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DEVELOPMENT OF ELASTIC ELEMENTS IN TISSUE CULTURES

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The genesis of connective tissue fibres is a much debated, and as yet unclarified, problem. According to one the widely accepted theories, fibres are formed from cells, while some authors suggest that they originate from the ground substance. The advocates of the ectoplasm doctrine, in their effort at finding a common denominator of the two theories, suggest that the ground substance would take its origin from the ectoplasm produced by cells, while the fibres are differentiated from this ground substance. *Huzella* [21] has put forward a peculiar theory suggesting that the ground substance is a cellular secretion product remaining in the organism, being neither eliminated by the glands nor passed into the circulation. He concluded from his studies of argyrophil fibres that these originate from the intercellular substance along the lines of bearing force. He agreed with the view that cells do participate in the formation of the fibre network. For detailed information, the papers by *Krompecher* [5], *Wassermann* [15], *Haas* [4], *Studnicka* [14] and by *Revutskaya* [13] should be consulted.

The causal-analytical theory of the differentiation of living matter considers histogenesis as a dependent differentiation. Without going into theoretical details, from the functional structure of connective tissue formations it appears most probable that in the genesis of connective tissue fibres mechanical factors play an important rôle. As to the genesis of elastic fibres, it has been claimed that the factor responsible for the differentiation of such fibres would be a periodically repeated contraction and relaxation, viz. a so-called intermittent traction (e. g. pulsation). In support of this view are the results of embryological, and some experimental studies. So, according to *Linser* [8], in the lung elastic fibres would be formed only after birth, i. e. after respiration has started. *Marcus* [9], on the other hand, has demonstrated elastic fibres in the lung of an embryo about 3 and a half months old. This, however, does not exclude the rôle of the mechanical factors, it being well-known that respiratory movements actually take place during intrauterine life. *Krompecher* [5], demonstrating the first appearance of elastic elements on the surface of elastoblasts in the carotid artery of a 24 mm. sheep embryo, and presenting

coloured microphotographs of this finding, wrote that «in accordance with vascular tension — which increases on each heart contraction — the elastic elements take up the form of a uniform circular membrane». Elastic fibres, on the other hand, are formed from the primitive pericellular membranes which are thickened at sites corresponding to the lines of force of contraction. Thus, *Krompecher* recognized as early as 1928 the influence of mechanical factors on the formation of the elastic substance, but failed then to establish a correlation between intermittent traction and the development of elastic elements. *Bloom* [3] observed the development of elastic fibres in pulsating heart cultures. He thought, however, that contractile forces were not absolutely necessary to the development of elastic fibres, as newformation of elastic elements occurs in aorta cultures too although he did not fail to observe that development is most marked where there is pulsation. *Bloom* found that the elastic fibres developing in tissue cultures were always extracellular, but presented no definite view as to the origin of these fibres. *Porta* [12] claimed on the basis of explantation experiments that mechanical factors have no part whatsoever in the genesis of elastic fibres. He, too, found the fibres extracellularly, but pointed out that they were invariably in a very close connection with the cells. *Odiette* [10, 11] observed formation of elastic elements in heart, aorta and skin cultures. According to this author, the elementary elastic fibres appear first in the ectoplasm of elastoblasts, then after penetrating into the cellular processes, anastomoze with the fibrils of adjacent cells. He also found that the formation of elastic fibres is accelerated on addition to the culture of certain amino acids (glycine, tryptophane, beta-phenylalanine, glutamic acid) and concluded that changes in the chemical composition of the medium affect the development of elastic fibres in the same way as do mechanical factors. In his work published in 1940, *Krompecher* [6] wrote the following about the conditions required for the formation of elastic elements: «Bei der Arterie scheint die pulsierende Wirkung, eine intermittierende dehnende Kraft die auslösende Ursache zu sein, wodurch die wandbildende Mesenchymzellen — zuerst zirkulär angeordnet — ihrer längsachse entsprechend gedehnt und nachher etwa auf die ursprüngliche Länge nachgelassen werden. Dieses Zerren, Anziehen scheint hier für die Elasticabildung der specifische Reiz zu sein». These conclusions were drawn from comparative histogenetical studies of various portions of the vascular wall, but at the same time it was emphasized that these assumptions would have to be substantiated experimentally.

