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THE NUCLEIC ACID METABOLISM OF LIVERS GRAFTED ON CHORIO-ALLANTOIC MEMBRANE

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In an earlier communication (Pósalaky, Törő, Barka 1953) we reported on histochemical investigations made in tissue cultures concerning the nucleic acid metabolism of the liver. Observing embryonic and adult liver cultures of chicks and rats, a close interrelation was found to exist between the proliferation of hepatic cells and their capacity to synthesize ribonucleic acid (RNA). The RNA invariably disappeared from explanted adult livers within 24 hours, and there was never a sign of growth in such grafts, whereas embryonic liver cultures always developed a marginal zone, the cells of which had retained their full RNA-content. These cultures grew always vigorously. The disappearance of the RNA seemed to be irreversible.

These observations have made it evident that, while the parenchyma cells of the embryonic liver retained the capacity to synthesize RNA, nothing but processes of decomposition occurred in adult hepatic cells. It seems that the nutritional and respiratory conditions of the tissue culture were adequate for the growth of embryonic liver but insufficient for that of the adult liver.

Our observations in question served as a base for the present experiments in which comparisons were made between the growth and nucleic acid content of livers grafted onto chorio-allantoic membrane. Explants transferred the chorio-allantois seem to invade the mesenchyma after a short time. Cells wander from the transplant into the mesenchyma, while capillaries arising in the latter invade the transplant, so that a very close contact is seen to develop between the transplanted tissue and its new surroundings. It is therefore obvious that the method of transplantation assures much better physiological and more favourable nutritional and respiratory conditions for the survival of the transferred tissues than tissue cultures do.

Methods

We employed Murphy's (1914) method with slight modifications.

Transilluminating eggs incubated for 9 to 10 days we marked on their shell that area — as a rule, the point at which a larger vessel was seen to ramify — which seemed to be most promising for the purposes of transplantation. After cleansing the marked area with petrol, 10 mm^2 of the shell were removed by means of a safety-razor blade, taking greatcare not to

injure the shell membrane which sits closely on the chorio-allantois. This done, a drop of Tyrode solution was deposited on the shell membrane which was then scraped with a fine needle. Due partly to the infiltration of the liquid, partly to the escape of the air, the two membranes separated. The chorio-allantoic membrane sank down so that it was easy to peel off the tigthly stretched shell membrane without impairing the former. Using Graefe's knife, a piece of liver tissue was then placed on the denuded chorio-allantoic membrane. Then the aperture was covered with cellophane, sealed with paraffin, and the egg was replaced into a 37° C incubator.

Embryonic and adult chick and rat livers were used in the experiments. The donors were either narcotized with ether, or bled, and their liver was enucleated immediately before transplantation. The excised pieces of liver were cut with a Graefe's knife into pieces of 2 mm in Tyrode solution containing 1000 U/ml of penicillin. Both the enucleation and the transfer were carried out with the utmost asepsis.

Opening the eggs after different periods of time (from 6 hours to 8 days), the transplants were excised together with the surrounding chorio-allantois, fixed in Carnoy's fluid, and embedded in paraffin. The 6 μ thick serial sections were stained with Unna's methyl green-pyronine. Dehydration was performed by means of tertiary butyl alcohol. Some of the sections were stained also with haematoxylin-eosin.

Experimental observations

The observations as described in the following are based on the results of 68 successful transplantations.

A) Embryonic liver

1. *Chick:* liver obtained from 10 to 12 day old chick embryos.

6-hour transplant. Six hours after the operation the transplanted piece of liver is seen to have become attached to the chorio-allantoic membrane. The morphological pattern is well preserved, although the lobules are somewhat loose. Stained sections reveal a marked contrast in staining between the centre and the marginal zone of the transplant. While the cells of the marginal zone, consisting of from 6 to 8 layers, still show unimpaired pyroninophilia, and contain intact and well-stained nuclei, the cells in the central part of the transplant take less pyronine, their nuclei are somewhat pycnotic and stain dark. Vacuoles are frequent in their cytoplasm.

