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Original Article

Chondrogenesis of mandibular mesenchyme from the embryonic chick is inhibited by mandibular epithelium and by epidermal growth factor

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ABSTRACT . This study documents the role of mandibular epithelium and epidermal growth factor (EGF) in the initiation, maturation and maintenance of Meckel's cartilage using percent ^{3}H-thymidine-labelled cells as an index of proliferative activity and distribution of labelled cells, chondrocyte size and relative amount of extracellular matrix as indices of chondrogenesis. Mandibular mesenchyme from embryos of H.H. stages 18, 22, 25 was cultured for 2 to 10 days (a) unseparated from mandibular epithelium, (b) in isolation, or (c) after recombination with mandibular epithelium in the presence or absence of 5-40ng/ml EGF. Epithelium delayed both initiation of chondrogenesis and maturation of already formed cartilage. The ^{3}H-thymidine-labelling index was reduced in cartilage that differentiated in the presence of mandibular epithelium. Epithelium influenced the timing of mesenchymal differentiation (a) by delaying cytodifferentiation through prolonging high levels of proliferation, and (b) by directly affecting differentiation itself. EGF, especially at 10-20ng/ml, affected both proliferation of mesenchyme and chondrogenesis in mesenchyme cultured with or without epithelium. All observed effects of epithelium on intact tissues could be duplicated by exposing isolated mesenchyme to EGF at 10ng/ml, i.e. a role for EGF in chondrogenesis is suggested.

KEY WORDS: *chondrogenesis, epidermal growth factor, epithelium, Meckel's cartilage, mitotic activity, epithelial-mesenchymal interactions*

Introduction

Considerable information is now available concerning epigenetic (primarily epithelial-mesenchymal) interactions in the differentiation of cartilage and bone (Hall, 1983; 1984; 1988). In particular, much is now known about chondrogenesis and osteogenesis of the neural crest-derived mesenchyme that forms Meckel's cartilage and the membrane bones of the mandibular arches in the embryonic chick.

Mandibular mesenchyme arises from mesencephalic neural crest at Hamburger and Hamilton (1951) (H.H.) stage 7 (26 hours of incubation). By H.H.15-16 the mandibular arches have their full complement of neural crest-derived mesenchyme. By H.H.18, mandibular mesenchyme is chondrogenic in the absence of any mandibular epithelial influence (Tyler and Hall, 1977), having undergone an earlier epithelial interaction (Hall and Tremaine, 1979; Bee and Thorogood, 1980). Chondrogenesis, as defined by the first appearance of chondroblasts and of extracellular matrix, begins at H.H. 26 (5 days of incubation), 3 1/2 days after the epithelial-mesenchymal interaction. Initiation of chondrogenesis is characterized by formation of a cellular condensation, decrease in mitotic activity, synthesis of type II collagen and of proteoglycans and deposition of these products into pericellular and extracellular cartilaginous matrices.

Studies primarily undertaken with cultures of dissociated chondrogenic limb mesenchyme have consistently shown that there is an obligatory requirement for formation of high density cell condensations before cytodifferentiation can begin (Umansky,1966; Matsutani and Kuroda,1980; Cottrill *et al.*,1987; Hall,1987). Cell to cell interactions required for initiation of chondrogenesis occur within such mesenchymal condensation and may be mediated by cAMP, hyaluronate, growth factors and/or factors that suppress mitotic activity (Toole *et al.*, 1972; Ahrens *et al.*, 1977; Solursh and Reiter,1980; Solursh *et al.*, 1982; Archer and Rooney, 1984; Shen *et al.*, 1985). A decline in mitotic activity within pre-chondrogenic limb mesenchyme at H.H. stage 22 corresponds to the initial synthesis of specific extracellular matrix products, with cytological and histological differentiation of cartilage occurring approximately ten hours later (Janners and Searls, 1970; Kosher *et al.*, 1986a, b). The same culture conditions that enhance chondrogenesis in prechondrogenic mesenchyme, inhibit chondrogenesis of differentiating chondroblasts (Elmer, 1983) emphasizing that the phases of chondrogenesis are subject to differing environmental controls.

The dependence upon an interaction with one or more epithelia has now been documented for cranial, facial and limb cartilages (Newsome,1972; 1976; Hall and Tremaine, 1979; Bee and Thorogood, 1980; Gumpel-Pinot, 1980; Hall, 1983; Smith and Thorogood, 1983). In part, these epithelial

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influences can be explained by a mitogenic effect on mesenchyme; epithelia promote growth and survival of prechondrogenic mesenchyme, ensuring that sufficient mesenchyme exists for the formation of condensations in which chondrogenesis can be initiated (Solursh, 1983). Control of proliferation of mandibular mesenchyme by epithelium and by epidermal growth factor has recently been documented (Hall and Coffin-Collins, submitted for publication, 1989).

Epithelia also affect the process of differentiation itself, for epithelia inhibit chondrogenesis in adjacent mesenchyme (McLoughlin, 1961; Gumpel-Pinot, 1980; Tyler and McCobb, 1981; Tyler, 1983; Solursh,1983; 1987). Epithelial inhibition may operate through epithelia maintaining a high rate of mitotic activity in adjacent mesenchyme, thereby preventing it from differentiating, or by a more direct inhibition of chondrogenesis through production of a differentiation inhibitor(s). The best documented epithelial inhibition of chondrogenesis is during development of the limb buds of the embryonic chick. This epithelium must be viable to inhibit chondrogenesis, an inhibition that is mediated by the production of an epithelial diffusible inhibitor (Solursh *et al.*, 1981; Solursh, 1984; Solursh *et al.*, 1984; Zanetti and Solursh, 1986). Thus, epithelia can, by exerting enhancing or inhibitory influences, determine whether or not, and where and when, chondrogenesis will be initiated during development (Hall, 1987).

In the present study we have investigated whether mandibular epithelium exerts any inhibitory influence(s) on chondrogenesis of Meckel's cartilage maintained *in vitro*. Specifically, (1) whether the proliferation of chondrogenic mandibular mesenchyme maintained *in vitro* is affected by coculture with mandibular epithelium, and (2) whether the epithelium affected cartilage differentiation. Proliferation was assessed by ^{3}H-thymidine-labelling and counting of labelled cells in autoradiographs, while chondrogenesis was assayed by quantifying the state of cytodifferentiation attained in vitro. Mandibular tissues from embryos of three different morphological stages were used; H.H.18, which is before initiation of chondrogenesis; H.H.22, when condensation of prechondrogenic mesenchyme has begun, and H.H.25 when overt differentiation has commenced. Furthermore, we utilized homochronic (same-age) and heterochronic (different-age) tissue recombinations between mandibular epithelia and mesenchyme to determine whether epithelial influences could be explained by the relative stage of chondrogenesis of the mesenchyme, and/or by the age of the epithelium.

Possible effects of epidermal growth factor (EGF) on chondrogenesis were also investigated. Epidermal growth factor is so named because it exerts a profound influence over the mitotic activity of epithelia. However, EGF also exerts a mitogenic influence on embryonic mesenchyme such as palatal mesenchyme from human and rodent embryos (Pratt, 1987 for review and references), dental mesenchyme from embryonic mice (Partanen and Thesleff, 1987; Topham *et al.*,1987) and dermal stroma from sheep (Panaretto *et al.*,1984). EGF also enhances mitotic activity and delays differentiation in chondrocytes (Gospodarowicz and Mescher, 1977; Kato *et al.*,1983; Mercola and Stiles,1988). Since the actions of EGF on chondrogenesis resemble those of epithelia we wondered whether actions of mandibular epithelium and EGF were related. Specifically, we investigated whether EGF exerted any influence on chondrogenesis in mandibular mesenchyme maintained *in vitro* in the presence or absence of mandibular epithelium. As in the determination of the action of the epithelium, we assessed the effects of EGF on mitotic activity and chondrogenesis. The doses of EGF used (5 to 40 ng/ml) were based on past studies utilizing embryonic mesenchyme, Pratt and his colleagues and Thesleff and her colleagues having found 10-20ng/ml to be optimal in their studies on palatal and dental mesenchyme (Hassell and Pratt, 1977; Tyler and Pratt, 1980; Pratt *et al.*, 1980; Thesleff *et al.*,1984; Turley *et al.*, 1985; Partanen *et al.*, 1985). These concentrations are within the physiological concentration of EGF found in fetal and adult mammalian tissues, serum, milk and urine (Carpenter,1978; Nexo *et al.*,1980; Adamson and Rees, 1981).

