Epithelial–mesenchymal tissue interactions guiding otic capsule formation: the role of the otocyst

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SUMMARY

The otocyst is the epithelial anlage of the membranous labyrinth which interacts with surrounding cephalic mesenchyme to form an otic capsule. A series of *in vitro* studies was performed to gain a better understanding of the epithelial–mesenchymal interactions involved in this process. Parallel series of otocyst/mesenchyme (O/M) and isolated periotic mesenchyme (M) explants provided morphological and biochemical data to define the role of the otocyst in organizing and·directing formation of its cartilaginous otic capsule. Explants were made from mouse embryos ranging in age from 10 to 14 days of gestation, and organ cultured under identical conditions until the chronological equivalent of 16 days of gestation. Expression of chrondrogenesis was determined by both histology and biochemistry. The *in vitro* behaviour of periotic mesenchyme explanted either with or without an otocyst supports several hypotheses that explain aspects of otic capsule development. The results indicate that (a) prior to embryonic day 12 the otocyst alone is not sufficient to stimulate chondrogenesis of the otic capsule within O/M explants; (b) the otocyst acts as an inductor of capsule chondrogenesis within O/M explants between embryonic days 12 to 13; (c) isolated mesenchyme within M explants taken from 13-day-old embryos are capable of initiating *in vitro* chondrogenesis, but without expressing capsule morphology in the absence of the otocyst; and (d) the isolated mesenchyme of M explants obtained from 14-day-old embryos expresses both chondrogenesis and otic capsule morphology in the absence of the otocyst. These findings suggest that the otocyst acts as an inductor of chondrogenesis of periotic mesenchyme tissue between embryonic days 11 to 13, and controls capsular morphogenesis between embryonic days 13 to 14 in the mouse embryo.

INTRODUCTION

Epithelial and mesenchymal tissues interact during the development of many organs (Sawyer & Fallon, 1983), and this interaction of tissues leads to specific alterations of either one or both of these tissues. Evidence for such a reciprocal interaction between epithelial and mesenchymal tissues during formation of a specific organ has been clearly demonstrated in wing development where Zwilling (1956), working with recombinants between the tissues of normal embryos and wingless mutants, postulated that a factor produced by wing bud mesenchyme is needed to maintain the integrity of the apical ectodermal ridge (AER), and an earlier study by Saunders (1948) had demonstrated that surgical removal of this

Key words: otocyst, tissue interaction, capsule formation, epithelium, mesenchyme, mouse embryo.

epithelial structure (AER) disrupted the normal development of most distal mesenchymally derived skeletal elements of the wing.

A stage-related capacity of limb bud mesoderm to initiate chondrogenesis *in vitro* is evident in micromass cultures obtained from both chick (Ahrens, Solursh & Reiter, 1977) and mouse (Owens & Solursh, 1981) embryos. Mesoderm taken from different positions within the same stage limb bud varied in its capacity to form cartilage in micromass cultures (Ahrens *et al.* 1979). Dibutyrlcyclic AMP treatment of micromass cultures derived from limb bud mesoderm that varied in both stage and position revealed a complex pattern of responsiveness that was both dependent on stage and position and correlated to the pattern of limb cartilage histogenesis in the species tested (Solursh, Reiter, Ahrens & Vertel, 1981*a*; Solursh, Singley & Reiter, 1981*b*).

The developing inner ear consists of epithelial and mesenchymal components, and the epithelial component (i.e. otocyst) has been shown to affect the development of the mesenchymal component (i.e. otic capsule) in both amphibian (Lewis, 1907; Spemann, 1918; Kaan, 1938) and avian species (Benoit, 1960). Only recently has an experimental method existed to test this interaction in a mammalian system. Van De Water & Ruben (1974) were the first to observe that otic explants would form a cartilaginous capsule when excised from mouse embryos that were 12 days of gestation or older. Van De Water (1981) has demonstrated that a critical volume of mesenchyme is essential for normal morphogenesis of explanted mouse otocysts developing *in vitro*. Recently it was proposed (Van De Water, 1983) that a continuing reciprocal interaction between otocyst and surrounding periotic mesenchyme is required for the development of the otic capsule and the labyrinthine sensory structures.

The production of the glycosaminoglycan components of the extracellular matrix has been shown to be closely related to the morphological changes observed in chondrogenesis (Minor, 1973). Production of the sulphated glycosaminoglycans (S-GAG) chondroitin 4-sulphate and chondroitin 6-sulphate is particularly indicative of cartilage formation (Searls, 1965). An autoradiographic study of the incorporation of $[^{35}S]$sulphate into S-GAG can therefore provide a semiquantitative profile of chondrogenesis and a better means for measuring the effect of an altered cellular environment on cartilage formation. McPhee & Van De Water (1985) applied the analysis of S-GAG to the formation of the cartilaginous otic capsule in mouse embryos. In this study it was shown that O/M explants develop in a manner comparable to *in vivo* development during the critical stages of capsule formation. Morphogenesis of the inner ear structures and its capsule follow the same pattern and chronology *in vitro* and *in vivo*. The synthesis of S-GAG measured by $[^{35}S]$sulphate incorporation showed some variation between *in vivo* and *in vitro* results, but the general pattern of incorporation is similar.

In our present study of the tissue interactions occurring between a mammalian otocyst and its periotic mesenchyme we attempt to establish whether these epithelial–mesenchymal interactions direct differentiation and morphogenesis of

the otic capsule. We also seek to determine whether the morphological and biochemical stages of development that have been observed to occur in other chondrifying systems (e.g. Ahrens *et al.* 1977) are also present during formation of the otic capsule. Interrelated to these two goals, we will attempt to define the role of the otocyst in the initiation of these stages of chondrogenesis. Explantation of parallel series of otocyst/mesenchyme complexes and periotic mesenchyme tissue isolated from the otocyst is our experimental system. Histological and biochemical methods are used to analyse morphogenesis and sulphated glycosaminoglycan (S-GAG) production during otic capsule formation.

MATERIALS AND METHODS

Experimental animals

CBA/J and C57 BL/6J mice strains (Jackson Memorial Labs, Bar Harbor, ME) were cross-mated to produce CBA/C57 hybrid embryos. Gestational age was estimated by the occurrence of a vaginal plug. Actual embryonic age for the first 13 days of gestation was determined by somite count (Rugh, 1968), and for older stages by somite count plus external features (Theiler, 1972). Microdissection was performed according to the procedures of Van De Water & Ruben (1974).

Organ culture

Explants of otocysts with adhering mesenchyme (O/M) or isolated periotic mesenchyme (M) were transferred to double-chambered plastic culture dishes 60×15 mm (Falcon). The culture chamber contained 0·15 ml of serumless medium (Newmann & Tytell, 1960) supplemented with 20 % foetal calf serum, 100 i.u. ml^{-1} penicillin and 0·01 % L-glutamine. Humidification of the culture dish was accomplished by filling the outer dish chamber with sterile distilled water. Sterile vaseline sealed the top of the culture dish. Cultures were incubated at 35 °C, with one half of the medium being exchanged every 48 h. Periotic mesenchyme was consistently excised from a region adjacent and ventral–caudal to the otocyst (Fig. 1). All mesenchyme explants were trimmed to uniform size (approximately 2×1×0·5 mm). No enzyme preparations were used in the isolation or preparation of either the periotic mesenchyme or otocyst/mesenchyme explants. Care was taken to avoid any tissue associated with the branchial arches, surface ectoderm or pharyngeal endoderm.

Histology

All specimens for histology were grown to the chronological equivalent of 16 days of gestation (e.g. 12-day-old explants were cultured for 4 days). Explants were fixed in 10 % neutral buffered formalin at 4 °C for 12–24 h, and then dehydrated in a 1:1 (v/v) mixture of methanol and ethylene glycol monomethyl ether. Infiltration and embedding was done with a wax mixture of 9:1 (v/v) polyethylene glycol 400 distearate and cetyl alcohol. All histological specimens were serial sectioned at 7 μm. Staining with toluidine blue was carried out following Johnson's method for metachromasia (Luna, 1968). This method imparts a pink to scarlet metachromasia to tissue containing acidic mucopolysaccharides which include all of the sulphated glycosaminoglycan moieties (S-GAG). A total of 16–20 *in vitro* specimens were studied histologically for each of the selected gestation ages.

