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MORPHOLOGICAL STUDIES OF FRACTURE HEALING IN TISSUE CULTURE

By

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The tissue culture technique has been used for morphologic studies of fracture healing on rare occasions by earlier authors (*Niven* 1931, *Krull* 1936, *Weil* 1951, *Bucher* 1952), and then only the regenerative properties of embryonic bone have been studied. Tissue culture is, however, a method of experimental biology which seems advantageous for such studies. Contrary to studies *in vivo*, the tissue culture technique allows the process of morphologic and biochemical differentiation of fracture healing to take place and develop in a simplified and well defined system, not influenced by local and general factors occurring in the experimental animal. Furthermore, the experimental conditions are more or less standardized during the healing process and can be modified at will.

The present communication is a report of an investigation undertaken to study the morphological process of regeneration of adult and embryonic bone in tissue culture. Furthermore, in a series of cultures an attempt has been made to influence the rate of the regenerative process by adding RNA to the culture medium. RNA has been reported (*Emanuelsson* 1960) to possess differentiation promoting biological properties.

METHODS AND MATERIALS

The "watch-glass" tissue culture method of *Fell & Robison* (1929) was used throughout. A solid homologous medium was used in all the cases and it was composed of rooster plasma and freshly prepared tissue extract derived from 12-day old chick embryos. The plasma and tissue extract were suspended in 1 % glucose–enriched Tyrode's solution in a proportion 12: 1: 3 respectively. The cultures were transferred to fresh medium 3 times a week and were incubated at 37° C.[1]

[1] For details regarding the tissue culture technique, the reader is referred to a previous work by the author (1958).

In one group of cultures a small amount (3 drops) of a 0.26 mg./ml. solution of yeast RNA was added to the medium.

The cultures were derived either from adult roosters or from chick embryos. Cultures from adult bone were prepared as follows: The ribs of young roosters were dissected free from adherent soft tissues and the majority of the periosteal membrane. Then they were divided by vertical sections in small pieces of about 2 mm. of length. Each piece of bone was further divided by transversal section into two bits. The bone fragments were first bathed for some minutes in a Tyrode's solution to remove small soft tissue particles, then the endosteal surfaces were pressed gently together and the adherent fragments were finally explanted to the previously prepared culture medium.

Embryonic bones were obtained from chick embryos at the 18th day of incubation. The ribs were also used in this case. They were dissected in a similar way and were divided in 3–5 mm. long pieces. Each piece of bone was then bent with forceps so that the cortical bone was broken on one side. In this way a "green-stick" type of fracture was produced. Explantation on the culture medium followed after rinsing the bone pieces for a few minutes in a bath of Tyrode's solution.

Preparation of the cultures was always performed under strict conditions of sterility. No antibiotics were added to the medium.

The migratory activity of fibroblastic cells was studied in all cases and recorded according to the same principles followed in an earlier investigation (*Sevastikoglou*, 1958).

Cultures were selected for sectioning every 5th day up to the 70th day of cultivation. They were fixed in a 10 per cent neutral formaldehyde solution and later decalcified for a short time with hydrochloric acid. The specimens were sectioned serially at 5 μ thickness, and haematoxylin-eosin and Azan stains were used.

Altogether 158 cultures were studied and they were divided into the following groups: a) 20 cultures derived from adult bones, b) 100 cultures derived from embryonic bones and c) 38 cultures derived from embryonic bones and grown in a medium containing RNA.

RESULTS

68 cultures in groups *b* and *c* were destroyed by infection or accident. No casualties occured in group *a*. The total of the remaining 90 cultures of all the groups were sectioned for histological studies. However, in one culture of group *a* and in seven of group *b* and *c* no signs of fracture could be found in the serial sections, probably because of sectioning in a wrong direction. Furthermore, 33 out of the 70 sectioned cultures of embryonic bone were destroyed by wrinkling and breaking of the specimens so that no details for comparative studies could be recorded in these cases. Thus only 19 cultures of group *a*, 21 of group *b* and 9 of group *c* were available for the histological study. Table 1 demonstrates the distribution of the material.