Results

Cartilage differentiation from mesenchyme cultured with and without epithelium, and/or EGF

DNA labeling indices

DNA labeling indices (DLI) of the cartilage were not affected by the epithelium, irrespective of the age of the embryo providing the mesenchyme (Figs. 1, 2; Table 1). EGF did influence mitotic activity of the differentiating cartilage, but only at the higher doses and in a manner that depended on the stage of the embryos from which the mesenchyme had been isolated. Thus, presence of 40ng/ml EGF at resulted in a four-fold enhancement over control levels (17 vs 4%) in cartilage differentiating in isolated mesenchyme from H.H. stage 18, but had no statistically detectable effect on labelling in cartilage differentiating in the presence of epithelium (Table 1). The same dose of EGF significantly depressed percent labelling in cartilage differentiating in mesenchyme from H.H. stage 22 embryos whether epithelium was presence or not (Table 1). Proliferation in cartilage differentiating from H.H. 25 mesenchyme was significantly increased in the presence of 20 ng/ml by EGF.

Distribution of dividing cells

Mitotically active cells are progressively limited to the edges of the condensation. Cultures were categorized as mature (mitotically active cells localized in the most peripheral cartilage, 4 being the most mature) or immature (labeled cells found throughout the cartilage, 1 being immature; Table 2).

The cartilages that differentiated in isolated mesenchyme from H.H. 18 embryos cultured for 10 days in the

TABLE 1

LABELLING INDICES (X ± SEM (N)) OF CARTLIAGE THAT DIFFERENTIATED *IN VITRO* IN MANDIBULAR MESENCHYME FROM H.H. STAGE 18, 22, OR 25 EMBRYOS IN THE PRESENCE OR ABSENCE OF EPITHELIUM AND/OR EGF FOR 10 DAYS

EGF	H.H. stage 18		H. H. stage 22		H.H.stage 25	
ng/ml	- epithelium	+ epithelium	- epithelium	+ epithelium	- epithelium	+ epithelium
0	4.3 ± 0.9(12)	6.1 ± 1.5(13)	7.7 ± 0.8(18)	9.8 ± 1.6(17)	7.5 ± 0.9(13)	11.9 ± 1.2(7)
5	9.5 ± 3.1(5)	7.5 ± 4.7(5)	2.8 ± 1.2(7)	4.8 ± 1.6(10)	-	-
10	6.9 ± 1.9(10)	7.3 ± 1.3(9)	7.8 ± 1.4(12)	7.9 ± 1.7(8)	10.9 ± 1.2(14)	13.0 ± 0.7(7)
20	7.2 ± 2.4(5)	4.6 ± 2.6(7)	5.2 ± 1.3(8)	12.7 ± 1.2(12)	11.1 ± 1.6(6)[c]	10.8 ± 1.0(7)
40	16.9 ± 1.0(3)[a]	7.4 ± 1.4(3)	3.5 ± 0.8(7)[b]	4.4 ± 1.9(9)[b]	-	-

a. Significantly different from O ng EGF, $P < 0.0005$
b. Significantly different from O ng EGF, $P < 0.005$
c. Significantly different from O ng EGF, $P < 0.025$

absence of EGF were mature with a score of 3.93 (Table 2; Fig. 1) . The index for cartilage that developed in H.H.18 mesenchyme cultured with epithelium was not significantly lower (3.54), i.e. presence of epithelium did not retard this index of chondrogenic differentiation. At each concentration of EGF, this index of cartilage differentiation was lower in mesenchyme cultured with epithelium than in mesenchyme cultured alone (Table 2). This pattern was also seen in the cartilage that differentiated in mesenchyme from embryos of H.H. 22 and 25, where the differences were statistically significant (Table 2).

The greatest effects of EGF and epithelium on retardation of this index of cartilage differentiation were seen in cartilage differentiating from H.H. 25 mesenchyme where EGF at 20ng/ml or presence of epithelium resulted in cartilage with indices of 1.0, the most immature pattern of distribution of labelled cells seen (Table 2). In fact, no H.H. 25 mesenchyme cultured with epithelium progressed beyond a score of 1.0 (Table 2). Therefore, epithelium and EGF retarded the maturation of chondroblasts from the proliferating to the differentiating state; EGF primarily exerted its retarding effect in the presence of epithelium, and primarily affected mesenchyme from embryos of H.H. 22 and 25.

Chondrocyte size

Relative size of the chondrocytes was also assessed and cartilages categorized as mature or immature on a four-point index (Table 2). As with cartilage maturation based on localization of dividing cells, it can be seen that cell size was lower (a) with increasing stage of the embryos providing the mesenchyme, (b) in the presence of epithelium and (c) when cultured with EGF (Table 2; Figs. 3-7). EGF at 20 or 40 ng/ml substantially retarded attainment of mature cell size from isolated H.H. 18 mesenchyme. (Figs. 3-7) Lower concentrations produced the same inhibition in H. H. 22 and 25 mesenchyme. Thus, mandibular epithelium and EGF both retarded chondrocyte maturation.

Amount of extracellular matrix

A determination of relative amount of extracellular matrix deposited by chondrocytes that differentiated in mesenchyme from embryos of the three H.H. stages was made (Table 2, Figs 3-7). Stage of embryo, presence of epithelium and concentration of EGF, all retarded accumulation of extracellular matrix. Mesenchyme from H.H. 22 embryos was most affected. The most effective concentration of EGF was 20ng/ml (Figs 3, 6; Table 2).

Effect of age of epithelium on cartilage differentiation from mesenchyme in the presence or absence of EGF.

Mesenchyme was cultured with its epithelium (intact), in isolation, or after recombination with mandibular epithelium from embryos of one of the three stages to determine whether different-aged epithelia influenced chondrogenesis to a greater or lesser extent than same-age epithelium, both in the absence and in the presence of EGF at 10 ng/ml.

DNA labeling indices

Neither age of the epithelium, nor EGF, had any significant effect on the DNA labeling indices of the cartilages

TABLE 2

MEAN VALUES (X± SEM) FOR THE STATE OF DIFFERENTIATION ATTAINED BY CARTILAGE BASED ON (a) DISTRIBUTION OF ^{3}H-THYMIDINE-LABELED CELLS, (b) CELL SIZE AND (c) AMOUNT OF EXTRACELLULAR MATRIX FOR CULTURES FOR CARTILAGE THAT DEVELOPED IN ISOLATED MESENCHYME, MESENCHYME CULTURED WITH EPITHELIUM AND/OR IN THE PRESENCE OF EGF [A]