Glycosaminoglycan analysis

Organ-cultured specimens for biochemical analysis were continuously exposed to radio-isotope by introduction of 5 μCi Sulphur-35 (^{35}S) ml^{-1} of culture medium (medium volume in culture dish = 0·15 ml; unlabelled free sulphate present = 7·5 μg in 0·15 ml).

The number of specimens per tissue sample for S-GAG analysis varied with the gestational age of the explants. 11-day-old explant samples consisted of twelve pooled specimens, 12- and 13-day-old samples of eight specimens, and 14-day-old and older samples of six specimens. S-GAG was extracted using the method of Saito, Yamagata & Suzuki (1968) with modifications by Daniel, Kosher, Hamos & Lash (1974). The liquid scintillation cocktail contained 15 % Biosolv and 0·6 % Omnifluor in scintillation grade toluene. Counts were made in a Beckman Liquid Scintillation Counter using a ^{14}C window with an error set of <5 % and reported in cts min^{-1} per μg of DNA.

The efficiency and accuracy of the procedure were verified using the method of Segen & Gibson (1982): 0·2 mg of a fluorescent-labelled S-GAG, methylumbelliferyl chondroitin sulphate (MUCS), was added to the homogenate produced in the first step of S-GAG extraction. After the extraction procedure was completed, a sample of each of the extracts was subjected to acid hydrolysis. The resultant concentration of methylumbelliferone was determined spectrofluorometrically using a 364 nm exciting light and read at 448 nm emission. The efficiency of the system was better than 92 % with an accuracy within 10 %.

DNA analysis

To compensate for variations in sample tissue volumes and cell densities, DNA analysis (Abraham, Scaletta & Vaughan, 1972) was carried out on each specimen pool to provide a common basis for comparison of the biochemical results.
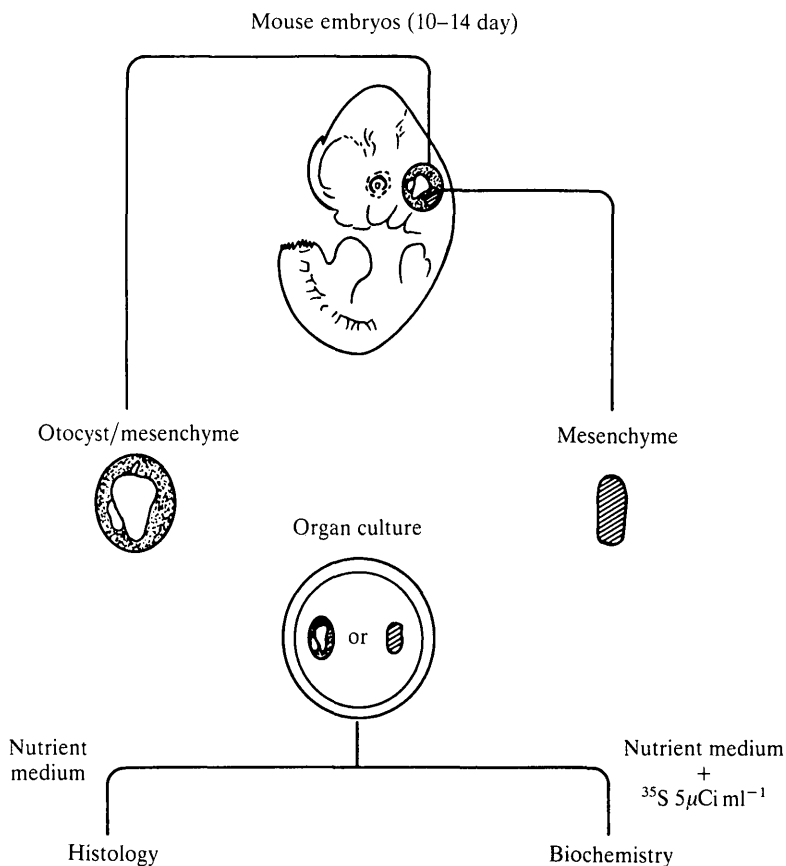
Mouse embryos (10–14 day)

Otocyst/mesenchyme   Mesenchyme

Organ culture

Nutrient medium

Nutrient medium
+
^{35}S 5μCi ml^{-1}

Histology   Biochemistry

Fig. 1. Schematic drawing of a mouse embryo (12 days), showing method of dissection for otocyst/mesenchyme (O/M) and isolated periotic mesenchyme (M) explants and experimental design.

Staging of capsule development

Based on the *in vivo* histology, development of the cartilaginous otic capsule was separated into five stages (McPhee & Van De Water, 1985). A condensed description of those five stages is given here and seen in Fig. 2.

Fig. 2. Histological cross sections of mouse embryo heads at the level of the inner ear anlage, representing stages I–V of otic capsule (*o*) formation: (A) 10-day-old mouse embryo (stage I) – prechondrogenic phase; (B) 11-day-old mouse embryo (stage II) – aggregation phase; (C) 12·5-day-old mouse embryo (stage III) – metachromatic phase; (D) 14-day-old mouse embryo (stage IV) – early chondrogenesis phase; (E) 16-day-old mouse embryo (stage V) – late chondrogenesis phase. Bar, 100 μm.
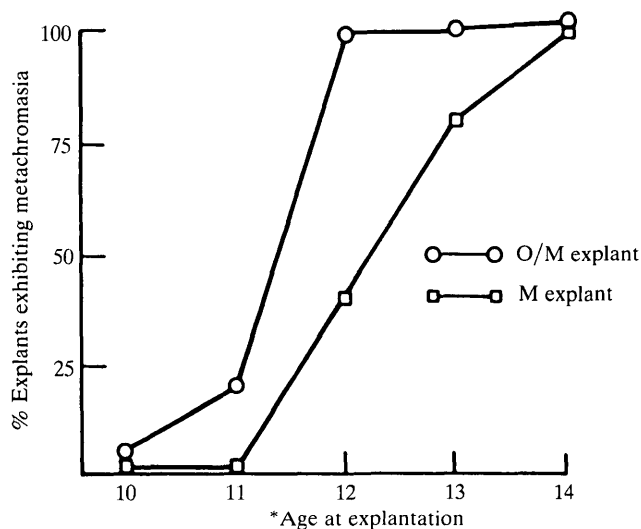
Fig. 3. Graphic presentation of the percentage of otocyst/mesenchyme (O/M) and isolated periotic mesenchyme (M) explants that exhibited tissue metachromasia at the end of the culture period. *All explants grown to the equivalent of 16 days of gestation.

Stage I. Prechondrogenic phase, days 9 to 10·5 (13–28 somites)

This is characterized by loose organization of the periotic mesenchyme; no apparent metachromasia or capsule differentiation (Fig. 2A).

Stage II. Aggregation phase, days 10·5 to 12 (29–42 somites)

Periotic mesenchyme has begun to aggregate; occasional areas of light pink metachromasia (Fig. 2B).

Stage III. Metachromatic phase, days 12 to 13 (43–51 somites)

Most otic structures are enveloped by condensed mesenchyme; distinct metachromasia (Fig. 2C).

Stage IV. Early chondrogenesis phase, days 13–14·5 (52–61 somites + morphological indices)

Capsular tissue is defined and intensely metachromatic (Fig. 2D).

Stage V. Late chondrogenesis phase, days 14·5–16·5 (morphological indices)

Mature cartilaginous capsule with a deeply metachromatic matrix. Chondrocytes in a well-defined lacuna are present throughout the capsule and a perichondrium surrounds most capsular cartilage (Fig. 2E).

RESULTS

Stage I. Capsule development in otocyst/mesenchyme and mesenchyme explants (9 to 10·5 days)

Stage I otocyst/mesenchyme (O/M) explants do not develop a cartilaginous otic capsule (Fig. 3; Table 1). The periotic mesenchyme remains diffuse, showing

no apparent areas of either condensation or metachromasia (Fig. 4). Labelled S-GAG production is limited to the first 24 h of culture. Incorporation of ^{35}S by these cultures is extremely variable, yielding no consistent values for the production of S-GAGs.