Our purpose in studying the genesis of elastic fibres was to gain information about fibre differentiation and to clarify the role of mechanical factors under various biological conditions. The present paper, representing the first part of a series of experiments on a large scale, will discuss the genesis *in vitro* of elastic fibres.

Materials and Methods

Cultures were made of the hearts of 3 to 7 days old chick embryos, according to *Maximov*'s method, in about 30 culture series, with 12 to 20 explantates in each series. The medium was chicken plasma + Tyrode + chicken embryo extract, mixed 1 : 1 : 1. As in a medium containing such a large amount of embryonic extract the intensity of pulsation of heart cultures diminishes rapidly and cannot be observed after 3 to 7 days, in one series no embryonic extract was added to the medium, or its amount was reduced to a minimum (1 : 1 : approximately 0,1). In this way pulsation was continued for several weeks. The cultures were bathed at 48 hour intervals in a Tyrode solution containing embryonic extract. Duration of pulsation was carefully recorded several times a day and after pulsation had ceased, the cultures were allowed to grow for different lengths of time. The cultures were fixed in Susa's fluid, embedded in paraffine and cut serially. Staining was with resorcin-fuchsine-alumcarmine-erythrosine.

Aorta cultures were made from material taken from embryos of the same age group. After these were allowed to proliferate for different periods of time, the material was worked up in a similar way as were the heart cultures.

In one series elastase *(Baló—Banga*, 2) was added to the medium in a concentration of 0,05 mg/ml. Since it has been reported *(Alin* and *Helander*, 1) that paraaminosalicylic acid is electively absorbed by elastic fibres, in a few experiments the pulsating heart cultures were treated with sodium paraaminosalicylate and examined in the living state under a luminescence microscope.

Results

a) *Heart cultures*

Irrespective of the actual age of the chick embryo, in pulsating heart cultures made with material taken between the 3d and 7th days of hatching newly formed elastic elements could be found in every case. Although in control sections made of the explanted hearts no elastic fibres could be detected, only the elastic elements in the proliferation zone were considered when evaluating the findings. We could not demonstrate any relationship between the duration of pulsation and the quantity of elastic elements formed. In some cases more elastic elements were detected in cultures pulsating for shorter periods than in those with prolonged pulsation. Nor was there a demonstrable correlation between the age of the chick embryo and the quantity of newly formed elastic elements. In cultures not pulsating at all after explantation no neoformation of elastic material could be detected.

The close connection between elastic elements and cells was evident in every case. The elastic elements appeared either as fine pericellular fibres or a pericellular membrane staining with resorcin-fuchsine. This membrane was split up into fibres in some areas (Fig. 1). Elastic fibres resulting from merged elastin granulae could be detected in no case. Older fibres detach themselves from the cells and take up an intercellular position (Fig. 2). No signs indicative of endocellular fibre formation were seen, nor could we detect any kind of elastic fibre formation in areas not containing cells.

Cultures made without, or with very small amounts of, embryonic extract pulsated energetically for prolonged periods of time (3 to 4 weeks), but proliferation was unusually scarce. In such cultures elastic elements were either totally