EGF	H. H. stage 18		H. H. stage 22		H. H. stage 25	
(ng/ml)	isolated mesenchyme	mesenchyme + epithelium	isolated mesenchyme	mesenchyme + epithelium	isolated mesenchyme	mesenchyme + epithelium
			(a) Distribution of 3H-thymidine-labelled cells			
0	3.93 ± .06(15)	3.54 ± .31(13)	2.67 ±.30(18)	1.82 ± .26(17)c	2.31 ± .26(13)	1.0± 0 (7)c
5	3.60 ± .24 (5)b	3.40 ± .40(5)	1.71 ± .28 (7)b	1.40 ± .16(10)	-	-
10	3.60 ± .26(10)	2.77 ± .97 (9)c	1.25 ± .17(12)b	1.62 ± .37 (8)	1.64 ± .84(14)	1.0± 0 (7)b,c
20	3.60 ± .24 (5)b	3.42 ± .36 (7)	1.25 ± .16 (8)b	1.08 ± .08(12)b	1.00 ± 0 (6)	1.0± 0 (7)b
40	4.00 ± 0 (3)	2.66 ± .66 (3)	1.71 ± .42 (7)b	2.11 ± .38 (9)	-	-
			(b) Cell size			
0	3.47 ± .13(15)	2.77 ± .12 (13)c	2.72 ± .10(18)	2.12 ± .16 (17)c	3.00 ± .11(13)	2.00±0 (7)c
5	2.80 ± .20 (5)b	2.40 ± .24 (5)	2.4 3± .20 (7)	2.30 ± .15 (10)	-	-
10	3.40 ± .22 (10)	2.55 ± .24 (9)c	3.00 ± 0 (12)b	2.12 ± .12 (8)c	2.64 ±.13 (14)b	1.86±.14(7)c
20	2.20 ± .20 (5)b	2.43 ± .20 (7)	1.75 ± .16 (8)b	1.58 ± .14 (12)b	1.67 ±.21 (6)b	2.00+.21 (8)
40	2.33 ± .33 (3)b	3.00 ± 0 (3)	2.14 ± .14 (7)b	2.33 ± .16 (9)	-	-
			(c) Amount of extracellular matrix			
0	3.33 ± .12(15)	3.31 ± .21(13)	2.61 ±.11 (18)	1.76 ± .10 (17)c	3.23 ± .16 (13)	2.57± .20 (7)c
5	2.80 ± .20 (5)b	2.40 ± .24 (5)b	2.57 ± .20 (7)	2.10 ± .18 (10)b	-	-
10	3.30 ± .26 (10)	2.67 ±. 41 (9)	2.50 ± .15 (12)	2.37 ± .18 (8)b	3.00 ± 0 (14)	2.85± .14 (7)
20	2.20 ± .20 (5)b	2.57 ± .20 (7)b	1.88 ± .12 (8)b	2.00 ± 0 (12)b	2.50 ± .22 (6)b	2.43± .20 (7)
40	3.00 ± 0 (3)	2.00 ± 0 (3)b,c	2.00 ± 0 (7)b	1.78 ± .14 (9)	-	-

a. State of cartilage differentiation based on a scale from 1 (immature) to 4 (mature; see Materials and Methods). Mesenchyme and epithelium isolated from embryos of H. H. stages 18 (cultured for 10 days), 22 and 25 (cultured for 7 days). EGF at concentrations of 5-40 ng/ml.
b. Significantly different from 0 ng/ml EGF
c. Significantly different from isolated mesenchyme of same H. H. stage

that differentiated (data not shown).

Distribution of dividing cells

Stage of embryo providing the mesenchyme, presence of epithelium or EGF all influenced the distribution of labeled cells. H.H.18 mesenchyme produced much more mature cartilage when recombined with H.H.18 epithelium (index of 4.0, Table 3) than when combined with epithelium from H.H.22 or 25 embryos (indices of 2.43 and 1.8, respectively). Presence of EGF further retarded all these indices for all three stages (Table 3).

Chondrocyte size

Although EGF had little effect on chondrocyte size (only H.H.18 intact and H.H. 22 isolated mesenchyme being significantly different from control; Table 3), both pres-

ence and age of epithelium affected chondrocyte maturation with chondrocytes becoming most mature when mesenchyme was cultured with epithelium from embryos of the same stage (homochronic; Table 3, Fig. 8).

Amount of extracellular matrix

As with chondrocyte size, EGF had little effect on the accumulation of extracellular matrix, except for H.H. 22 intact mesenchyme and epithelium where EGF enhanced, and H.H.18 mesenchyme recombined with H.H.22 epithelium where it retarded, matrix accumulation (Table 3). On the other hand, presence and age of epithelium did significantly influence matrix accumulation, particularly in cartilage that developed in mesenchyme from H.H.18 and 22.

From an analysis of the trends seen in Table 3 it can be concluded that 10ng/ml EGF delayed chondrogenesis in a manner that resembled recombination of mesenchyme with epithelia, with both age of mesenchyme and age of epithelium affecting the extent of the effect. EGF potentiated changes in chondrogenesis initiated by the epithelium.

Time course of the effects of epithelium ± EGF on chondrogenesis in vitro.

Mesenchyme obtained from H.H.18 embryos was cultured either in isolation or with mandibular epithelium in the presence or absence of10ng/ml EGF and indices and the three indices of cartilage differentiation determined at two-day-intervals for 10 days.

Onset of chondrogenesis

Cartilage appeared earlier in mesenchyme cultured intact with epithelium (in one specimen after only 2 days *in vitro* ; Table 4) than in isolated mesenchyme. EGF accelerated the appearance of cartilage in intact cultures (57 vs 17% with cartilage after 2 days, Table 4) but this cartilage was immature procartilage (Table 2 and see below). After 4 days *in vitro* all cultures except those of intact mesenchyme and epithelium had formed cartilage. Thus, the only effects of epithelium or EGF on initiation of chondrogenesis were seen very early in the culture period (0-4 days) and the EGF-effect was only seen in the presence of epithelium.

DNA labeling indices

The ^{3}H-thymidine-labeling index declined with time as the cartilage differentiated. The index was very high in those cartilages that had already differentiated in intact mesenchyme and epithelium after 2 days *in vitro* (Table 4, Fig. 9). These cartilages were also at a very immature state of procartilaginous differentiation (see below). After 4 days of *in vitro* cultivation, labeling indices had declined substantially (11.5-20.7% vs 68 and 54%), although they were still higher in intact than in isolated mesenchyme (significantly higher in the absence of EGF, Table 4, Fig.9). After 10 days *in vitro* the labeling indices had declined even further (4.3-7.3%).

Distribution of dividing cells

Cartilage in cultures of intact mesenchyme and epithelium after 2 days was very immature on the basis of this and of the other two maturation indices below (Table 4). Cartilage that differentiated in isolated mesenchyme was mature (index of 3.93) after 10 days of *in vitro* cultivation (Table 4). Epithelium delayed chondrocyte maturation at all days; intact mesenchyme did not proceed beyond the lowest index (1.0) until after 4 days of *in vitro* incubation (Table 4).

EGF further lowered the index for distribution of labeled cells, both for isolated and for intact mesenchyme. Cartilage differentiating in intact mesenchyme was still at an index of only 1.12 after 6 days *in vitro* (Table 4).

Chondrocyte size

Presence of the epithelium slowed chondrocyte maturation as assessed by cell size, the indices being significant lower after 6 and 10 days of incubation (Table 4). EGF had no significant effect on chondrocyte size.

Amount of extracellular matrix

Accumulation of extracellular matrix was slowed significantly in intact mesenchyme over the first 6 days of*in vitro* cultivation, but equal to values for isolated mesenchyme thereafter, i.e. the inhibitory effect of the epithelium was either short-lived or compensated for by the differentiating mesenchyme. There was a trend toward reduction of matrix accumulation in the presence of EGF.

The three indices of cartilage maturation have been combined into a single score in Figure 10 to illustrate the trends over time

Discussion

In summary, (a) the ^{3}H-thymidine-labelling index of cartilage was enhanced when mesenchyme was cultured with mandibular epithelium; (b) epithelium delayed both the onset and the maturation of chondrogenesis (Fig.9), (c) EGF, especially 10-20ng/ml, affected proliferation and the initiation and maturation of chondrogenesis in mesenchyme cultured either in the presence or absence of epithelium, (d) in isolated mesenchyme the effect of EGF resembled the epithelial action seen in intact tissues (Fig.9).