Periotic mesenchyme explants (M) from this embryo stage consistently fail to develop (Fig. 3; Table 1) and usually degenerated within 48 h of culture. As a result, no S-GAG analysis was possible for mesenchymal segments excised from embryos of a gestation age earlier than 11 days.

Stage II. Capsule development in otocyst/mesenchyme and mesenchyme explants (10·5 to 12 days)

Early prechondrogenic changes occur in stage II O/M explants excised from gestation day 11 embryos that develop for 5 days *in vitro*. 20 % of these explants produce light metachromasia in the extracellular matrix of their periotic mesenchyme (Figs 3, 5A; Table 1). Chondrogenesis is not completed in these specimens (Fig. 5A); cells only attain an immature stage of cartilage formation, with the areas of positive metachromasia lacking the histological characteristics of mature, hyaline cartilage. Labyrinthine morphogenesis, including the formation of a partially coiled cochlea, utricle and semicircular ducts, is observed in 20 % of the explants in which capsule development advanced to the early metachromasia stage. The overall appearance of the capsule approximates that associated with stage III of capsule formation *in vivo*, whereas the otocyst has developed to the level of stage IV of capsule formation. In the 80 % of the explants (Fig. 3; Table 1) where no tissue metachromasia occurs, the otocyst exhibits some growth and

Table 1. *Metachromasia in otocyst/mesenchyme (O/M) and mesenchyme (M) explants*

Type of explant	Age*	Stages†	No. of specimens	No. positive‡	% Positive
O/M	10–6	I/I	16	1	6
M	10–6	I/–	20	0	0
O/M	11–5	II/III	20	4	20
M	11–5	II/I	20	0	0
O/M	12–4	III/V	20	20	100
M	12–4	III/III	20	8	40
O/M	13–3	IV/V	20	20	100
M	13–3	IV/V	20	16	80
O/M	14–2	IV/V	20	20	100
M	14–2	IV/V	20	20	100

 * Gestational age at explantation – days *in vitro*.
 † Stages of otic capsule formation; at explantation/at end of culture period. The dash (–) equals degeneration of explant tissue.
 ‡ Specimens are defined as positive if they exhibit tissue condensation with metachromasia (early chondrogenesis).
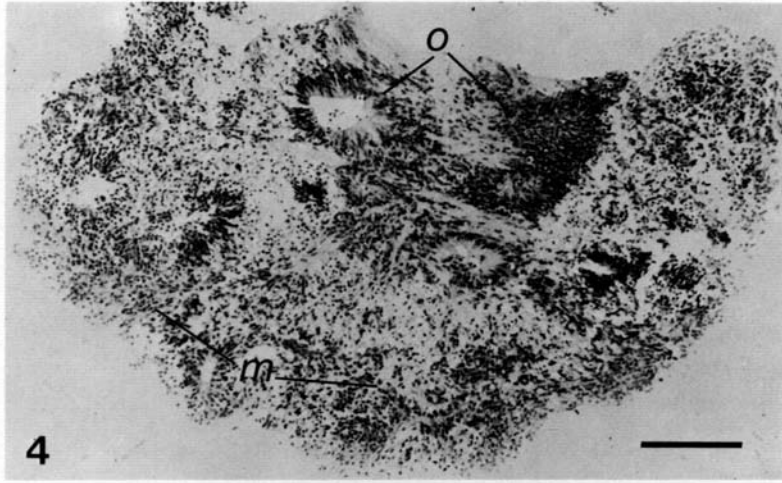
Fig. 4. 10-day-old otocyst/mesenchyme explant – 6 days *in vitro*. Neither the otic epithelium (*o*) nor the periotic mesenchyme (*m*) advanced in cytodifferentiation. Bar, 200 μm.

broadening, but no distinctive cochlear or vestibular morphology. Analysis of S-GAG labelling in pooled 11-day-old O/M explants demonstrates that moderate amounts of S-GAG have been synthesized by these tissues over the 5 day period (Table 2; Fig. 6), consistent with the histological observations. The total number of labelled counts for the *in vitro* growth period is $2135 \pm 175 \, \text{cts min}^{-1} \, \mu\text{g}^{-1}$ DNA. The average daily rate of S-GAG labelling increase/synthesis for the period in culture is, therefore, approximately $427 \, \text{cts min}^{-1} \, \mu\text{g}^{-1}$ DNA day^{-1}.

Isolated mesenchymal explants excised from gestation day 11 embryos and cultured for 5 days (Fig. 5B) show no metachromasia or cellular aggregates typical of prechondrogenic tissue (Fig. 3; Table 1). Measurement of the amount of ^{35}S incorporation into S-GAG during the *in vitro* growth of an 11-day-old mesenchyme specimen (Table 3) shows an initial rate of labelling comparable to that observed in the O/M explants of equivalent age during the first 48 h (Fig. 4). However, subsequent measurements taken at the 3rd to the 5th day *in vitro* exhibit a decline in the total counts from a high of $1199 \pm 249 \, \text{cts min}^{-1} \, \mu\text{g}^{-1}$ DNA on the second day to $638 \pm 91 \, \text{cts min}^{-1} \, \mu\text{g}^{-1}$ DNA after 5 days, compared to the continued rise of labelled S-GAG present in the 11-day-old O/M specimens (Fig. 6; Table 2). Since these M explants are continuously exposed to label, the decline in counts indicates little or no *de novo* labelling occurring after the initial 24 h in culture.

Stage III. Capsule development in otocyst/mesenchyme and mesenchyme explants (12 to 13 days)

12-day-old O/M explants follow the pattern of otic and capsular morphogenesis observed to occur *in vivo*. All of these specimens produced a morphologically complete inner ear after 4 days of culture. Some vestibular structures that developed *in vitro* are evident in a cross section of a 12-day-old explant (Fig. 7A).

A large segment of metachromatic, mature cartilage envelops the otic structures and is bounded by the partially formed perilymphatic spaces of the vestibule. No perichondrium is present at the interface between the cartilaginous otic capsule and the perilymphatic spaces of these cultures. The S-GAG production of these 12-day-old O/M specimens is presented in Table 2 and Fig. 8. The total amount of S-GAG labelled during the 4 days of organ culture is $4489 \pm 233 \, \text{cts} \, \text{min}^{-1} \mu\text{g}^{-1}$ DNA. A sharp increase in incorporation of ^{35}S into S-GAG occurs after the second day of *in vitro* growth, corresponding to stage IV of otic capsule development. The rate of labelling increase during this stage is approximately $1108 \, \text{cts} \, \text{min}^{-1} \mu\text{g}^{-1}$ DNA day^{-1}, almost seven times greater than the rate observed in 11-day-old O/M explants at this stage *in vitro* ($166 \, \text{cts} \, \text{min}^{-1} \mu\text{g}^{-1}$ DNA day^{-1}).
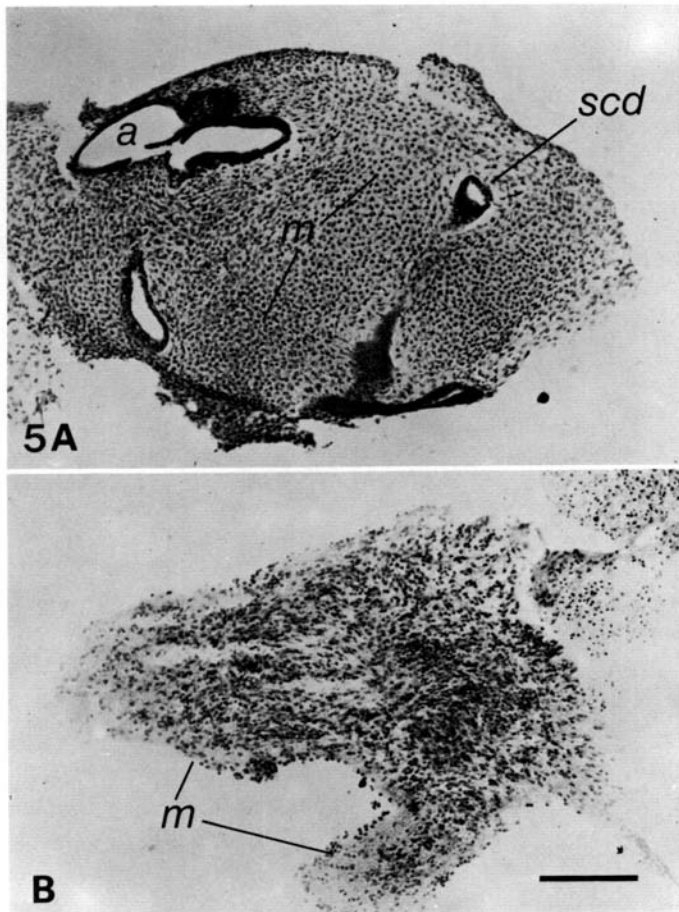

