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Skin inhibits cartilage proliferation and calcification

Tissue culture of fetal mice bones

In an in vitro study, fetal mice metatarsal bones were cultured with skin or muscle fragments at some distance. We found that skin fragments inhibit growth in length and progress of calcification. Muscle fragments had no significant effects. Apparently, skin cells may produce factors that inhibit the development of metatarsal bones.

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Epithelial-mesenchymal interactions play a large role in morphogenesis, development, and repair in many systems (for reviews, see Hay 1981, Kleinman et al. 1981).

In the early stages of development of the chick embryo, epithelial tissues of a certain age induce membranous bone formation in neural crest-derived mesenchyme (Bee & Thorogood 1980, Hall 1981). In vitro, chick embryo-wing mesoderm differentiates into cartilage only in the presence of ectoderm, but after a certain stage the presence of ectoderm is no longer necessary (Gumpel-Pinot 1980).

One of us (C.W.Th.) observed dedifferentiation of osteoblasts in fetal long bones cultured in vitro with skin fragments. To extend this preliminary observation, we examined the influence of skin and muscle tissue on developing long bones.

Materials and methods

All tissues were taken from 18-day-old fetuses of Swiss albino mice. The mother and embryos were killed by decapitation. The second, third, and fourth metatarsal bones were immediately dissected under aseptic conditions. Skin and tendons were removed from the metatarsus, and the metatarsal bones were separated from each other by using a dissecting microscope and cataract knives. Skin and muscle fragments were taken from the same leg from which the metatarsal bones were dissected. The mean size of the skin and muscle fragments was 0.8 (0.5–1.5) mm and 1.2 (0.6–1.7) mm, respectively.

Experimental design. A total of nine culture experiments were performed. In each experiment, 15–18 metatarsal bones were evenly distributed in three groups: Group 1. Metatarsal bones cultured alone; Group 2. Metatarsal bones cultured with skin; and Group 3. Metatarsal bones cultured with muscle. The second, third, and fourth metatarsal bones were uniformly present in the three groups; and only tissues of the same fetus were cultured together.

Each metatarsal bone was placed on a 0.7 x 0.7-cm piece of perforated cellophane (Serva, Heidelberg, West Germany). In Groups 2 and 3, a tissue fragment was placed at some distance on both sides of the midshaft of the metatarsal bone (Figure 1). In both groups the mean distance to the bone was 2 (1–6) mm.

Organ culture . Tissues were cultured for 7 days in a culture dish (diam. 32 mm, three cultures per dish) containing 1 ml of a semisolid medium consisting of 20 per cent cock plasma, 10 per cent extract from 11-day-old chick embryos, and 10 per cent rat serum in Minimum Essential Medium (alpha medium without ribonucleosides and deoxyribonucleosides, Gibco) in a humidified 5 per cent CO_2 atmosphere at 37°C. The medium was changed every 2 or 3 days.

At the fetal stage of 18 days, a metatarsal bone consists of cartilage with a calcified zone in the midshaft occupying about 27 per cent of the total length (Table 1). During culture the total length of the long bone increases and calcification progresses towards both ends of the metatarsal bone. In the midshaft the marrow cavity enlarges. In the phase-contrast mi-

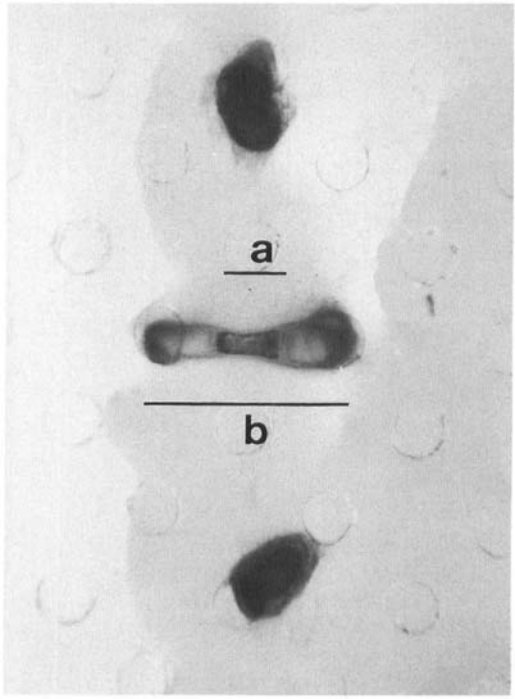

Figure 1. Living culture of a metatarsal bone with a skin fragment on both sides at some distance after 7 days of culture. The perforated cellophane on which the tissues are cultured is visible.
a: length of calcified zone. b: total length (phase contrast, ×20).

croscope, it can be observed that outgrowth of fibroblast-like cells occurs both from the metatarsal bones and from the soft-tissue fragments. These cells form layers, which spread over the cellophane.