With respect to cartilage differentiation, EGF at 40ng/ml, but not presence of epithelium, influenced proliferation within the cartilage (Table1). Thus, EGF enhanced percent labeling (mitotic activity) in cartilage differentiating in uncondensed mesenchyme from the H.H.18 embryos, inhibited labelling in cartilage differentiating from condensing H.H. 22 mesenchyme, and only at higher doses (20ng/ml) effected cartilage that was already differentiating at the beginning of the culture (H.H.25). This stage-

TABLE 3

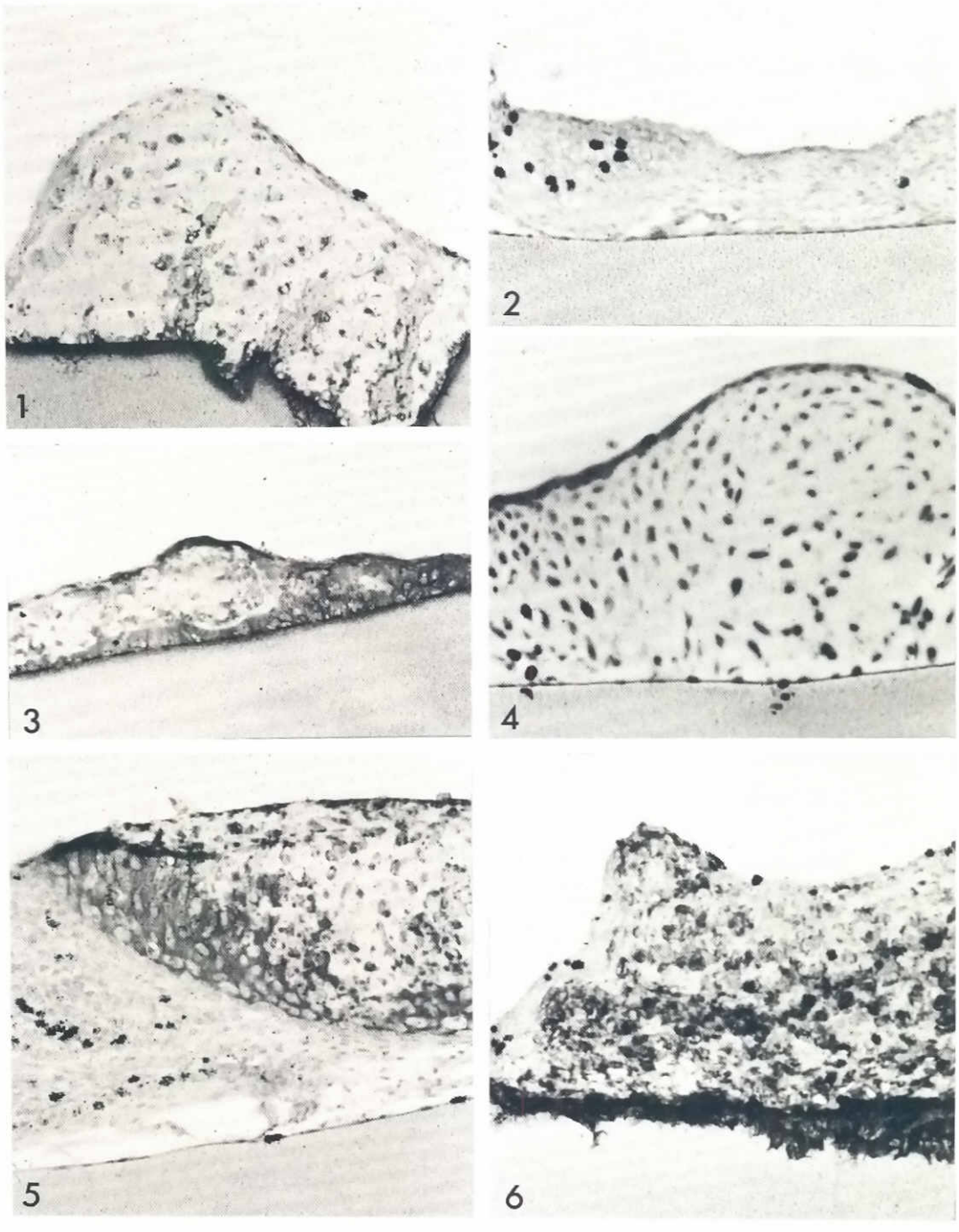
MEAN VALUES (X± SEM) FOR TISSUE RECOMBINATIONS INVOLVING MESENCHYME AND EPITHELIUM FROM H. H. STAGES 18, 22 AND 25 THAT ATTAINED THE MATURE STATE OF DIFFERENTIATION AS SHOWN ON THE BASIS OF (a) DISTRIBUTION OF ^{3}H-THYMIDINE-LABELLED CELLS, (b) CELL SIZE AND (c) AMOUNT OF EXTRACELLULAR MATRIX DEPOSITED. [A]

EGF (ng/ml)	Isolated mesenchyme	Intact mesenchyme and epithelium	Epithelium from embryos of H. H. stages 18	22	25
		(a) Distribution of ^{3}H-thymidine-labelled cells			
		H.H. stage 18 mesenchyme			
0	2.67 ± .18 (15)	2.31± .27 (16)	4.00 ± 0 (2)[a,b]	2.43± .52 (7)	1.80±.49 (5)[a]
10	2.81± .29 (21)	2.00± .27 (17)[a]	3.67± .33 (3)[b]	2.14±.45 (7)	1.25 ± .25(4)[a]
		H.H. stage 22 mesenchyme			
0	2.67 ± .30(18)[c]	1.82± .26 (17)[a]	2.44± .37 (9)[c]	1.88± .47 (8)	-
10	1.21 ± .15 (14)	1.63± .37 (8)	1.33± .33 (9)	1.60 ± .30(10)	-
		H.H. stage 25 mesenchyme			
0	2.31± .26 (13)[c]	1.00 ± 0 (7)[a]	2.00± .54 (5)[b]	-	3.00± .54 (5)[b,c]
10	1.50± .23 (12)	1.00 ± 0 (7)	1.29± .18 (7)[b]	-	1.29 ± .28(7)
		(b) Cell size			
		H.H. stage 18 mesenchyme			
0	3.27± .15 (15)	3.06± .23(16)[c]	3.00± 0 (2)	2.43± .20 (7)[a]	2.80± .20 (5)
10	2.86± .18 (21)	2.59± .15 (17)	2.67± .33 (3)	1.86± .34 (7)[a,b]	2.50±.29 (4)
		H.H. stage 22 mesenchyme			
0	2.72± .14 (18)[c]	2.12± .17 (17)[a]	2.89± .20 (9)[b]	3.00 ± 0 (8)[a,b]	-
10	3.00 ± 0 (14)	2.12± .12 (8)[a]	2.89± .11 (9)[b]	2.70± .15(10)[a,b]	-
		H.H. stage 25 mesenchyme			
0	3.00± .11 (13)[c]	2.00± 0 (7)[a]	2.80± .20 (5)[b]	-	3.00± 0 (5)[b]
10	2.67± .14 (12)	1.86± .14(7)[a]	2.86± .14 (7)[b]	-	3.00± 0 (7)[a,b]
		(c) Amount of extracellular matrix			
		H.H. stage 18 mesenchyme			
0	3.27± .15 (15)	2.88 ± .22(16)	3.00 ± 0 (2)[b]	3.00± .21 (7)[c]	3.40± .24 (5)[b]
10	2.86± .19 (21)	2.35± .22 (17)	3.33± .33 (3)[b]	2.14± .26 (7)[a]	3.00± 0 (4)
		H.H. stage 22 mesenchyme			
0	2.61± .11 (18)	1.76± .10(17)[a,c]	2.78± .14 (9)[b]	3.10± .12(8)[a,b]	-
10	2.57± .13 (14)	2.37± .18 (8)	2.67± .16 (9)	3.00± .21 (10)[a,b]	-
		H.H. stage 25 mesenchyme			
0	3.23± .16 (13)	2.57± .20(7)[a]	3.20 ± .37(5)	-	3.00 ± .31(5)
10	3.00± 0 (12)	2.85± .14 (7)	3.00 ± .21(7)	-	2.43 ± .20(7)[a]

a. Significantly different from isolated mesenchyme. **b**. Significantly different from intact mesenchyme and epithelium. **c**. Significantly different from 10ng EGF.

dependent effect demonstrated that EGF acted differently on the proliferation of prechondrogenic and chondrifying mesenchyme.