Fig. 5. 11-day-old explants – 5 days *in vitro*. (A) An otocyst/mesenchyme explant in which the otic epithelium has advanced in labyrinthine morphology, a semicircular duct (*scd*) and ampulla (*a*) are seen in this cross section. The periotic mesenchyme has condensed and produced light metachromasia – no cartilage is present. (B) A mesenchyme explant with no advances in cytodifferentiation. Bar, 200 μm.
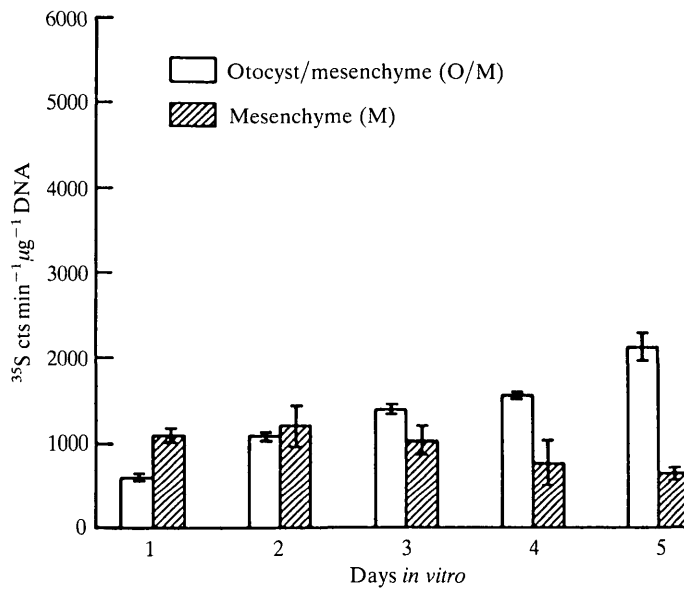
Fig. 6. 11-day-old otocyst/mesenchyme and mesenchyme explants – cumulative amounts of sulphated glycosaminoglycans produced in culture. Based on ^{35}S labelling data (mean values) of Tables 2 and 3.

Table 2. ^{35}S labelling* of sulphated glycosaminoglycans (S-GAG) in otocyst/mesen-chyme (O/M) explants

Age†	Sample I (cts min^{-1} μg^{-1} DNA)	Sample II (cts min^{-1} μg^{-1} DNA)	Sample III (cts min^{-1} μg^{-1} DNA)	Mean ± s.d. (cts min^{-1} μg^{-1} DNA)	Rate‡ (cts min^{-1} μg^{-1} DNA day^{-1})
11–1	584	667	597	616 ± 45	616
11–2	997	1062	1134	1087 ± 41	471
11–3	1485	1319	1458	1399 ± 72	312
11–4	1573	1554	1575	1565 ± 11	166
11–5	2318	2118	1970	2135 ± 175	570
12–1	952	886	905	914 ± 34	914
12–2	1297	1446	1316	1353 ± 81	439
12–3	2529	2378	2476	2461 ± 77	1108
12–4	4606	4221	4640	4489 ± 233	2028
13–1	904	799	837	847 ± 75	847
13–2	1751	1774	1595	1707 ± 97	860
13–3	3618	3030	3190	3279 ± 304	1572
14–1	1906	2031	1908	1948 ± 72	1948
14–2	4217	4267	4179	4221 ± 44	2273

* Continuous exposure to radioisotope.
† Gestational age at explantation – days *in vitro*.
‡ Extrapolated from the cumulative mean values.

Light metachromasia developed in 40 % of the mesenchymal explants from 12-day-old embryos after 4 days *in vitro* (Figs 3, 7B; Table 1). The differentiated tissue has more of a prechondrogenic tissue appearance than was observed in the periotic mesenchyme of 11-day-old O/M explants after 5 days *in vitro* (Fig. 5A). A few lacunae with chondrocytes were present in only the most advanced explants. None of the specimens of this group had a perichondrium present. The amount of labelled S-GAG extracted from the pooled, 12-day-old mesenchyme specimens during their 4 days in culture is illustrated in Table 3 and Fig. 8. The level of S-GAG does not appear to increase or decrease, with the specimens containing $767 \pm 102\,\mathrm{cts\,min^{-1}\,\mu g^{-1}}$ DNA after 24 h in culture and $789 \pm 130\,\mathrm{cts\,min^{-1}\,\mu g^{-1}}$ DNA after 4 days in culture. This is in marked contrast to the sharp increase of ^{35}S label incorporation into S-GAG (Fig. 8) that is observed in the cultured O/M explants taken from embryos of equivalent age.

Stage IV. Capsule development in otocyst/mesenchyme and mesenchyme explants (13 to 14·5 days)

13-day-old O/M explants during 3 days in culture develop in a pattern similar to 12-day-old explants (Fig. 9A). The capsule fully chondrifies and perilymphatic spaces are formed. The extent of the perilymphatic spaces formed in these explants appears to be greater than that observed at an equivalent stage (gestation

Table 3. ^{35}S *labelling* * *of sulphated glycosaminoglycans (S–GAG) in mesenchyme (M) explants*

Age†	Sample I (cts min^{-1} μg^{-1} DNA)	Sample II (cts min^{-1} μg^{-1} DNA)	Sample III (cts min^{-1} μg^{-1} DNA)	Mean ± s.d. (cts min^{-1} μg^{-1} DNA)	Rate‡ (cts min^{-1} μg^{-1} DNA day^{-1})
11–1	884	1180	1212	1092 ± 181	1092
11–2	1359	1076	862	1199 ± 249	107
11–3	1247	956	886	1030 ± 191	(169)
11–4	886	945	458	763 ± 266	(267)
11–5	714	663	538	638 ± 91	(125)
12–1	697	884	720	767 ± 102	767
12–2	967	750	1039	919 ± 150	152
12–3	979	814	992	928 ± 99	0
12–4	819	647	901	789 ± 130	(139)
13–1	789	821	541	717 ± 153	717
13–2	1445	1292	1184	1307 ± 131	590
13–3	4989	2529	3135	3551 ± 1282	2244
14–1	2850	2278	2323	2484 ± 318	2484
14–2	5783	7459	4962	6068 ± 1273	3584

Numbers in parentheses represent a net loss of label from day 1 *in vitro* – daily rate is at or near zero.

* Continuous exposure to radioisotope.

† Gestation age at excision – days in culture.