12 to 24-hour transplant. By this time the graft has somewhat sunken into the chorio-allantoic mesenchyma. The difference between the centre and the edge is much more pronounced. The central part stains faintly; the hepatic structure is completely destroyed; sinusoids and lobules are unrecognizable, the cells are isolated or in small groups. Against this, the peripheral cells are intensely pyroninophilic with well-stained granules and nucleoli and well-stained nuclei. Cell-rows, and between them are the sinuses well observable (Fig. 1).

48-hour transplant. The liver is already in the mesenchyma of the chorio-allantoic membrane and the line dividing them is indistinct due to the wandering of cells. It seems as if capillaries, coming from the mesenchyma, penetrated the

transplant : this cannot, however, be claimed with certainty since the hepatic cells proper are also full of nucleated erythrocytes. The separation of the central and marginal zone is still more marked. The pyroninophylia of the central zone is so slight that this looks nearly empty. Only occasional hepatic cells are stained, full stainability being more or less restricted to blood cells and nuclei in the sinusoids. The nuclei of the hepatic cells are shrunken and fragmented ; normal hepatic tissue is almost completely absent, the cell borders are blurred ; individual cells are undistinguishable. The marginal zone offers a sharp contrast to this picture : the cells in this zone are completely intact, their cytoplasm contains intensely pyroninophilic granules ; the nuclei are light, with well-staining and very conspicuous nucleoli in them. The cells are arranged in bundles, with wide capillary networks among them. The whole picture presents the typical pattern of hepatic tissues (Fig. 2).

4 to 6-day transplant. These sections no longer reveal a marked difference between central and marginal zone. The entire transplant takes the methyl-green-pyronine-stain, although the central part remains somewhat fainter. There are still many vacuolated cells in this part. The nuclei are intact and stain well with pyronine and so do the granules in the cytoplasm but the nucleoli do not stain. The marginal zone is similar to that in the 48-hour transplant.

8-day transplant. No difference whatsoever between centre and periphery is seen. The hepatic structure is well recognizable throughout the transplant. The hepatic cells are arranged in bundles ; they stain well and there is no sign of degeneration (Fig. 3).

2. *Rat livers* were obtained from 18 to 20-day old embryos.

Six hours after transplantation, also the embryonic rat liver reveals the notorious difference between central and marginal zone. The subsequent development follows the same lines as that of the chick embryo. Eight-day transplants take the pyronine stain also in this case in their entirety. The cells are completely intact and are in rows.

Liver tissue taken from 1 to 6-day old *new-born* rats showed on chorio-allantois the same behaviour as embryonic rat livers. Vascularization of the transplant was well-observable, as the blood vessels arising from the chorio-allantoic mesenchyma and filled with nucleated red blood cells were easy to distinguish from the transplant's own vessels filled with non-nucleated erythrocytes. Vascularization became particularly pronounced on the 2nd day.

B) Adult liver

1. *Chick:* livers taken from hens ranging in weight between 1500 and 2000 g were used.

Hen liver behaves in every respect as embryonic chick liver. The difference between central and marginal zones disappears likewise on the 8th day, the whole