The stimulatory effect of EGF on proliferation of chondrogenic mesenchyme isolated from H. H.18 embryos delayed chondrogenesis, as especially evident in the relative acumulation of extracellular matrix but also in the distribution of ^{3}H-thymidine- labeled cells and in chondrocyte size (Table 2). The inhibitory action of EGF and of epithelium on cartilage differentiation was even more evident when mesenchyme from H.H. 22 or 25 was used as the starting material (Table 2). EGF and epithelium retarded chondrogenesis by slowing chondrocyte maturation from the pro-



Autoradiographs of cultures exposed to ^{3}H-thymidine for the last 4 hours of the culture period. Focus is on the silver grains unless otherwise indicated.

Fig. 1. *H. H. stage 18 isolated mandibular mesenchyme cultured for 10 days has almost all differentiated into cartilage (cf. with the amounts of cartilage in Figures 2 and 3). The one or two ^{3}H-thymidine-labelled cells are confined to the periphery of this mature cartilage. x 215.*

Fig. 2. *H. H. stage 18 mandibular mesenchyme and epithelium cultured for 10 days. Note the small amount of cartilage on the left (cf. Fig. 1) and the larger number of labelled cells (black). x 215.*

Fig. 3. *H. H. stage 18 isolated mandibular mesenchyme cultured for 10 days in the presence of 20ng/ml EGF. A small amount of cartilage has differentiated along the supporting Millipore filter. x 215.*

Fig. 4. *H. H. stage 22 isolated mandibular mesenchyme cultured for 7 days differentiates into a large mass of mature cartilage. x 218*

Fig. 5. *H. H. stage 22 mandibular mesenchyme and epithelium cultured for 7 days. 3H-thymidine-labeled cells are primarily localized in the unchondrified mesenchyme and in the epithelium. x 218*

Fig. 6. *H. H. stage 22 mandibular mesenchyme cultured for 7 days in the presence of 20ng/ml EGF. Note the large number of labeled cells. x 218.*

TABLE 4.

(A) PERCENT (N) OF CULTURES WITH CARTILAGE AND MEAN VALUES (X± SEM) FOR (B) THE ^{3}H-THYMIDINE-LABELING INDEX AND FOR THE STATE OF DIFFERENTIATION ATTAINED BY CARTILAGE BASED ON (C) DISTRIBUTION OF ^{3}H-THYMIDINE-LABELLED CELLS, (D) CELL SIZE AND (E) AMOUNT OF EXTRACELLULAR MATRIX [A] DEPOSITED FOR ISOLATED MESENCHYME AND MESENCHYME + EPITHELIUM OBTAINED FROM H. H. STAGE 18 EMBRYOS AND CULTURED FOR 2-10 DAYS IN THE PRESENCE OR ABSENCE OF EGF. [B]

Days *in vitro*	Isolated mesenchyme	Intact mesenchyme and epithelium	Isolated mesenchyme + EGF	Intact mesenchyme and epithelium + EGF
		(a) Percent of cultures with cartilage		
2	0 (0)	17 (6)	0 (8)	57 (7)
4	100 (6)	67 (6)	100 (3)	100 (10)
6	100 (7)	100 (6)	100 (11)	100 (9)
8	100 (8)	100 (10)	-[c]	-
10	100 (15)	100 (13)	100 (10)	100 (9)
		(b) ^{3}H-thymidine labeling indices		
2	-	67.9 (1)	-	53.9±2.7(4)
4	11.5±2.5(6)[e]	20.7±1.7(4)	16.5±2.0(3)	18.7±1.7(10)
6	15.9±1.3(7)	12.7±1.2(6)	10.0±2.5(9)	12.9±1.4(7)
8	7.4±1.3(8)	9.1±1.4(9)	-	-
10	4.3±0.9(12)	6.1±1.5(13)	6.9±1.9(10)	7.3±1.3(9)
		(c) Distribution of ^{3}H-thymidine-labeled cells		
2	-	1.00 (1)	-	1.00±0 (4)
4	1.33±.81(6)	1.00±0(4)	1.00±0(3)	1.50±.26(10)
6	2.29±.18(7)	1.66±.33(6)	2.09±.39(11)[d]	1.12±.12(8)
8	3.00±.26(8)	2.70±.33(10)	-	-
10	3.93±.06(15)	3.54±.31(13)	3.60±.26(10)[d]	2.77±.32(9)
		(d) Cell size		
2	-	1.00(1)	-	1.00(4)
4	2.33±.21(6)	2.00±.40(4)	2.33±.66(3)	2.20±.20(10)
6	3.14±.14(7)[e,f]	2.33±.3(6)	2.36±.20(11)	2.62±.18(8)
8	3.37±.26(8)	3.50±.22(10)	-	-
10	3.47±.13(15)[e]	2.77±.12(13)	3.40±.22(10)[d]	2.55±.24(9)
		(e) Amount of extracellular matrix		
2	-	1.00(1)	-	1.00(1)
4	2.33±.21(6)[e]	1.50±.28(4)[d]	2.00±.57(3)	2.00±0(10)
6	3.14±.14(7)[e,f]	2.33±.42(6)	2.45±.24(11)	2.00±0(8)
8	3.37±.26(8)	3.20±.20(10)	-	-
10	3.33±.12(15)	3.31±.20(13)	3.30±.26(10)	2.67±.40(9)

a. Based on a scale from 1 (immature) to 4 (mature; see Materials and Methods). **b**. EGF at 10 ng/ml. **c**. No data **d**. Significantly different from intact mesenchyme and epithelium + EGF. **e**. Significantly different from intact mesen chyme and epithelium. **f**. Significantly different from isolated mesenchyme + EGF

liferating to the differentiating state, and accumulation of cartilaginous extracellular matrix. EGF, although it retarded maturation of cartilage differentiating from isolated mesenchyme, chiefly exerted its retarding effect via the epithelium, primarily in mesenchyme from embryos of H.H. 22 and 25.

10ng/ml EGF delayed chondrogenesis in a manner that resembled recombination of mesenchyme with epithelia, with both age of mesenchyme and age of epithelium affecting the extent of the effect (Table 3). EGF potentiated changes in chondrogenesis initiated by the epithelium.