‡ Extrapolated from the cumulative mean values.

day 16) *in vivo*. There is almost no increase in the rate of labelled S-GAG synthesis (Fig. 10; Table 2) in these specimens during the second day *in vitro* (stage IV of capsule development *in vivo*). S-GAG synthesis during this 24 h period of the second day in culture is 860 cts min^{-1} μg^{-1} DNA day^{-1} (Table 2), lower than that observed in the 12-day-old otocyst/mesenchyme specimens for the same 24 h period (1108 cts min^{-1} μg^{-1} DNA day^{-1}), or for the level of S-GAG synthesized by the 14-day-old explants for this stage of capsule development (1948 cts min^{-1} μg^{-1} DNA day^{-1}). An almost twofold increase in S-GAG synthesis is evident during the third day of culture (i.e. 1572 cts min^{-1} μg^{-1} DNA).
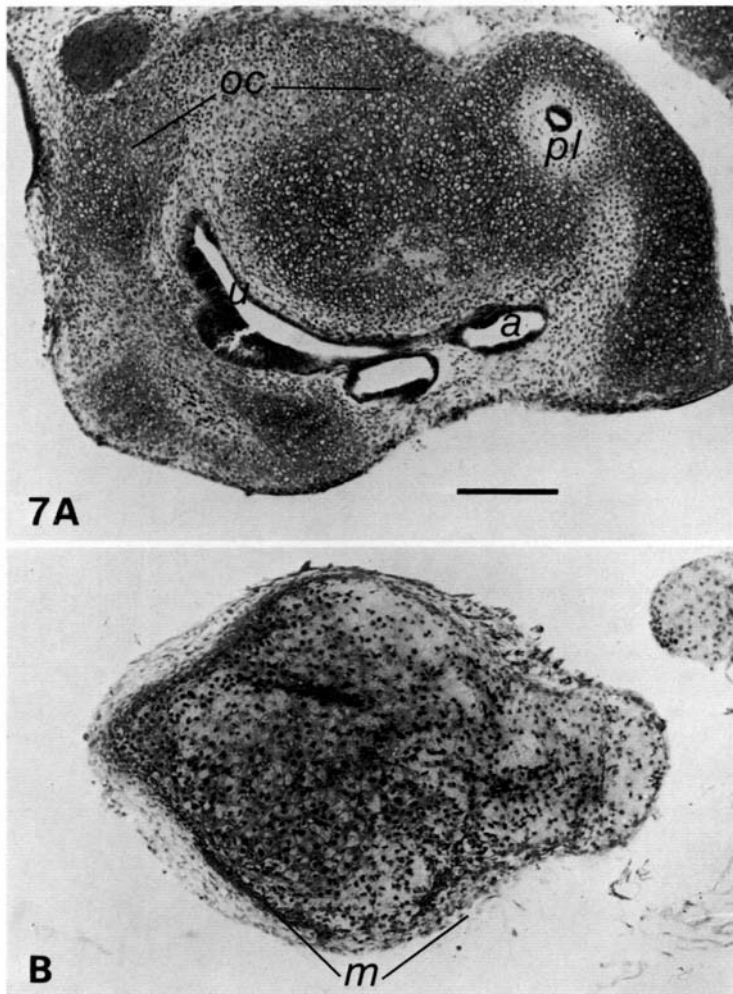
Fig. 7. 12-day-old explants – 4 days *in vitro*. (A) An otocyst/mesenchyme explant that formed a cartilaginous capsule (*oc*) with early stages of perilymphatic space (*pl*) formation. The sensory areas of the macula utriculus (*u*) and a semicircular duct ampulla are present in cross section. (B) A mesenchyme explant with matrix production and light metachromasia, but not cartilage formation. Bar, 200 μm.
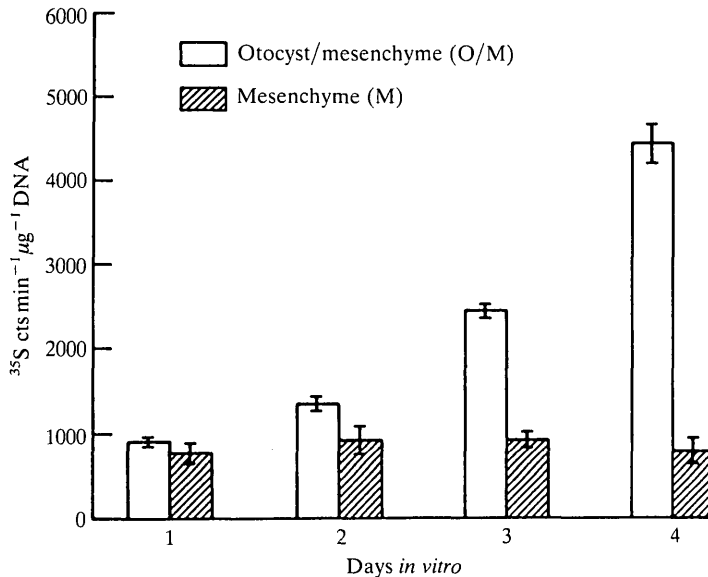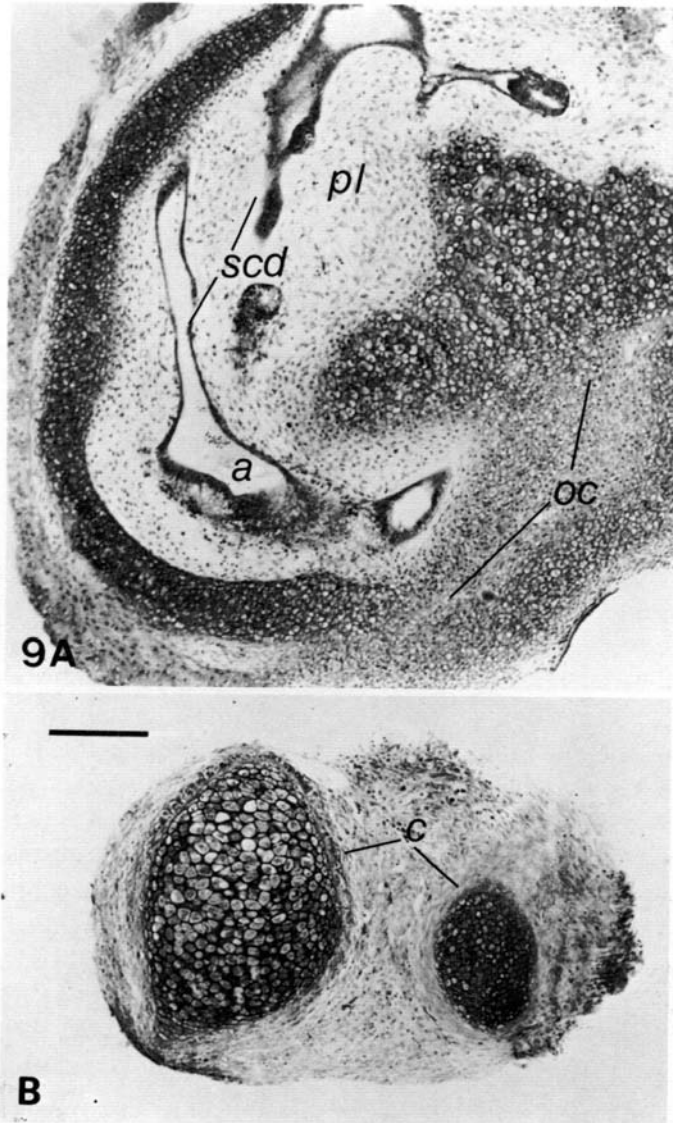
Fig. 8. 12-day-old otocyst/mesenchyme and mesenchyme explants – cumulative amounts of sulphated glycosaminoglycans produced in culture. Based on ^{35}S labelling data (mean values) of Tables 2 and 3.

Isolated mesenchyme excised at 13 days of gestation produces recognizable cartilage in 80 % of the specimens after 3 days *in vitro* (Fig. 3; Table 1). Cartilage-positive specimens contain foci of deeply stained, highly metachromatic tissue, with well-defined lacunae and perichondrium (Fig. 9B). These foci of cartilage (Fig. 9B) do not have the characteristic organotypic morphology seen in mature capsular cartilage surrounding an intact inner ear (Fig. 9A). The most common morphology of these foci is two or more globular clusters, usually formed near the centre of the explant. Biochemical analysis of these mesenchyme explants for labelled S-GAG yields (Table 3; Fig. 10) is in marked contrast to what is observed in the 11- and 12-day-old isolated mesenchyme explants (Figs 6B, 8B, respectively). Beginning on the second day of culture (equivalent to stage IV of capsule formation), the rate of ^{35}S incorporation into GAG increases considerably. This increase continues through the third day of culture with a total count for the entire period *in vitro* of 3551 ± 1282 cts min^{-1} μg^{-1} DNA, and an average daily rate of incorporation of 1184 cts min^{-1} μg^{-1} DNA day^{-1} (the large deviation observed in the final count is thought to be the result of variation in the number of positive specimens contained in each pool). Taking the variability of these results into account, the rate of S-GAG production for the isolated periotic mesenchyme exceeds that observed for the O/M explants of these 13-day-old explants (Fig. 10; Tables 2 and 3).