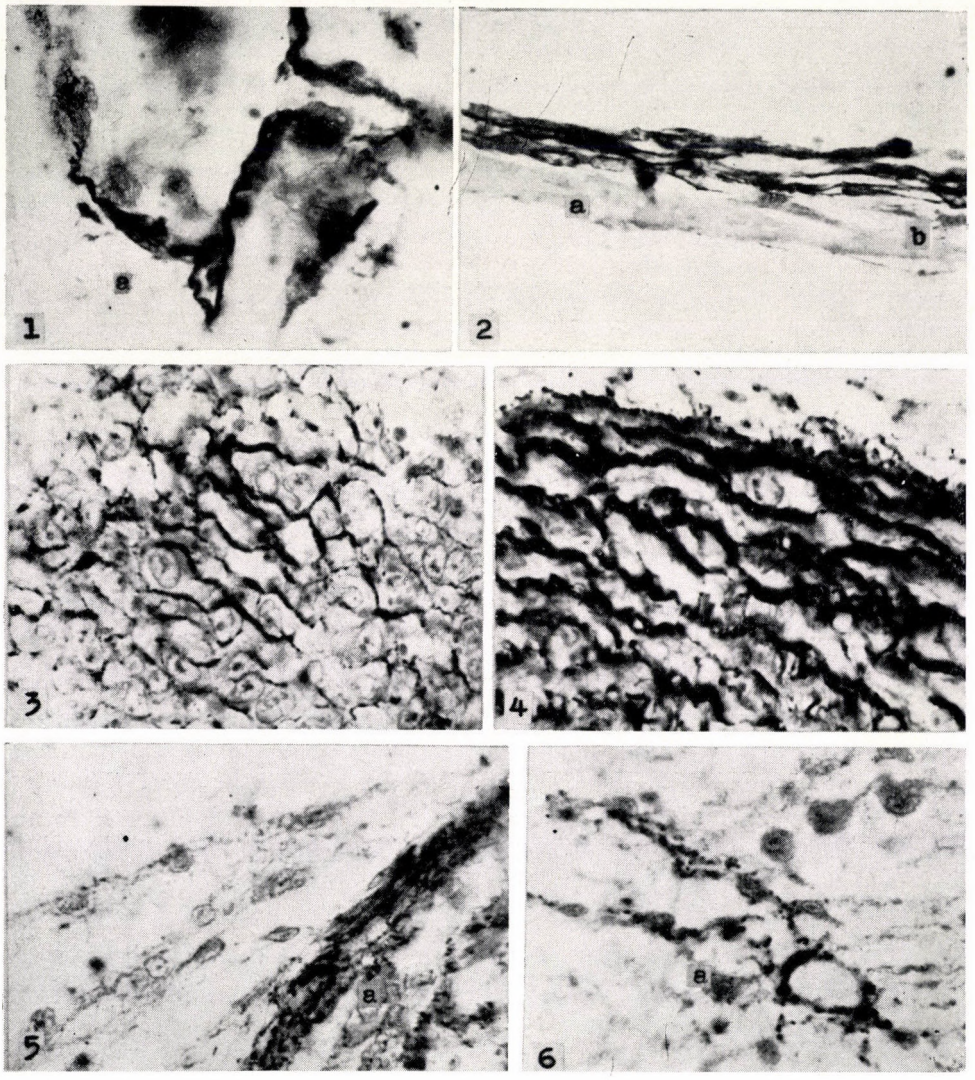

Fig. 1. Culture from the heart of a 7-day old chick embryo. Pulsated for 6 days. a.: pericellular elastic membrane split up into fibres. Staining resorcin-fuchsine-alum carmine-erythrosine. Leitz Periplan 10 × eyepiece ; Leitz H. I. 100× lens

Fig. 2. Culture from the heart of a 7-day old chick embryo. Pulsated for 3 days. Elastic fibres in the zone of proliferation in connection with cells (a) and independent of cells (b). Leitz Periplan 10× eyepiece ; Zeiss apo. 40× lens. Staining as in Fig. 1

Fig. 3. Aorta from chick embryo hatched for 6 days. Staining as in Figs 1 and 2. Leitz Periplan 10× eyepiece ; Leitz H. I. 100× lens

Fig. 4. Aorta from chick embryo hatched for 7 days. Magnification and staining as in Fig. 3

Fig. 5. Aorta from 7-day old chick embryo after 3 days of growth in culture. a.: elastic fibres showing granular disintegration. Magnification and staining as in Fig. 3

Fig. 6. A section from the proliferation zone in Fig. 5. a.: elastin granules adsorbed onto cell surfaces. Magnification and staining as in Fig. 3

absent or could be detected by the above described method only in very small amounts.