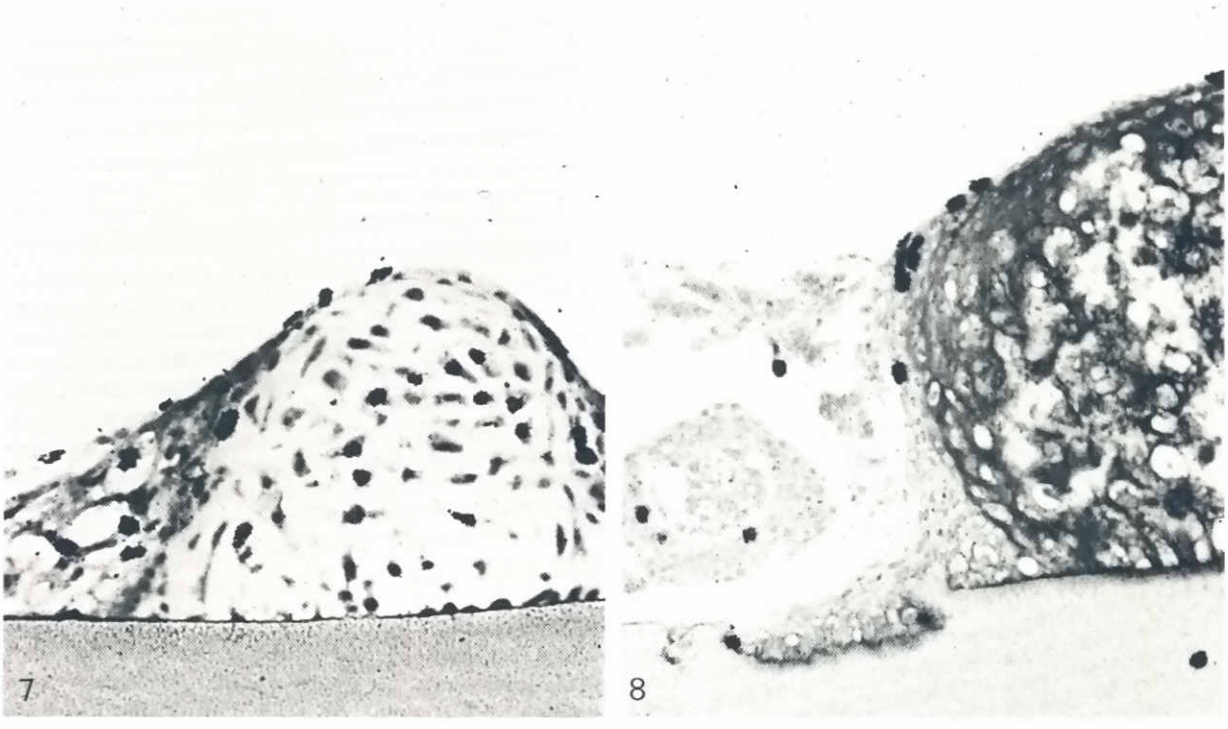


Fig. 7. *H. H. stage 18 mandibular mesenchyme cultured for six days (exposed to ^{3}H-thymidine for the last 4 hours) differentiated into cartilage with labelled cells localized both in the periphery and in the body of the cartilage. x 255.*

Fig. 8. *H. H. stage 22 mandibular mesenchyme recombined with H. H. stage 22 epithelium and cultured for 7 days (exposed to ^{3}H-thymidine for the last 4 hours) underwent extensive chondrogenesis (right). Labeled cells are confined to the perichondrium, adjacent mesenchyme and epithelium (left). x 242.*

The time course of the chondrogenesis-inhibiting action of epithelium and EGF was further analysed by examining maturation of cartilage that formed over a ten day culture period (Table 4). Epithelium delayed all aspects of chondrogenesis, especially over the first 4 days of the culture period. Chondroblasts appeared later, their proliferation was significantly stimulated and cell enlargement and matrix accumulation were delayed. Given that epithelium initially significantly stimulates mitotic activity in the mesenchyme (Hall and Coffin-Collins, submitted for publication, 1989) it is evident that presence of the epithelium progressively delays all stages of cartilage cytodifferentiation (Fig. 2). Delayed matrix production correlated with high levels of chondroblast mitotic activity supports reports that chondrocytes generally do not exhibit high levels of proliferation when they are undergoing or maintaining differentiation (Cahn and Lasher,1967; George *et al.*, 1983).

Although the influence of the epithelium on chondrogenesis could be related to a generalised mitogenic stimulus to prechondrogenic mesenchyme and/or early chondroblasts, the epithelium also inhibited initiation and progression of the differentiated state directly (Table 3). Further support for a differentiation-stage dependent influence of the epithelium comes from the data in which aspects of differentiation affected in intact mesenchyme and epithelium varied according to the age of the mesenchyme (Fig.11). All aspects of chondrogenesis were equally affected in the youngest tissues because of the cumulative effect of the epithelium on all ongoing and subsequent events. In older tissue, the epithelial inhibition was primarily on differentiation (Table 3, Fig. 11). Other studies have documented that the differentiative stages of chondrogenesis are susceptible to a variety of inhibitory or stimulatory influences (Von der Mark and Conrad,1979; Gallandre and Kistler,1980; Takigawa *et al*,,1980; Nathanson,1983; Burch and McCarthy, 1984; Mirsky and Silbermann,1984; Zimmermann and Tsambaos,1985). The current findings of an inhibitory epithelial influence on chondrogenesis is consistent with the findings of McLoughlin (1961), Solursh *et al* .(1981, 1984) and Tyler (1983). We should also note that other investigations have suggested that chondrocytes in cell culture can regulate their differentiation through the production and progressive accumulation of autostimulatory peptides (Azizkhan and Klagsbrun,1980; Solursh *et al.*,1982; Shen *et al.*,1985).

A mitogenic effect of EGF on chondrocytes in cell culture has been previously reported (Gospodarowicz and Mescher,1977; Carpenter,1978; Gospodarowicz *et al.*,1979; Kato *et al.*,1983; Madsen *et al.*,1983). That 10ng/ml EGF

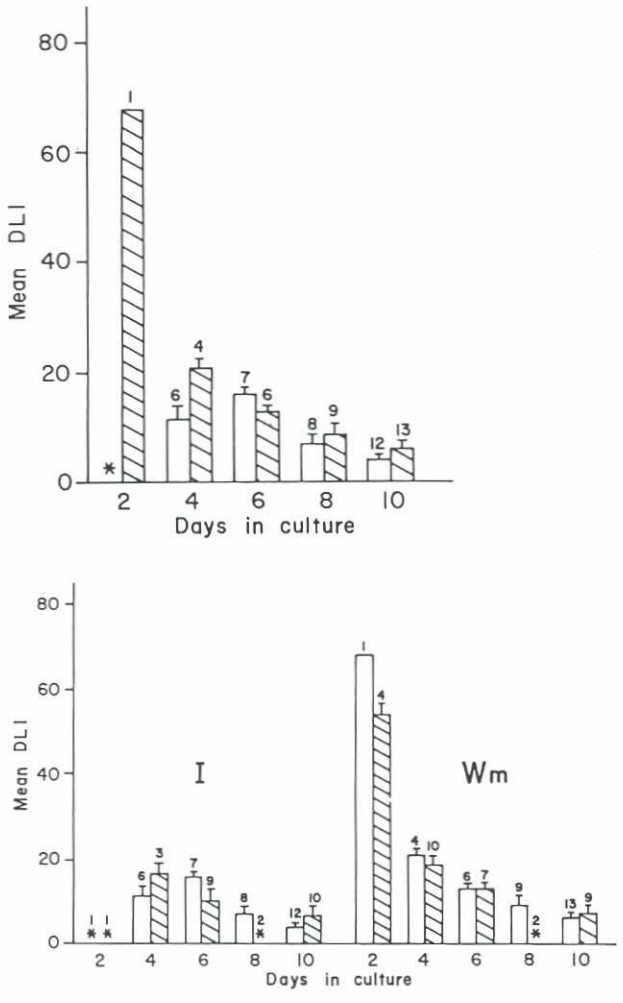


Fig 9. *Top. Mean daily labelling indices (DLI) of cartilage differentiating in H. H. 18 mesenchyme cultured for 10 days either isolated from (open boxes) or in contact with epithelium (hatched boxes). Note the dramatic decline in DLI between 2 and 4 days, and the progressive decline thereafter. Bottom. Mean DLI of cartilage differentiating in H. H. 18 mesenchyme cultured for 10 days either in isolation (I) or with epithelium as whole mandibles (Wm) in the presence (hatched) or absence (open boxes) of 10ng/ml EGF. The epithelial effect is much greater than the EGF effect. For both Figures, vertical bars = 1 standard error of the mean; numbers = sample sizes, * = no cartilage present after 2 days or no data for 8 days.*

delayed both the decline in chondroblast proliferation and the rate of cytodifferentiation when assesed over a ten day culture period (Table 4, Fig. 10) provided evidence for the mitogenic activity of EGF on chondrocytes in *tissue or organ culture*. All concentrations of EGF tested delayed cartilage maturation (Table 2). At H.H.18 and 25 the effect of EGF could be related to its mitogenic activity; at H.H. 22 it could be more readily interpreted as acting directly on differentiation processes themselves, specifically accumulation of products of the extracellular matrix.

EGF has been shown to influence expression of cell-type specific products in other cells; inhibition of type I but stimulation of type III collagen synthesis by bone cells (Canalis and Raisz, 1979; Hiramatsu *et al.*, 1982; Canalis, 1983; Kumegawa *et al.*, 1983; Hata *et al.*, 1984); stimulation of production of collagen degrading enzymes by osteoclasts (Tashjian and Levine, 1978; *Raisz et al.*,1980; Chikuma *et al.*,1984); stimulation of synthesis of type V collagen by palatal mesenchyme (Silver *et al.*,1984); stimulation of the synthesis of collagen by liver-derived, epithelial cell lines (Kumegawa *et al.*,1982). The latter effect, as well as modulation of extracellular matrix products, can occur in the absence of any mitogenic influence of EGF (Canalis,1985), a dissociation also shown in the action of EGF on steroid hormone production by ovarian and testicular mesenchymal cells, glycogen production by cervical cells, fibronectin by fibroblasts, prostaglandin by kidney cells and growth hormone and prolactin by pituitary cells (see Coffin-Collins,1987 for details). EGF should therefore be considered as both a mitogen and a regulator of differentiation in the absence of any effect on proliferation.