Otocyst/mesenchyme explants from gestation day 14 embryos already possess some capsular morphology. Explants after 2 days in culture have a capsular extracellular matrix that is deeply metachromatic with lacunae, a perichondrium and well-defined perilymphatic spaces (Fig. 11A). The perilymphatic spaces formed in

these explants are similar in configuration to those formed at the equivalent *in vivo* stage (gestation day 16) of capsule development. The rate of S-GAG labelling is high from the onset of *in vitro* exposure to ^{35}S (Fig. 12; Table 2), as would be expected from the results obtained from the 12- and 13-day-old O/M explants, which both show high levels of labelling (1108 and 860 cts min^{-1} μg^{-1} DNA day^{-1}, respectively) at the *in vitro* equivalent of stage IV of capsule formation.

Fig. 9. 13-day-old explants – 3 days *in vitro*. (A) An otocyst/mesenchyme explant that presents a well-formed cartilaginous capsule (*oc*) with extensive perilymphatic spaces (*pl*) surrounding a semicircular duct (*scd*) and its ampulla (*a*). (B) A mesenchyme explant with two globular islands of cartilage (*c*), but without organotypic morphology. Bar, 200 μm.
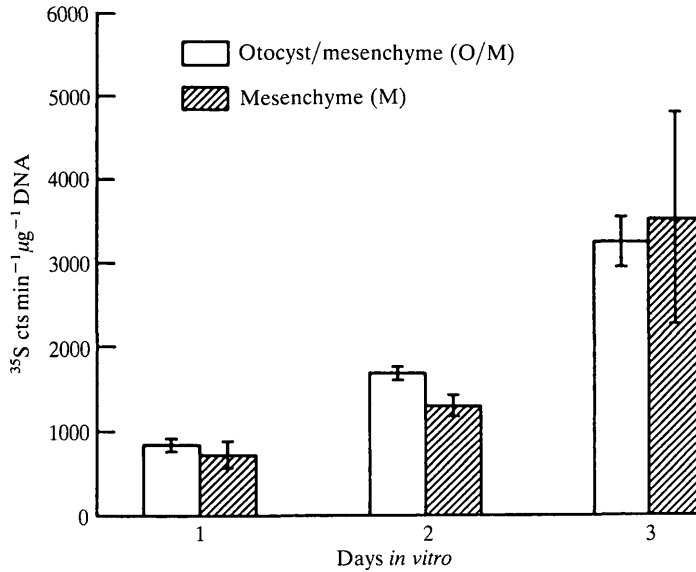
Fig. 10. 13-day-old otocyst/mesenchyme and mesenchyme explants – cumulative amounts of sulphated glycosaminoglycans produced in culture. Based on ^{35}S labelling data (mean values) of Tables 2 and 3.

14-day-old mesenchyme explants after 2 days of *in vitro* development all form mature cartilage (Fig. 3; Table 1). The intensity of metachromasia observed in these explants is identical to that observed in the 13-day-old mesenchyme explants after 3 days *in vitro*. The 14-day-old mesenchymal explants tend to show a greater variation in the form of their cartilaginous foci, varying from globular clusters, similar to those observed in 13 day cartilage-positive segments (Fig. 9B), to more commonly observed, narrow, arc-shaped bars (Fig. 11B). These cartilaginous bars reproduce, to some extent, the morphology of the cartilaginous structures appearing in the otic capsules of O/M explants (Figs 7A, 9A, 11A). Production of labelled S-GAG in these specimens is extremely high, starting from the very first day of culture (Fig. 12; Table 3). Total labelled S-GAG production is 6068 ± 1273 cts min^{-1} μg^{-1} DNA, with a daily rate of 3034 cts min^{-1} μg^{-1} DNA day^{-1}, the highest recorded for any of the periotic mesenchyme isolates analysed in this study. The level of S-GAG production in 14-day-old mesenchyme explants exceeds that observed in O/M explants from the same and earlier embryonic ages (Table 2; Fig. 12).

DISCUSSION

Excision of otic (O/M) explants at different stages of development results in dramatic alterations in the pattern of both inner ear and cartilaginous otic capsule formation. The otocyst with periotic mesenchyme, isolated from preaggregation and aggregation stages (stages I and II), develops abnormally, with metachromasia occurring in only a small percentage of these O/M specimens (Fig. 3). Otic

explants excised prior to gestation day 11 fail to undergo any of the sequence of otic capsule development (Fig. 4). The limited development seen in these early O/M explants is confined to moderate enlargement of existing epithelial structures, with otic capsule development apparently arrested at stage I, or, in those 6 % of the explants positive for metachromasia, perhaps as late as stage II. This
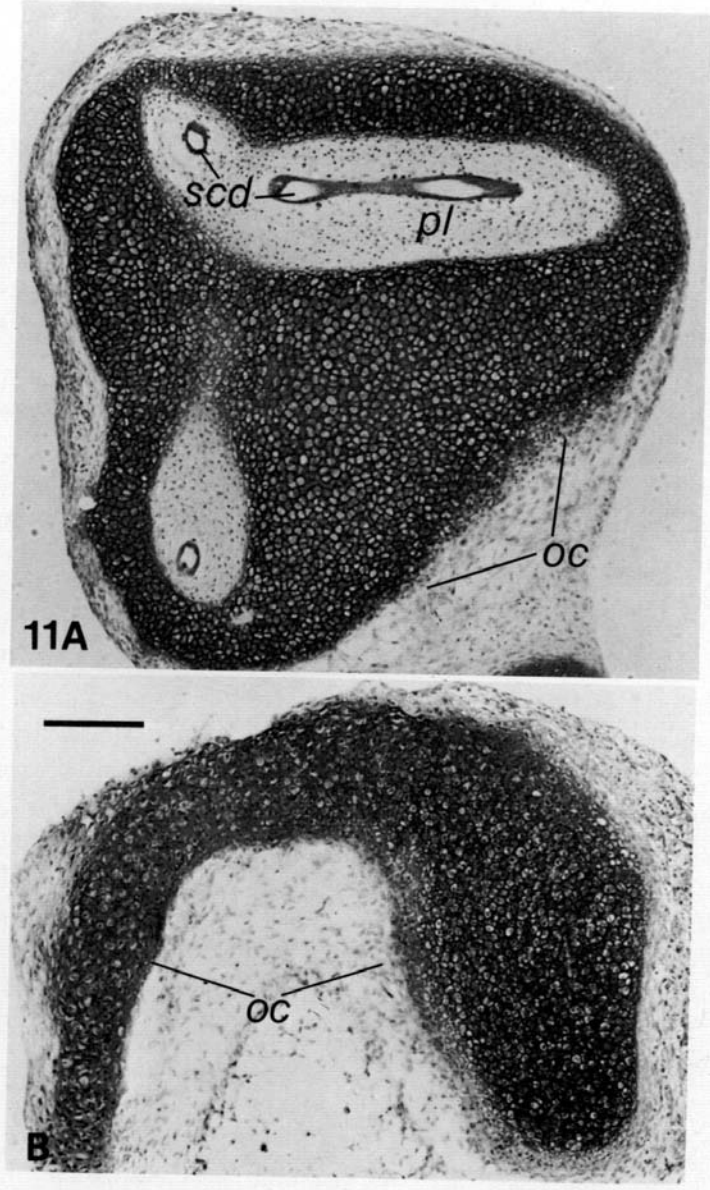