Migratory activity of the cells appeared very early in the cultures of group *a*. Already during the first subcultivation—3rd day—there were

clear signs of radial migration of cells from the greater parts of the surface of 15 out of 20 cultures of this group. At the end of the 3rd week a thick halo of migrating cells was present in all the cases.

TABLE 1
Distribution of the Material and Results.

Culture group	No of cultures	No of studied cultures	Formation of periosteal capsule	Presence of osteoid tissue
a	20	19	18 (94.6 %)	10 (55.5 %)
b	100	21	10 (47.5 %)	7 (70.0 %)
c	38	9	6 (66.6 %)	0

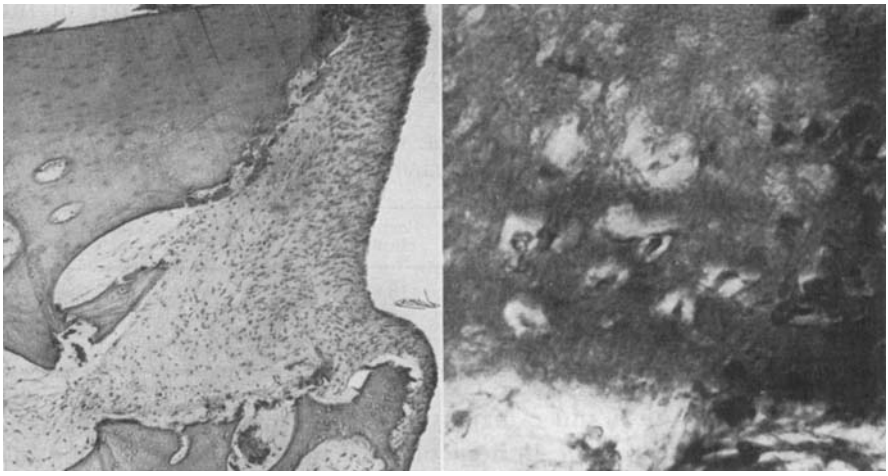
Cultures of group *b* and *c* displayed a poorer activity of migrating cells than those of group *a*. In none of them was a real halo of migrating cells present throughout the period of cultivation. In 90 out of the total of 138 cultures of both groups no migratory activity of cells could be registered during the period of cultivation. In the remaining cases only radial migration from a limited area of the periphery of the cultures, and in very few cases from the greater parts of their periphery could be registered.

Histological studies on cultures from adult bone: In histological sections the specimens are composed by two pieces of mature bone placed on each other. Compact bone is identified on the outer surface of the fragment and cancellous bone on their sides fronting each other. An interspace exists between the fragments in which small pieces of broken bone trabeculae are often found.

In the specimens grown in tissue culture for 5 days the bone fragments are composed in their greater part by living bone but small areas of devitalized bone were recognized in some places. A thin layer of fibrous capsule was visible on the superficial surfaces of the fragments. Numerous migrating fibroblasts were found within the interspace between the bone fragments. These cells formed a thin network, the appearance of which remained unchanged throughout the time of cultivation.

By increasing the time of cultivation the areas of devitalized bone increased and at about the end of the 2nd week both fragments were often found almost entirely devitalized.

The formation of the fibrous layer soon increased and both fragments were encased in a well developed capsule in all specimens after about 10 days of cultivation. At this stage the fibrous capsule had a



a. *b.*

Fig. 1.

Adult bone culture in tissue culture for 59 days.

a. The two bone fragments are connected by the fibrous capsule. Osteoid tissue arising from the inner layer of the capsule fills the one end of the "fracture" interspace and fuses the fragments. The bone has devitalized appearance. Haematox.-eosin. × 65.

b. The osteoid tissue under high magnification. Azan. × 650.