EGF by delaying chondrogenesis, resembled the similar effect of mandibular epithelium. EGF at 10-20 ng/ml delayed chondrogenesis in isolated mesenchyme to the same extent as when mesenchyme was cultured with epithelium (Table 2, Fig. 11). Both EGF and epithelium delayed the decline in proliferation, and subsequent chondrogenesis.

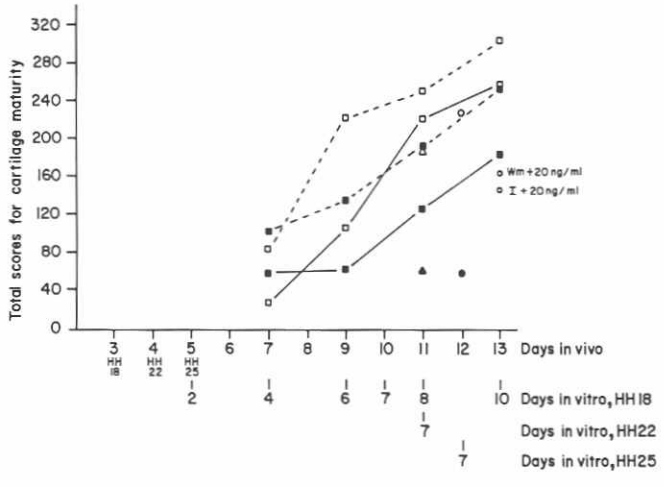


Fig. 10. *A summary of total scores for maturation of cartilage that differentiated in H. H. 18, 22 or 25 mesenchyme over 7 or 10 days* in vitro *. Total maturity score is based on distribution of 3H-thymidine-labelled cells, chondrocyte size and relative amount of extracellular matrix (each out of100, i.e. total possible maturity index is 300). The time axis relates days of* in vitro *cultivation to ages of the embryos providing the mesenchyme and to equivalent days* in vivo. ❑ - - ❑ = *H. H. 18 mesenchyme cultured in isolation;* ❑— ❑ = *H. H. 18 mesenchyme cultured with epithelium;* ■ - - ■ = *H.H.18 isolated mesenchyme cultured in 10ng/ml EGF;* ■ — ■ = *H. H. 18 mesenchyme + epithelium cultured in 10ng/ml EGF;* △= *H.H. 22 mesenchyme cultured in isolation;* ▲ = *H. H. 22 mesenchyme cultured with epithelium;* O = *H. H. 25 mesenchyme cultured in isolation;* ● = *H. H. 25 mesenchyme cultured with epithelium; 0 I + 20ng/ml or 0 Wm + 20ng/ml = H. H. 18 isolated mesenchyme (I) or mesenchyme + epithelium (Wm) cultured for 10 days in 20ng/ml EGF.*

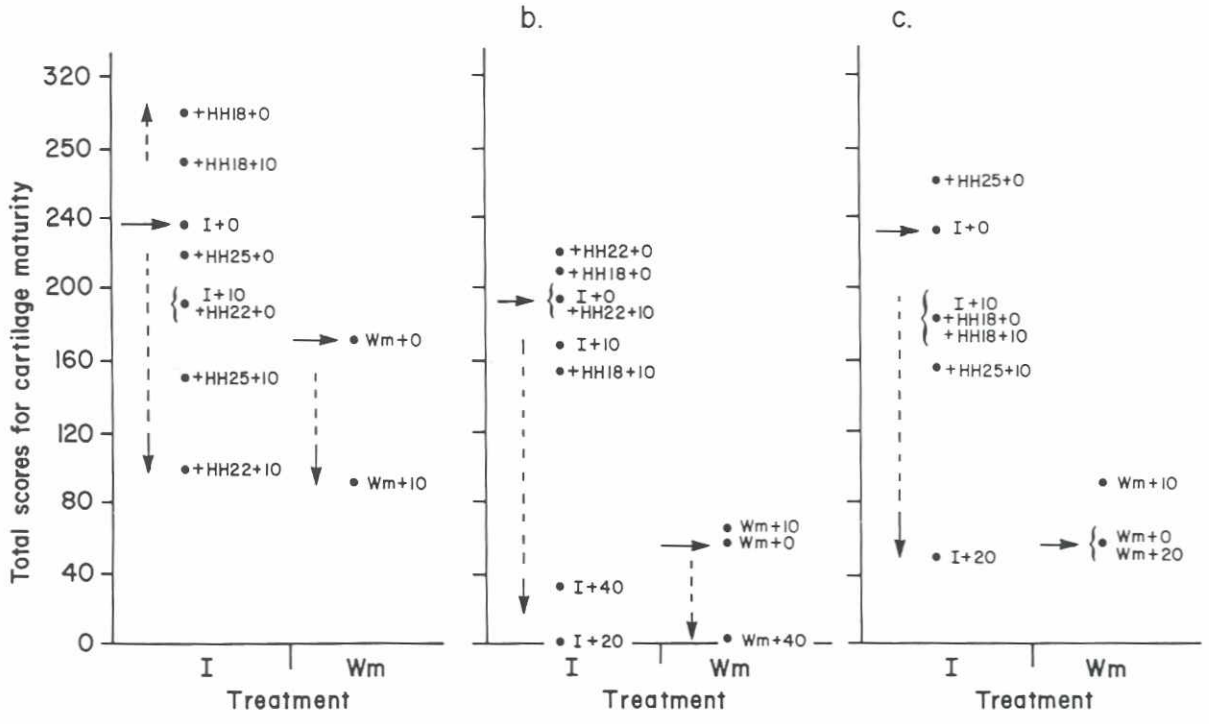


Fig. 11. *A comparison of total scores for cartilage maturity (calculated as described for Figure 2) for various treatment of H. H. 18 (a), 22 (b) or 25 (c) mesenchyme cultured for 7 days. Horizontal arrows indicate baseline levels for isolated mesenchyme (I) or for mesenchyme cultured with epithelium (Wm), both in the absence of EGF. Vertical arrows indicate treatments that significantly enhanced (↑) or inhibited (↓) chondrogenesis relative to the baseline levels. +0, = 0 ng/ml EGF (control); + 5, 10, 20 or 40 = cultured in the presence of 5-40 ng/ml EGF; + HH 18, 22 or 25 = mesenchyme cultured with epithelium from embryos of these three stages.*

Combining the effects of epithelium and EGF provided further insights. 10ng/ml EGF in combination with epithelium enhanced the minor effect of epithelium on promoting initial cytodifferentiation in H.H.18 mesenchyme (Fig.3). The combination of EGF and epitheliumt produced the earliest cartilage differentiation, relative to all other treatment (Table 4, Fig. 9). This initial promotion of cytodifferentiation was replaced by a later inhibition of ongoing differentiation mediated by delayed accumulation of extracellular matrix (Table 4, Fig.11).