Fig. 11. 14-day-old explants – 2 days *in vitro*. (A) An otocyst/mesenchyme explant with two semicircular ducts (*scd*) encompassed by well-defined perilymphatic spaces (*pl*) and a cartilaginous otic capsule (*oc*). (B) A mesenchyme explant with an arc-shaped bar of cartilage that exhibits the organotypic morphology associated with the cartilaginous otic capsule (*oc*). Bar, 200 µm.
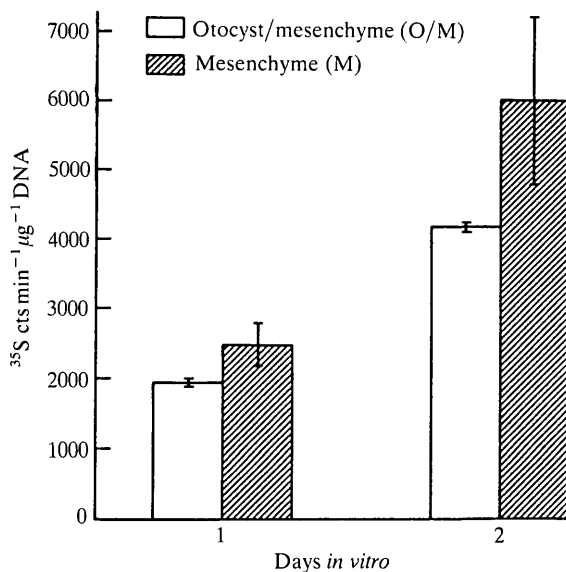
Fig. 12. 14-day-old otocyst/mesenchyme and mesenchyme explants – cumulative amounts of sulphated glycosaminoglycans produced in culture. Based on ^{35}S labelling data (mean values) of Tables 2 and 3.

stage of capsule formation corresponds to the stage of limb chondrogenesis described by Ahrens *et al.* (1979) and Owens & Solursh (1981), where limb bud cells explanted to micromass culture exhibited condensation, but not chondrogenesis. The influence of the otocyst at this stage is either lacking in some critical factor, or some other influence or condition is required in order for the otocyst to function as an inducer of capsule formation.

Tissue condensation and metachromasia in the mesenchyme of 11-day-old O/M explants is distinctly abnormal (Fig. 5A). The cartilage-free spaces that form the internal auditory meatus and the oval and round windows are not present. Synthesis of S-GAG occurs throughout the entire culture period of the O/M explants, but does not exhibit any sharp increase in synthesis beginning on the *in vitro* chronological equivalent of gestation day 14, as observed *in vivo* (McPhee & Van De Water, 1985). Recently, Nathanson (1983) noted in his study of rat skeletal muscle cultivated on bone matrix that S-GAG can be synthesized in these cultures for several days, even though no cartilage is produced. He suggested that the observed synthesis of S-GAG in the chondroitin 4-sulphate (C4S) form '... represents little more than the sustained synthesis of initially evoked C4S.' Nathanson's observation (1983) and ours demonstrate that production of an S-GAG can occur in embryonic tissue without completion of chondrogenesis.

Periotic mesenchyme, isolated from 11-day-old embryos, fails to advance in development and regresses to stage I of capsule development. Examination of this tissue after 5 days in culture shows a fibroblastic cell population with no organization or metachromasia. Isolated periotic mesenchyme explants from this stage of development do not possess the capability of differentiating into cartilage.
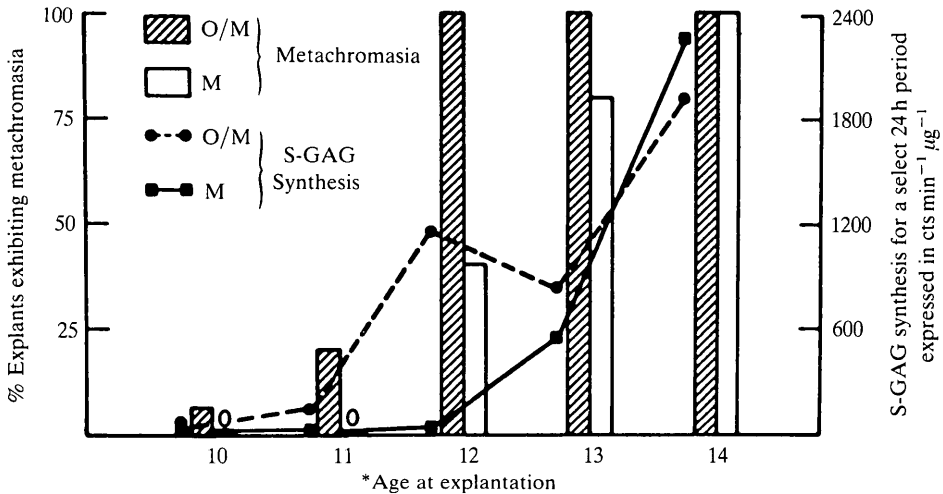
Fig. 13. A summary of *in vitro* results relating age of otocyst/mesenchyme (O/M) and isolated mesenchyme (M) explants to production of tissue metachromasia at end of culture period and rate of sulphated glycosaminoglycan production during the *in vitro* equivalent of gestation day 14–15 in culture (based on data taken from Tables 1–3). *All explants grown to the equivalent of 16 days of gestation.

Periotic mesenchyme in the parallel otocyst/mesenchyme cultures (Fig. 5A) does undergo, in some instances, cellular aggregation and produces a metachromatic matrix. The otocyst must, therefore, be exerting an inductive influence on the periotic mesenchyme to which the mesenchyme only partially responds. Hall (1983) indicated that inductive influences are rarely unilateral and that in epithelial–mesenchymal interactions the tissues 'must be of a specified ancestry, and the interaction is reciprocal.' It would appear in this case that the otocyst at embryonic day 11 can provide the necessary inductive 'signal' to the periotic mesenchyme, but that the mesenchyme is not yet fully competent to act upon it and initiate chondrogenesis. This observation is supported by the report of Grobstein & Holtzer (1955) that the 11-day-old murine otocyst could induce chondrogenesis in cultures of competent somitic mesoderm.

Otocyst/mesenchyme explants from 12-day-old embryos follow the stages and chronology of membranous labyrinth and otic capsule formation observed *in vivo*. A comparison of the gross morphology of the inner ear structures grown *in vivo* and *in vitro* shows no significant differences (Van De Water & Ruben, 1974) during the period under study. The first appearance of mature cartilage in the 12-day-old explants occurs approximately 72 h after explantation (the chronological equivalent of the 15th day of gestation *in vivo*), as evidenced by the sharp increase in the rate of S-GAG synthesis (Fig. 13; Table 2). This is identical to the pattern of chondrification observed *in vivo* (McPhee & Van De Water, 1985). Quite clearly, a dramatic change has taken place in the otocyst and its periotic mesenchyme between gestation days 11 and 12 (stages II and III, respectively). This change in the competence of mesenchyme between stages II and III could be the results of a change in the composition of its cellular population. Noden (1978) proposed that

avian neural crest cells are responsible for the bulk of skeletal formation in the head region and that '. . . the interactions leading to the expression of the cartilage phenotype are subject to environmental influences of the area into which they migrate.' This is further corroborated by the studies of Bee & Thorogood (1980) who also report on the ability of neural crest cells to form cartilage under the influence of cranial ectoderm. Noden (1984) has further shown in the chick embryo that the mesenchyme that forms the otic capsule is a mixture of cephalic and perichordal mesoderm. It could be that the same cell populations ultimately form the otic capsule in the mouse and that the perichordal mesoderm migrates and mixes with the cephalic mesodermal component of the periotic mesenchyme between stages II and III. This mixing of mesodermal cell populations could, therefore, be postulated as a mechanism for the observed change in periotic mesenchyme competence which occurs between stages II and III of otic capsule development. If the migration of these cells into the periotic mesenchyme occurs prior to stage II of capsule formation, as appears to be the case in avian embryos, then the arrival of these cells might be the necessary prerequisite for the induction of capsule chondrogenesis by the otocyst.

Isolated periotic mesenchyme explanted from 12-day-old embryos has limited ability to differentiate (Fig. 7B). Cellular aggregation and pale metachromasia occur, but cartilage formation is not complete and the synthesis rate of S-GAG remains low. This mesenchyme has an appearance similar to the mesenchyme of 11-day-old otocyst/mesenchyme explants at the end of their culture period (Fig. 3A). An additional 24 h of interaction with the otocyst in the embryo produces a tissue that is now capable of undergoing the first stages of chondrogenesis by itself, but not yet capable of producing mature cartilage.