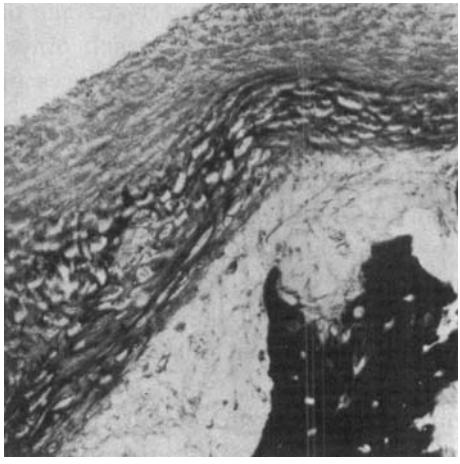


Fig. 2.

Adult bone culture grown in tissue culture for 18 days. Osteoid tissue laid down by the periosteum. Azan. × 260.

typical morphologic character of the periosteum with an inner osteoblastic and an outer fibrous layer.

After about the 10th day of cultivation osteoid tissue usually appeared

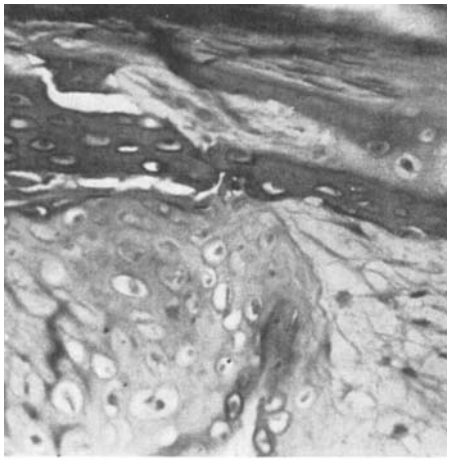


Fig. 3.
Embryonic bone culture grown in tissue culture for 10 days. The fractured embryonic trabecula is composed by living bone. The periosteal capsule is visible at the top. A network of migrating fibroblasts (down right) and part of the cartilage cone (down left) fills the medullary cavity. Haematox.-eosin. × 260.

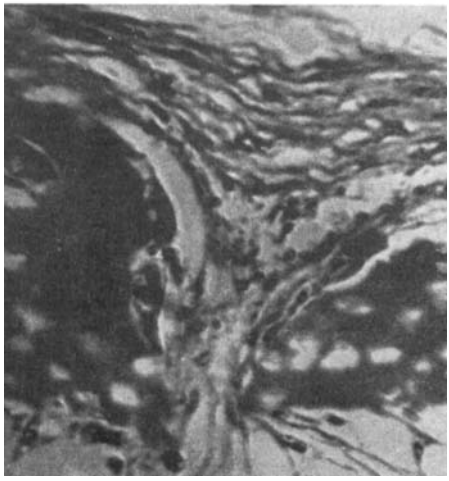


Fig. 4.
Embryonic bone culture grown in tissue culture for 20 days. Migrating cells arising from the periosteal capsule fill the fracture space. Azan. × 260.

at the ends of the interspace. The osteoid tissue originated from the osteoblastic layer of the periosteal membrane and tended to fuse the bone fragments at their ends (Fig. 1). In a few cases some osteoid tissue was laid down under the periosteum, on the superficial surfaces of the fragments (Fig. 2).

In 10 out of the 18 specimens in this group which developed a periosteal capsule, osteoid tissue could be recognized in one or both poles of the interspace.