The synergistic overall inhibitory effects of EGF and epithelium could be interpreted as intact mesenchyme and epithelium containing residual levels of endogenous EGF or EGF-like molecules when dissected from the embryos. Consistent with such an interpretation is the observation that with all ages of tissues used, the inhibitory effect of low concentrations (10ng/ml) was greater in intact mesenchyme and epithelium than in isolated mesenchyme and that the effect of 10ng/ml on intact tissues could be duplicated in isolated mesenchyme with higher concentrations of EGF (Table 2, Fig. 11). The tissue recombinations could also be interpreted as EGF influencing the intact tissues via endogenous EGF (Table 3). Only two recombinations in the absence of EGF delayed chondrogenesis relative to that seen in isolated mesenchyme-H.H.22 epithelium with H.H.18 mesenchyme and H.H.18 epithelium with H.H. 25 mesenchyme (Table 3, Fig. 11). In these recombinations the inhibitory action of the epithelium was not as great as in intact mesenchyme and epithelium and could be duplicated by treating isolated mesenchyme with 10ng/ml EGF. The more pronounced inhibitory influence of the epithelium in the intact tissues could be duplicated by culturing isolated mesenchyme of any stage in higher concentrations of EGF. That EGF affected chondrogenesis in the absence of epithelium, could mimic epithelial influences and was influenced by the presence of epithelium all point to the epithelial influence being mediated by endogenous EGF. Such a conclusion is consistent with the pattern of action of EGF on developing palate and teeth (see Introduction).

In summary, levels of proliferation within chondrogenic and chondrifying mandibular mesenchyme are highest during initial differentiation and decline progressively thereafter. Mandibular epithelium influences the timing of the differentiation of mandibular mesenchyme (a) by delaying cytodifferentiation because of prolonging high levels of mitotic activity and (b) by directly affecting differentiation itself. A functional role for EGF in regulating chondrogenesis from mandibular mesenchyme is therefore suggested. All observed effects of epithelium in intact tissues

could be duplicated by exposing isolated mesenchyme to EGF at 10ng/ml. Thus, this study provides direct evidence that EGF affects chondrogenesis of mandibular mesenchyme *in vitro,* and suggests a role for EGF *in vivo.*

Materials and Methods

Incubation of eggs and staging of embryos

Embryos used were from the White Leghorn strain of the common fowl, *Gallus domesticus,* obtained from Cook's Hatchery Ltd., Truro, Nova Scotia. Fertile eggs were incubated at 37°C and 50-60% relative humidity. After appropriate periods (3 to 5 days), the embryos were removed, placed into sterile saline and staged according to the morphological criteria established by Hamburger and Hamilton (1951). Embryos of H. H.18, 22 and 25 (3, 4 and 5 days of incubation) were used.

Isolation, separation, and recombination of mandibular processes

Mandibular processes were dissected from staged embryos and placed into sterile saline. All mandibles were then placed into a solution of 2.57% trypsin and 0.43% pancreatin (BDH Chemicals Ltd., Montreal, Quebec) in Ca^{++} and Mg^{++}free Tyrode's solution for 45-60 mins at 4°C, and then placed into a 1:1 solution of BGJ_b (a synthetic culture medium) and horse serum (Grand Island Biological Co., Montreal, Quebec) to slow any further enzymatic digestion. Mandibular processes were then either left intact as controls, or separated into their epithelial and mesenchymal components by microdissection using sharpened needles. Intact, enzyme- treated mandibular processes, isolated mandibular mesenchyme, or mesenchyme recombined with its own age epithelium (homochronic recombinations) or with epithelium from embryos of another age (heterochronic recombinations) were then established in tissue culture.

Tissue culture procedures

Isolated tissues were placed onto sterile, black Millipore, filters (0.45μm porosity,125-150 μm thin). Intact mandibles and isolated mesenchyme received no further treatment. To establish epithelial-mesenchymal recombinations, the mandibular epithelium was flattened onto the filters, secured by gentle pressure at the edges and mesenchyme then placed onto the epithelium .

Tissues were placed onto stainless steel supports and transferred to 35mm presterilized plastic Petri dishes containing 1.5 ml of culture medium; the tissues were cultured at the medium : atmosphere interface. Culture medium consisted of BGJ_b, plus 15% horse serum and 150μg/ml ascorbic acid (Matheson, Coleman and Bell, Norwood, Ohio). When required, 5,10, 20 or 40 ng/ml (0.8×10^{-11} to 1.5×10^{-10}M) epidermal growth factor (Collaborative Research Inc., Waltham, Mass), was added to the medium at the beginning of the culture period and replaced at each medium change. Cultures were maintained in a water-jacketed CO_2 incubator at 37°C in an atmosphere of 5% CO_2 in air for 2 to 10 days. Medium was completely changed every second day.

^{3}H-thymidine labeling and autoradiography

Four hours before termination of the culture period, tissues were provided with 1.5 ml of fresh medium containing 0.2ml (10μCi) of ^{3}H-thymidine (specific activity 64 Ci/mmol; New England Nuclear Canada Ltd., Lachine, Quebec). Four hours exposure to 3H-thymidine was chosen to ensure that no cell would have divided more than once during the labelling period, the duration of the S phase of embryonic craniofacial mesenchymal cells being of the order of eight hours (Hall,1978) and 5.5 hours in maxillary mesenchyme of embryos of H.H. stages 24 to 26 (Minkoff, 1984). After exposure to 3H-thymidine the tissues were rinsed in Ca^{++} and Mg^{++} free Tyrode's solution and immersed in a solution of BGJb-15% horse serum for ten minutes.

Tissues were fixed in neutral buffered formal saline, dehydrated, cleared, embedded, and serially sectioned at 5-6 μm. Sections were dried overnight at 37oC. Slides were dewaxed in xylene, brought to distilled water in a descending ethanol series and stained with Mayer's Haematoxylin, Alcian Blue 8GN and Phosphomolybdic Acid, a sequence that stained nuclei red-purple and cartilage matrix blue. The slides were placed in tap water and, coated with Kodak NTB3 nuclear track emulsion (Kodak Canada, Ltd) diluted 1:1 with double distilled water, air dried for 60 mins, and placed into light-tight boxes containing silica gel for 5 days at 4°C.

Autoradiographs were developed for 2 mins in Kodak D-19 developer, rinsed in tap water and stopbath for 30 secs each, fixed in Kodak Rapid Fixer for 5 mins, and washed in running tap water for 20 mins. The slides were then removed from the darkroom, stained with Chlorantine Fast Red, crash dehydrated in absolute ethanol, cleared in xylene and coverslipped using DPX, a synthetic resin mounting medium (BDH Chemicals, Montreal, Quebec).

Determination of DNA labeling indices and location of labeled cells

Slides were examined under x100 oil immersion. Background labeling levels were determined by counting grains in areas of the sections devoid of tissue but equivalent in area to 12-14 mesenchymal cells. Such background levels were at or below the equivalent of 0.5 grains/nucleus. However, only nuclei where 3 or more silver grains could be seen in a nuclear profile were counted and considered to have incorporated 3H-thymidine during DNA synthesis. The nuclei in differentiating cartilage were counted in every tenth section of each sample, yielding an average count of 800 to 900 nuclei per sample. The DNA labeling index was calculated as the total number of labeled nuclei divided by the total number of labeled and unlabeled nuclei X 100. Mean labeling index and standard errors of the mean were calculated for each sample and treatment and analysed using the Standard Student t test.

Location of labeled cells within the cartilage was also determined. The first cells to cease dividing and differentiate are those in the centre of the developing cartilage; mature cartilage has dividing cells only around the periphery. Disappearance of labeled cells from central regions was therefore used to assess the progress of cytodifferentiation, those with labelled cells confined to the periphery were graded 4 (mature), those with only centrally-located labelled cells were graded 1 (immature). Mean scores for treatments were determined and compared statistically.

Quantification of state of cartilage differentiation

In addition to the determination of DNA labeling indices as just described, two cytological parameters were used to assess the relative state of differentiation of the cartilage that formed. The two were (i) relative cell size (cell size increases as mesenchymal cells differentiate into prechondroblasts, chondroblasts and finally into chondrocytes) and (ii) relative amount of extracellular matrix (amounts of matrix increase as differentiation progresses) as evidenced by Alcian Blue 8GX- staining of cartilage matrix in mid-sections of cartilage in the autoradiographs.

Each culture was scored on a four point scale with 1 being immature, 4 mature, and 2 and 3 the 33rd and 66th percentiles.

The mean score for cartilage developing in each of the treatment conditions was determined and the treatments compared using Student t tests for any effects of epithelia and/or of EGF on relative cartilage differentiation.

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