Measurements. Total length of the metatarsal bones and length of the calcified zone were measured in a projection microscope at the time of dissection and after 7 days of culture. The calcified zone appears as a dark zone in the metatarsal bones. It consists of the bony shaft and the calcified cartilage matrix on both sides of the enlarging marrow cavity (Figure 1). By staining the metatarsal bones with alizarine red after 7 days of culture, we confirmed that the dark area measured was indeed calcified.

The relative increase in length of both the calcified zone and the total bone was calculated from each metatarsal bone by dividing the values on Day 7 by the values on Day 0 (Day 0 = 100).

To determine whether calcified-zone growth and total length growth were influenced to the same extent, the ratio calcified zone/total length on Day 7 was also calculated for each metatarsal bone.

Histology. After culture, the metatarsal bones were either fixed for 24 hours in a 4 per cent neutral formaldehyde solution and block stained with alizarine red S (Searle Diagnostic), which selectively stains calcium, or were fixed and decalcified in Bouin Hollande (Romeis 1968) for 14 days after which longitudinal paraffin sections of 5 μm were cut and stained with hematoxylin and eosin.

The proximal and distal ends of metatarsal bones of 18-day-old fetal mice are composed of small chondrocytes; the intermediate zones consist of flattened chondrocytes; and the midshaft consists of a calcified cartilage with hypertrophic chondrocytes. The midshaft is surrounded by a thin collar of bone. A small marrow cavity is developing as a result of the resorptive activity of invading osteoclasts.

Statistics. Differences between the groups were analyzed by the Student's t test for unpaired data. The P values given were found by the two-tailed tests and the differences were considered significant if $P < 0.05$.

Table 1. Measurements of metatarsal bones (MB) at the time of dissection and after 7 days of culture[a].

	Control MB	Cultured MB	
		with muscle	with skin
	(N = 53)	(N = 43)	(N = 51)
Day 0			
length of total MB	1.71 (0.13)	1.68 (0.12)	1.72 (0.11)
length of calcified zone	0.46 (0.06)	0.45 (0.07)	0.46 (0.06)
Day 7			
length of total MB	2.37 (0.23)	2.40 (0.17)[b]	2.22 (0.18)[c]
length of calcified zone	1.07 (0.15)	1.02 (0.15)[b]	0.87 (0.18)[c]

[a] Values are given as mean (SD) mm.
[b] No significant differences were found between the muscle group and the control group.
[c] All values of the skin group were smaller than those in the control group and muscle group ($P < 0.01$).

Results

After 7 days of culture the total lengths of the metatarsal bones were smaller in the skin group than in the control and muscle groups (Table 1). The relative growth in length of each metatarsal bone in the skin group was smaller than in the muscle and control groups (Figure 2).

The lengths of the calcified zones and the relative calcified zone growth were smaller in the skin group than in the muscle and control groups.

The ratio calcified zone length/total length on Day 7 was smaller in the skin group (0.39 ± 0.09) than in the muscle group (0.43 ± 0.06) and the control group (0.45 ± 0.05). Whereas the difference from the control group was highly significant ($P < 0.001$), the difference from the muscle group was only marginally so ($P < 0.05$).

Histology. After 7 days of culture, the cells in the metatarsal bone had a normal healthy appearance (Figure 3). No qualitative differences between the metatarsal bones of the different subgroups were seen. The two growth plates moved away from the center. The enlarging marrow cavity was filled with loose and fibrous tissue and several osteoclasts were observed. The shaft consisted of a zone of residual cartilage, which was covered on both sides with bone matrix and a layer of basophilic osteoblasts (Figure 3). The marrow cavity appeared somewhat smaller in the skin group.