Histological studies on cultures from embryonic bone: The sectioned cultures both in group *b* and *c* were in all the cases composed of healthy

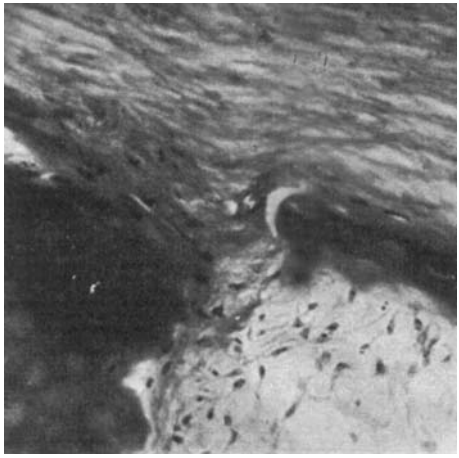


Fig. 5.
Embryonic bone culture grown in tissue culture for 15 days. Osteoid tissue arising from the periosteal capsule (upper) fills the fracture space. Azan. × 260.

embryonic bone trabeculae. No signs of devitalization of the bone were observed in these cultures even after growth in tissue culture for longer periods up to 70 days. The medullary cavity was found in the majority of the specimens to include marrow elements and some migrating fibroblasts. In some cases, however, it was filled by hypertrophic and degenerated chondrocytes of the cartilaginous cone, while resting, flattened, chondrocytes were not present in any specimens (Fig. 3).

As the "fracture" was produced by bending the bone, the specimens of these groups had a special appearance. A rupture of the continuity of the periosteal bone, usually composed by a single embryonic bone trabecula, was seen in the convexity. A slight diastasis between the fragments of the trabecula was often present. A compression and breaking of the trabecula, without dislocation, was usually present in the concavity. In some cases the trabecula in this side was bent but not broken. This characteristic appearance could not, however, be identified in all the specimens, probably because of sectioning through different planes.

A fibrous layer began to form very early in the culture of these groups and it was appreciably thicker at the site of the concavity. In a great number of the studied specimens a well developed capsule was present after 5 to 10 days of cultivation. The fibrous capsule had the characters of periosteum and the inner osteoblastic layer had already differentiated at this stage. Migrating fibroblasts were often seen within the fracture space (Fig. 4).

Osteoid tissue also appeared at early stages of cultivation as a very

thin layer localized under the periosteum in the convexity of the specimen near the fracture. At later stages of cultivation the fractured trabecula was bridged over by osteoid tissue, which in some cases filled the fracture space (Fig. 5). There were no signs of activity of the endosteal elements, the appearance of the medullary cavity remaining unchanged throughout the period of cultivation.

As a whole the number of sectioned cultures of group *b* which developed a periosteal capsule was rather low. Thus, in only 10 out of 21 of the specimens in this group could a well-developed capsule be recognized. The respective figure for cultures of group *c* was 6 out of 9 studied specimens. Osteoid tissue was found to exist in 7 out of 10 specimens of group *b* in which a capsule had developed. In the 6 cultures of group *c* in which formation of a periosteal layer was present no signs of osteoid tissue could be identified in the serial histological sections. Both the formation of periosteal capsule and the development of osteoid tissue appeared in these groups as early as the 5th day of cultivation. These activities were, however, appreciably rarer in specimens of later age of cultivation.

DISCUSSION

The results reported above demonstrate that there are several differences in the behavior of the cultures in the various experimental groups. Likewise there are differences between the cultures of the various experimental series in the development of the process of fracture healing during growth in tissue culture.

Contrary to cultures of group *b* and *c*, those belonging to group *a* displayed an early and intense activity of migrating cells. Migrating cells probably originated from periosteal remnants, which had remained adherent on the surface of the bone fragments despite careful dissection. Fibrocytes of the periosteal remains migrate into the medium as fibroblasts and by proliferation form a fibrous capsule covering the culture. The capsule soon differentiates into a fibrous and an osteoblastic layer and a new periosteum is thus formed. As periosteum is more adherent to adult bone it can be assumed that cultures of embryonic bone had less periosteal remnants and therefore they displayed a poorer migratory cell activity. On the other hand it is also possible that the very thin embryonic bone used in group *b* and *c* was exposed under dissection to comparatively larger mechanical injury than the adult bone used in group *a*. An impairment of the regenerative capacity of the embryonic bone can therefore be suspected.