Cultures treated with *Baló—Banga* elastase showed no substantial differences. This is thought to be explicable in two ways : either the amount of enzyme used was insufficient, or its effect was inhibited by some agent present in the medium.

Luminiscence microscopy failed to bring evaluable results. All that could be found was that the cultures treated with paraaminosalicylic acid showed a pale, diffuse fluorescence.

b) *Aorta cultures*

In Figs 3 and 4 are shown sections from the aorta stained with resorcin-fuchsine ; the one is from a 6-day old and the other from a 7-day old chick embryo. In both photographs the elastic elements staining very intensely can be excellently visualized as they appear amoung the densely arranged cells. Elastin granules are absent. The pointlike structures staining with resorcin-fuchsine scattered over the preparation are transversal sections of fibres, as indicated by the «Pünktchenwanderung» phenomenon. Figs 5 and 6 show 3-day aorta cultures, both from 7-day old embryos. As compared to the noncultured aorta (Figs. 3 and 4), here the elastic fibres have disintegrated into granulae during growth. At sites, disintegrated elastic elements are seen adsorbed onto cell surfaces.

A piece of aorta left to grow in connection with a pulsating heart culture presents a different pattern. As seen in Fig. 7, the elastic tissue of the aorta remains intact, no sign of disintegration can be observed. On the contrary, at the proliferating end of the aortic tissue newformation of elastic elements is detectable (Fig. 8), in the form of pericellular elastic membranes and elastic fibres.

Discussion

Studies of the development *in vitro* of elastica has, to our knowledge, been studied by *Bloom* [3], *Porta* [12], and *Odiette* [10, 11]. *Bloom* does not think that pulsation would be an absolute necessity in the formation of elastic elements and does not make mention of the connection between elastic fibres and cells. *Porta* refuses to accept that mechanical factors play a role in the genesis of elastic fibres. We have found newly formed elements only in pulsating cultures, but not in aorta cultures or in non-pulsating heart cultures. On the contrary, in aorta cultures granular disintegration of elastic fibres could be demonstrated.

Another observation was that elastic fibres could be detected only in the presence of cells, either in the form of pericellular membranes, or as developed

elastic fibres. (In this we refer to the reports and figures published by *Krompecher* in 1928 and in 1940). In areas containing no cells there is no elastogenesis. Two possibilities should be taken into account. It may be assumed that the elastin

