (E.h., B.s. × 800.)

Fig. H. Culture of aggregated scleral cells. The sheet formed by these cells in the liquid medium was bunched so as to give this aggregate the shape of a nodule which was then grown on a plasma clot for 4 days. Notice the presence of a folded, sheet-like, scleral structure within the clump. Due to their size these explants frequently showed considerable necrosis. (E.h., B.s. × 120.)

Fig. I. Part of a reconstituted scleral sheet grown in vitro for 7 days. In spite of marked growth and expansion, the characteristic shape of the tissue was retained. (E.h., B.s. × 30.)

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