The periosteal capsule was formed in a parallel way with the migratory activity of the cell. Cultures of group *a* which showed early and intense migratory cell activity developed a capsule very soon and in a higher percentage than cultures of group *b* and *c*.

Once the periosteal capsule had developed the process of healing began in the cultures and resulted in the formation of osteoid tissue of periosteal origin. A relationship between the development of the periosteal capsule and the production of osteoid tissue is supported by the fact that no osteoid tissue could be found in specimens which did not develop a periosteal capsule. The frequency of periosteal capsule formation in the three groups was, 94.6 % of the cultures in group *a*, 47.5 % in group *b*, and 66.6 % in group *c*. The frequency of osteoid formation in the cultures showing a periosteal capsule was highest in group *b* (70 %) followed by group *a* (55.5 %). No osteoid tissue was seen in any of the sectioned specimens in group *c*, thus there was no evidence of osteogenic healing properties in this group. (See table 1).

The number of the studied cultures in the three groups is too small to allow definite conclusions. Furthermore the comparative value of the results described is reduced because the material used and the type of injury applied were different in the cultures of the various groups. However, the differences of the results reported here may indicate that although cultures of adult bone show an intense activity of migrating cells and form a well developed periosteal capsule in a high percentage, cultures derived from embryonic bone and grown under identical experimental conditions (group *b*) show a stronger regenerative activity and form osteoid tissue more frequently. This fact must be attributed to a greater regenerative power in cultures of embryonic bone.

Cultures derived from embryonic bone, but grown in a medium containing yeast—RNA failed to display development of osteoid tissue in spite of a high incidence of periosteal capsule formation. This is remarkable in view of the observation reported above, that a relationship exists between capsule formation and osteoid tissue production. Apparently capsule formation is a requisite for the production of osteoid tissue, but the capsule may or may not do so. It seems that a well developed periosteal capsule can be inhibited from producing osteoid tissue.

Another difference between cultures of group *a* and these of group *b* and *c* was the observation that extensive areas of the bone in group *a* suffered devitalization already during the early stages of cultivation. Cultures of the latter groups were composed exclusively by vital bone even at the latest stages of cultivation. The reason for this difference

is probably the different size of the cultures in the two groups. Cultures of adult bone were considerably larger than these of embryonic bone. The conditions for nutrition in tissue culture of small bone fragments in group *b* and *c* were much more favourable than for the large fragments in group *a*.

By adding RNA in culture medium in group *c* the intention was to promote the rate of the regenerative process of the cultures. However under the prevailing experimental conditions, the RNA had an inhibitory rather than promoting effect on the fracture healing activity of the cultures, but no generalized conclusions should be drawn from the reported results regarding the action of RNA on the regenerative properties of the cultures. It has been proved that pure RNA in itself has the capacity of stimulating growth and differentiation in early chick embryos growing in tissue culture, the effect being dependent, however, on the origin of the RNA used (*Emanuelsson* 1960). The RNA used in the present experiments was probably a denaturated unpure product neither organ-specific nor homologous. Weil (1951) found that heterologous "Ossopan", i.e., powdered whole bones, added to the culture medium showed a clear promoting effect on the healing rate of fractured chicken embryonic bones grown in tissue culture. Her results may be attributed to the existence of heterologous organ-specific RNA in the used preparation.

The healing process in the studied specimens of group *a* and *b* was restricted to the formation of osteoid tissue. It has been stated earlier that formation of mature architecturally recognizable bone cannot be obtained in tissue culture (*Amprino* 1956, *Sevastikoglou* 1958). On the other hand in short-termed experiments of fracture repair in tissue culture, healing by formation of mature bone has been reported when embryonic bone of early stages of incubation, 5 to 16 days, was used (*Nivon* 1931, *Krull* 1936, *Weil* 1951 and *Bucher* 1952). Since the material and methods used by these authors were almost identical to those used in the present study an explanation for the discrepancy in the results could be the age of the embryonic bone used. Perhaps the regenerative capacity of the embryonic bone varies considerably during the different stages of embryonic development, and experimental investigation of this possibility is required.