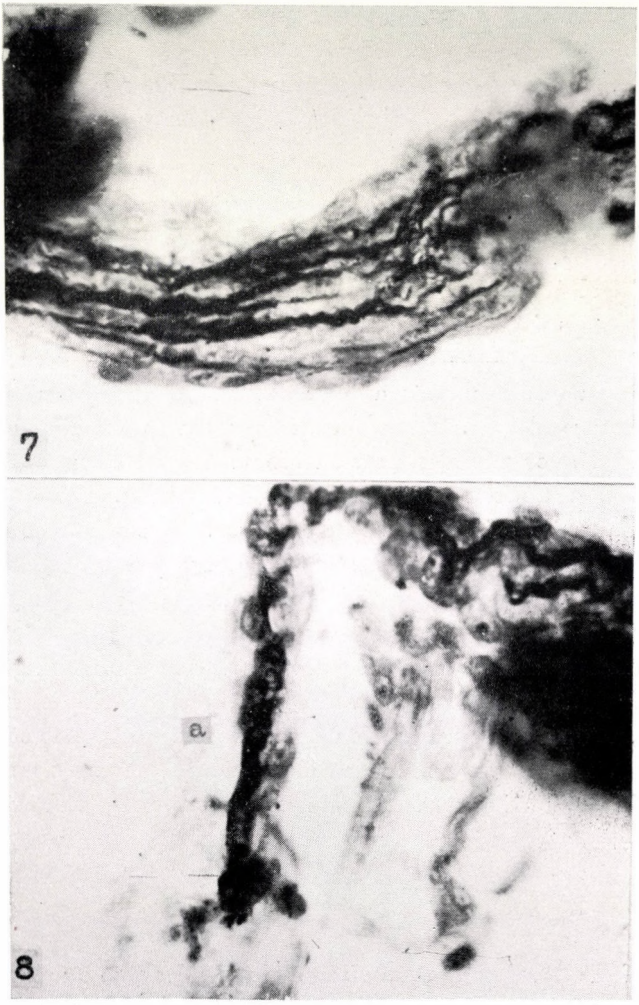

Fig. 7. Aorta from 7-day old chick embryo cultured in connection with pulsating heart culture. (Pulsated for 7 days.) The elastic fibres in the aorta remained intact. Magnification and staining as in Fig. 3

Fig. 8. The proliferating end of the above culture. *a)* Newly formed elastic elements. Magnification and staining as in Fig. 3

present in the intercellular substance is adsorbed onto the surface of cells, or what we deal with are such cells of mesenchymal origin as are capable of forming elastic elements either by formative or by enzymatic action, according to the actual environmental conditions. The fact that no elastic elements occur in

epithelial or nerve tissue, in spite of the conditions for surface adsorption being given, discredit the theory of elastin adsorption onto the surface of cells. We have therefore arrived to the conclusion that the cells of mesenchymal origin play an outstanding role in the genesis of elastic fibres. In support of this view are some more recent results of experimental studies on fibre formation. So, for instance, *Revutskaya* [13] suggests that when an exudate is made to settle, the ectoplasm of the settled fibroblasts forms a syncytium and 10 to 30 minutes after sedimentation preargyrophil fibres differentiate from the ectoplasm. We observed the elastic membranes to form on cell surfaces and therefore believe that cells should be attributed some role in the genesis of fibres. As regards the mechanism of fibre formation and the formative, enzymatic, or other functions presumably attributable to cells, we are unable to make any statements at present. Extensive histogenetical and histochemical studies must be carried out before anything revelant and reliable could be established in this respect.

Mention should be made of the tissue cultures in media containing no embryonic extract. In spite of the fact that they had been pulsating for several weeks, in such cultures, as mentioned above, no newly formed elastic elements could be demonstrated. The above cited results of *Odiette* [11] appear to indicate that a change in the chemical composition of the medium may affect the development of fibrous structures. The phenomenon might be explained as follows. In cultures containing sufficient quantities of embryonic extract the dedifferentiated myoblasts undergo in consequence of the pulsation such a differentiation which would give rise to the formation of elastic elements even after pulsation has ceased. The absence of elastogenesis in cultures containing minute amounts, or no embryonic extract might be explained by assuming that in these cultures the cells remain in the myoblast stage and as such are unsuitable for elastogenesis. The possibility that dedifferentiated myoblasts may be involved in fibrogenesis has been pointed out by *Levi* [7], on the basis of Olivo's work.

In the light of the experiments reported upon in this paper we are unable to support the view of those authors (*Gardner* [16], *Kervily* [17], *Törő* [19], *Szépe* [20]) who consider elastic fibres as structures resulting from confluent elastin granules. Elastin granules, or, more precisely, detritus of elastic material could be detected only associated with degeneration, in accordance with the observation by *Porta* [12] that in aorta cultures of chick embryo digestion of elastic elements takes place. In contrast with this, the observation that in the piece of aorta grown in connection with a pulsating heart culture the elastic fibres remain intact or even show new formation, indicates that pulsation not only plays a predominant role in elastogenesis, but is also the stimulus required for the maintenance of elastic elements.