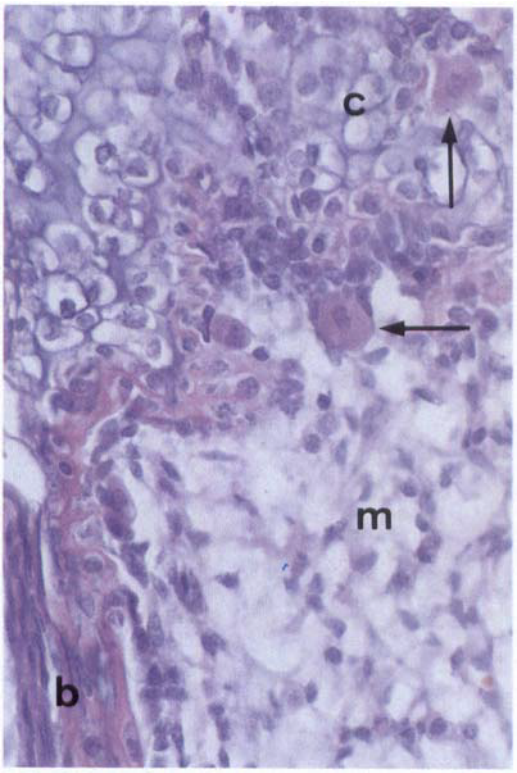

Figure 3. Metatarsal bone cultured for 7 days with skin fragments. m marrow cavity with stromal cells. c calcified cartilage in growth plate with osteoclasts (arrows). b bone collar with periosteum. Hematoxylin-eosin, x200.

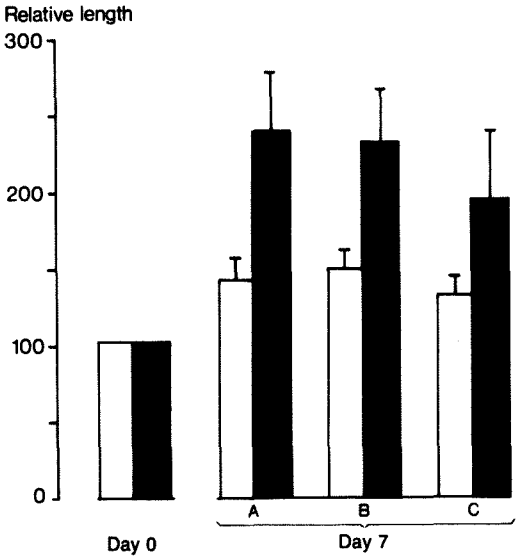

Figure 2. Relative growth of total length and calcified zone length (length Day 7/length Day 0), Day 0 = 100. Empty columns represent the total length and filled columns the calcified zone length; standard deviations are indicated by bars. A control group, B muscle group, and C skin group.

From the skin fragments, layers of fibroblast-like cells spread over the cellophane. The fragments formed spherical structures. Skin structure was maintained and a stratum corneum was formed. No necrosis of tissue was observed (Figure 4).

Cells of the muscle fragments dedifferentiated into fibroblast-like cells and spread over the cellophane (Figure 5).

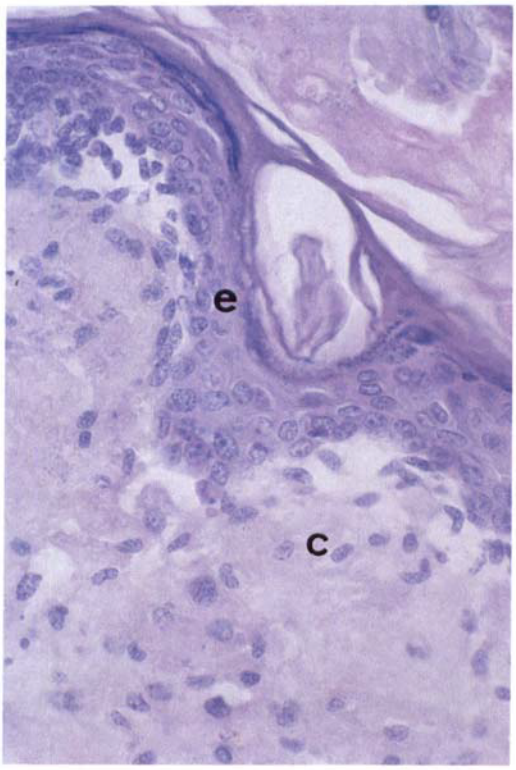

Figure 4. Detail of a skin fragment cultured for 7 days with preserved structure. e epidermis. c corium. Hematoxylin-eosin, x200.