The described results suggest that under the prevailing experimental conditions the process of bone regeneration of the adult as well as the embryonic bone can develop in tissue culture, in the absence of periosteum, nerves and vessels and independently of central healing mechan-

isms. The process is, however, limited to the formation of osteoid tissue, and it, therefore, might be assumed that the final phase of bone regeneration in tissue culture is dependent on local or general mechanisms which act *in vivo* but are not available in tissue culture.

SUMMARY

Experimental fractures in adult and embryonic bone displayed a regenerative activity in tissue culture. Under the prevailing experimental conditions the healing process was limited to the development of a periosteal capsule and the formation of osteoid tissue of periosteal origin. The process developed in spite of the lack of nerve and vessel supply to the bone fragments and without the influence of other local or systemic mechanisms occuring in the living organism.

Fractures produced in embryonic bone showed a higher incidence of osteoid tissue formation. Adult bone cultures developed a periosteal capsule earlier and more frequently. No osteoid tissue was observed in cultures which did not develop a periosteal capsule.

The regenerative activity of the cultures was displayed by the formation of osteoid tissue of periosteal origin. The bone fragments showed no active participation in the healing process.

The addition of non-specific RNA to the nutrient medium had an inhibitory effect on the differentiation of the periosteal capsule and the formation of osteoid tissue.

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RESUME

Les fractures expérimentales chez les adultes et dans les os embryonnaires manifestent une activité régénérative dans les cultures de tissu. Dans les conditions expérimentales du moment, le processus a été limité au développement d'une capsule périostale et à la formation de tissu ostéoïde d'origine périostale. Le processus se développe malgré l'absence de nerfs et de vaisseaux pour approvisionner le fragment osseux et sans l'influence d'autres mécanismes locaux ou systémiques apparaissant dans l'organisme vivant.

On a pu constater que la formation du tissu ostéoïde était plus fréquente lorsqu'il s'agissait d'une fracture d'un os embryonnaire. Dans

les cultures d'os adulte, il se développe plus tôt et plus fréquemment une capsule périostale. Aucun tissu ostéoïde n'a été observé dans les cultures où il ne s'est pas formé de capsule périostale. L'activité régénérative de la culture s'est manifestée par la formation de tissu ostéoïde d'origine périostale. Il n'est pas arrivé que les fragments d'os avaient une participation active au processus de la guérison.

L'addition de RNA non-spécifique au milieu nutritif a un effet inhibitif sur la différenciation de la capsule périostale et la formation de tissu ostéoïde.

ZUSAMMENFASSUNG

Experimentelle Brüche von Erwachsenem- und Embryoknochen zeigten eine regenerative Aktivitet in Gewebskulturen. Unter den vorherrschenden Versuchsbedingungen war der Heilungsprozess auf die Entwicklung einer periostalen Kapsel und der Bildung von osteoidem Gewebe periostalen Ursprungs begränzt. Der Prozess entwickelte sich trotz des Fehlens einer Nerven- und Gefässversorgung zum Knochenfragment und ohne den Einfluss anderer lokaler oder Systemmechanismen, die im lebenden Organismus vorhanden sind.

Brüche, die an Embryoknochen hervorgerufen wurden, zeigten ein häufigeres Vorkommen von osteoider Gewebsbildung. Gewebskulturen von Erwachsenen entwickelten eine periostale Kapsel frühzeitiger und häufiger.

Die regenerative Aktivitet der Kulturen wurde durch die Bildung von osteoidem Gewebe periostalen Ursprungs aufgezeigt. Die Knochenfragmente wiesen keine aktive Teilnahme am Heilungsprozess auf.

Die Hinzufägung von unspezifischem RNA zu dem Ernähfungsmedium hatte eine hemmende Wirkung auf die Differenzierung der periostalen Kapsel und die Bildung von osteoidem Gewebe.

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