On the basis of the results obtained it may be stated that elastogenesis takes place in connection with cellular function and under the influence of mechanical

factors (pulsation). The role of pulsation is, however, by no means exclusive. Intermittent contraction is considered, along with other, as yet unknown, biological influences, one of the eliciting factors under the experimental conditions employed.

Summary

The behaviour of elastic elements, as well as the appearance of newly formed elastic elements have been studied in cultures of embryonic chicken heart and aorta. In pulsating heart cultures (chicken embryo hearts from chick embryos of various ages cultured according to *Maximov* in a medium consisting of hen plasma + Tyrode + chick embryo extract, 1 : 1 : 1) elastic fibres developed in the proliferation zone. No correlation could be established between duration of pulsation and amount of elastic elements formed. In cultures not pulsating at all after explanation no newly formed elastic elements could be detected, nor were such formed in media containing no embryonic extract, although in such cases (along with slight proliferation) pulsation could be prolonged for several weeks.

In aorta cultures no newformation of elastic elements occurred and the elastic fibres of the explantate underwent granular disintegration. In pieces of aorta grown in connection with a pulsating heart culture the elastic elements did not disintegrate ; on the contrary, new ones were being formed.

Newly formed elastic elements appeared always in the presence of cells. In the opinion of the authors the genesis of elastic elements is bound to mesenchymal cell function, which, depending on the actually prevailing environmental conditions, form elastic elements either by formative or by enzymatic action.

No exclusive role in elastogenesis is attributed to pulsation which is thought to be one of the eliciting factors along with a number of unknown factors. On the basis of experiments with aorta cultures it is suggested that pulsation should be considered as an adequate stimulus for the maintenance of elastic elements.

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ОБРАЗОВАНИЕ ЭЛАСТИЧЕСКИХ ЭЛЕМЕНТОВ В ТКАНЕВЫХ КУЛЬТУРАХ

ДЬ. ЛЕЛЬКЕШ и Л. КАРМАЖИН

Авторы исследовали в культурах зародышевого сердца цыплят и аорты поведение эластических элементов, также как и появление новообразованных эластических элементов. Они наблюдали в пульсирующих сердцевых культурах (сердца зародышей цыплят различного возраста, культура по методу Максимова, питательная среда : куриная плазма + раствор Тироде + сок зародыша цыплят 1 : 1 : 1) в зоне разрастания появление эластических волокон. Авторы не находили связи между продолжительностью пульсирования и количеством возникших эластических волокон. В культурах, которые после высаживания совершенно не пульсировали, авторы не наблюдали новообразований эластических элементов. Эластические элементы не образовались также и тогда, когда в питательной среде не было зародышевого экстракта, хотя в таких случаях (при незначительном разрастании) возможно было достигнуть пульсации на протяжении нескольких недель.

Новообразований эластических элементов в культурах аорты не наблюдалось, эластические элементы высаженного кусочка распадались зернисто. В кусочке аорты, культивированном в связи с пульсирующей культурой сердца, эластические элементы сохраняются и явления новообразований также можно наблюдать.

Новообразование эластических элементов наблюдалось всегда только в присутствии клеток. По мнению авторов возникновение эластических элементов связано с деятельностью мезенхимальных клеток, которые образуют, — соответственно данным условиям окружающей среды, — эластические элементы путем формативной или ферментативной функции.

Авторы не приписывают пульсации исключительной роли при образовании эластических элементов, они рассматривают ее, — при условиях данной экспериментальной установки, — одной из разрешающих причин, наряду с другими, до сих пор неизвестными факторами. На основании вышеупомянутых исследований, проведенных над культурами аорты, пульсацию необходимо рассматривать как адэкватное раздражение сохранения эластических элементов.

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