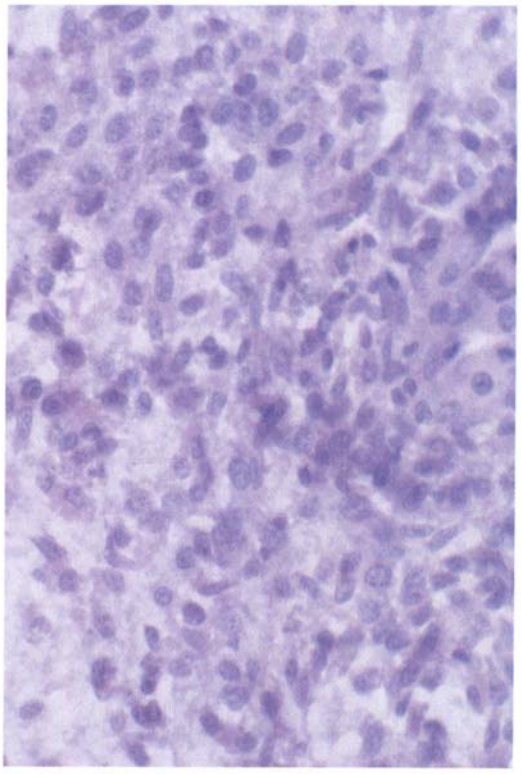

Figure 5. Detail of a muscle fragment cultured for 7 days. Muscle cells have dedifferentiated into fibroblast-like cells. Hematoxylin-eosin, x 200.

Discussion

This study reports an inhibititing effect of fetal skin, but not of muscle, on the total length growth and calcification in vitro of 18-day-old fetal mouse metatarsal bones.

The observed effects could be caused nonspecifically by withdrawal of nutrients or accumulation of waste products. The cells of the skin fragments could use certain nutrients in the medium more than cells of muscle fragments, causing a deficiency, which influences the growing metatarsal bones. They could also produce more waste products. We think, however, that it is unlikely that the observed effects were all caused by such factors. The amount of medium we used can support the normal growth of six metatarsal bones and soft-tissue fragments (Thesingh & Burger 1983): all tissues had normal histology, and there were no differences between the muscle group and the control group, even though the muscle fragments were larger than the skin fragments.

Our results therefore suggest that skin produces factors that are responsible for the inhibition; it is uncertain whether the factors from the skin fragments were produced in the epidermis or the dermis.

It is difficult to compare our results with other studies performed in earlier embryonic stages. The membranous bone induction in the maxillary region of chick embryos occurs in a special tissue (neural crest-derived mesenchyme) and only epithelial tissues of a certain age have this inductive effect (Hall 1981).

Ectoderm induces cartilage formation in vitro in wing mesoderm of young chick embryos; but after a certain stage of cartilage development, the presence of ectoderm is no longer necessary (Gumpel-Pinot 1980). The role of ectoderm in chondrogenesis is unclear (Nathan-

son 1985). Little is known about the influence of skin on developing long bones.

The results of our study indicate that factors from fibroblasts or epithelial cells of the skin fragments delay the growth and differentiation of fetal long bone models consisting mostly of chondrocytes.

A recent hypothesis (Hulth 1980) suggests that the slower healing of fractures with extensive soft-tissue damage is caused by competing healing factors: soft tissue heals with fibrous scar tissue and fractures heal by bone regeneration. This hypothesis postulates that the damaged tissues produce factors that stimulate undifferentiated mesenchymal cells to differentiate: factors from soft tissue stimulate differentiation into fibrous tissue, factors from bone stimulate differentiation into bone-forming tissue. In fractures with extensive soft-tissue damage, both factors compete with the risk of delayed healing due to dominance of cells differentiating into fibrous tissue. It is possible that skin not only inhibits chondrocytes in developing long bones, but also inhibits chondrocytes in callus, and thus plays a role in delayed fracture healing.

Although care must be taken in comparing primitive cells in developing bones to primitive cells involved in fracture healing and in extrapolating results of in vitro experiments to the in vivo situation, our results are some support for the hypothesis of competing healing factors.

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