Explants of otocysts with adhering periotic mesenchyme, excised from 13-day-old mouse embryos, readily differentiate cartilaginous otic capsules with fully formed inner ears. However, the rate of S-GAG production for these 13-day-old O/M explants is consistently lower than the rates observed in either the 12- or 14-day-old O/M specimens (see Fig. 13; Table 2). A possible explanation for this observation of a decrease in the rate of S-GAG synthesis is that the tissue of these 13-day-old explants is particularly sensitive to explantation at this stage of capsule chondrogenesis (stage IV). Explantation trauma would, therefore, reduce the rate of S-GAG synthesis. If correct, this begs the question, what makes this particular stage so sensitive to manipulation? It is unlikely that it is due to the physical disruption of the explant, since the more fragile 12-day-old explants (stage III) should be far more susceptible to this type of disturbance, yet S-GAG synthesis in these explants equals, or in some cases exceeds, the daily rates of S-GAG synthesis recorded for the 13-day-old O/M explants (Table 2). A more plausible explanation is that the otocyst and, or, the periotic mesenchyme is at a critical development stage that is partly dependent on the maintenance of tissue integrity and stability of the extracellular microenvironment. The appearance of globular clusters of cartilage formed from 13-day-old mesenchyme explants demonstrates that determination of the periotic mesenchyme to form cartilage has been

completed by this stage of development. However, these clusters of cartilage do not resemble the morphology of the chondrifying capsule observed in either *in vivo* (McPhee & Van De Water, 1985) or *in vitro* O/M explants (Figs 7A, 9A, 11A). Apparently, instruction of the mesenchyme by the otocyst during stage III establishes the sequence of events leading to the chondrogenesis of the periotic tissue, but not capsular morphology.

Epithelium has been shown not only to induce mesenchyme to initiate chondrogenesis (Gumpel-Pinot, 1980; Smith & Thorogood, 1983), but also to play a direct, but negative, role in chondrogenesis by inhibiting this process (Solursh, Singley & Reiter, 1981*b*). If the otocyst/mesenchyme tissue interaction that influences the formation of the perilymphatic spaces begins on day 13, then it is possible that this interaction may be perturbed by explantation at that time, resulting in either the lack of, or greater than usual, development of the perilymphatic spaces. The 13-day-old O/M explants (Fig. 9A) in our study do appear to have larger than normal perilymphatic spaces when compared to either the 12- or 14-day-old O/M explants (compare Figs 7A, 9A, 11A). This observation is consistent with the observed, lower rate of S-GAG synthesis for 13 day explants, compared to that observed for either the 12- or 14-day-old O/M explants (Table 3; Fig. 13). This finding of lower S-GAG synthesis would be consistent with the suggestion by Solursh *et al.* (1981*b*) that hyaluronate (a non-sulphated GAG) accumulation in the mesenchyme might mediate the inhibitory effect of epithelia on chondrogenesis. Increased levels of hyaluronate production would not be detected by our analysis of S-GAG production and additional mesenchyme involved in hyaluronate synthesis would decrease the rate of S-GAG production for the otocyst/mesenchyme explants, whereas this would not be evident in the isolated mesenchyme explants.

Stage IV otocysts (i.e. gestation days 13 and 14) already possess a fairly well-defined capsular region showing marked aggregation and light metachromasia. In this stage the major morphological changes include the development of a perichondrium and formation of the perilymphatic spaces. We postulate that there is a close interaction between the otocyst and its surrounding periotic mesenchyme at this stage in order to establish the morphology of the otic capsule. This is partly corroborated by the study by Van De Water (1981), where periotic mesenchyme volume was varied at different stages of development, resulting in marked changes in labyrinthine geometry. There is extensive evidence for epithelial–mesenchymal interaction that results in specific morphological changes in other cartilage-forming tissues (Lash, 1963; Kosher, Savage & Chan, 1981; Bradamante & Hall, 1980; Gumpel-Pinot, 1980; Vasan & Lash, 1979; Bee & Thorogood, 1980; Smith & Thorogood, 1983). Periotic mesenchyme explanted during late stage IV (i.e. 14-day-old) produces cartilage with characteristic organotypic morphology (Fig. 11B). These lenticular-shaped regions of cartilage possess a well-defined perichondrium. This is in marked contrast to the globular clusters of cartilage found in early stage IV mesenchymal explants (Fig. 9B). This apparent lack of structural morphology in cartilage derived from mesenchymal isolates points to a

dual role for the otocyst in capsule development. Acting first as an inducer of chondrogenesis during stages II and III, the otocyst alters its role in stage IV to a modeller of the presumptive capsule. This latter role requires that the otocyst be capable of selectively inhibiting in its immediate vicinity, while allowing cartilage formation to progress normally at a little further distance. A possible mechanism for this inhibitory effect might be found in the results of studies on the apical ectodermal ridge of limb buds. Solursh *et al.* (1981*b*) have reported that apical ectodermal ridge as well as other nonridge epithelia were capable of inhibiting chondrogenesis selectively in peripheral limb mesenchyme. It was further pointed out that this could occur without direct cell contact, at a distance of up to $25 \mu m$. The accumulation of hyaluronate or some other component of the extracellular matrix in the limb periphery mediates the inhibitory effect of epithelia on chondrogenesis. Production of such a diffusible inhibitor by the neuroectoderm of the otocyst would permit the formation of the perilymphatic spaces, while allowing chondrogenesis to proceed normally beyond the range of its effect. The existence of such an inhibitor secreted by neuroectoderm appears to be further evidenced by the lack of formation of an internal auditory meatus in the capsule of inner ears grown *in vitro*. This structure is apparently formed by the inhibition of capsule chondrogenesis by the $VIII_n$ root.

S-GAG production by both the explanted otocyst and the isolated mesenchyme is highest during late stage IV and stage V. This is identical to our *in vivo* observations (McPhee & Van De Water, 1985). The sharp increase in S-GAG synthesis starting at day 14 and continuing into day 15 occurred in all of the *in vitro* chondrifying otic explants (Fig. 13). A direct correlation between S-GAG synthesis and chondrogenesis in limb and somites is an established fact (Kosher & Lash, 1975; Kosher & Searles, 1973; Vasan & Lash, 1979; Minor, 1973). Even though all contact with the embryo has been severed as much as 72 h prior to the increase in S-GAG synthesis corresponding to chondrification during stages IV/V, the periotic mesenchyme possesses the inherent ability to carry out a complex series of biochemical steps leading to chondrogenesis, following a set chronology. This ability indicates that regulation of the sequence of events leading to both inner ear and otic capsule formation resides within the otocyst and its surrounding mesenchyme after gestation day 12 and does not occur as a result of extraneous factors. The only exception to this observation is the formation of the internal auditory meatus, which may require the presence of axons of the statoacoustic ganglion in order to form properly.

In summary, the results of our O/M and M explants (Fig. 13) indicate that during otic development the otocyst exerts several different influences on otic capsule formation. In stage I (Fig. 4) of otic capsule development, O/M explants are not yet capable of initiating any advances in capsule formation and interaction with additional factors (e.g. rhombencephalon and notochord) is still necessary for advances in otic capsule development. During stage II (Figs 5, 6) of capsule formation, the otocyst initiates prechondrogenic changes in the periotic mesenchyme of cellular aggregation and S-GAG synthesis, while isolated mesenchyme

cannot yet initiate any changes without the presence of the otocyst. Stage III of otic capsule development is the period during which the otocyst induces its mesenchyme to initiate the complete sequence of chondrogenesis. By the end of stage III, the ability of the periotic mesenchyme to form cartilage (Fig. 9B), but not capsular morphology, is determined. The results of S-GAG production and perilymphatic space formation in 13-day-old O/M explants (Figs 9A, 13) suggest that the action of the otocyst on periotic mesenchyme for the formation of perilymphatic space and the modelling of the capsule cartilage begin at this stage. In stage IV of capsule development, the otocyst exerts its final function in controlling the morphology of the cartilaginous otic capsule (Fig. 11B).

Further definition of the induction capability and tissue competence of the otocyst and its periotic mesenchyme is needed. Elucidation of the biochemistry of capsule formation is also necessary before the mechanism of capsule induction by its otocyst can be fully understood. The pattern and rate of synthesis of the other major constituents of cartilage, hyaluronic acid and the collagens will be of particular interest in future studies.

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