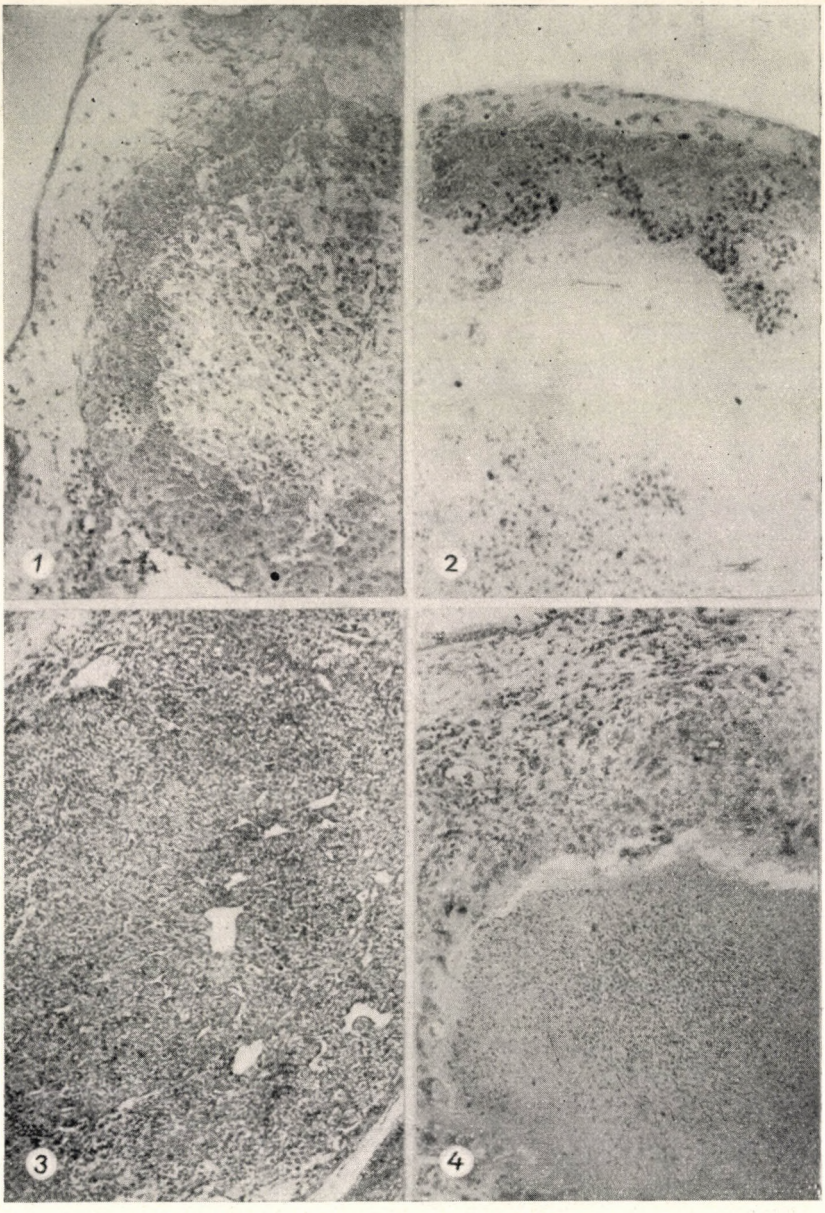

Fig. 1. Embryonic chick liver, 24-hour transplant. Difference in staining between marginal and central zone. Methyl-green-pyronine stain

Fig. 2. Embryonic chick liver, 48-hour transplant. Central zone unstained, cell borders hardly perceptible. Marginal zone composed of brightlystaining normal hepatic cells. Methyl-green-pyronine stain

Fig. 3. Embryonic chick liver, 8-day transplant. The entire transplant stains well and reveals a typical hepatic structure. Methyl-green-pyronine stain

Fig. 4. Adult rat liver, 8-day transplant. Transplant completely necrosed. Its place has been occupied by an uniform tissue mass. Haematoxylin-eosin stain

transplant being intensely pyroninophilic and composed of rows of hepatic cells of normal appearance.

2. *Rat:* liver were obtained from rats weighing between 160 and 180 g. During the first days there is no difference between the behaviour of the adult and that of the embryonic rat liver. Yet, by the 4th to 6th day — notwithstanding the fact that the transplant appears to be well-vascularized by this time — a process of degeneration seems to have ensued: the cytoplasm of the cells is filled with vacuoles ; the nuclei show shrinkage and deformation ; no intact hepatic cells can be found, and it is only the nuclei of the blood cells which stain with methyl-green-pyronine. Degeneration goes on without interruption, so that—by the 8th day—the transplant will have deteriorated into a structureless necrotic tissue (Fig. 4).

Discussion

While part of our observations was quite in line with the phenomena seen in tissue cultures, another part of what we saw was very different. *Embryonic liver* proliferates vigorously both in tissue culture and on the chorio-allantoic membrane. Yet, while the explant itself degenerates and perishes in tissue cultures, it is seen to be composed of intact hepatic cells *in its entirety* on the 8th day if grafted onto chorio-allantois. OAKLEY (1938), transplanting chick embryo liver onto chorio-allantoic membrane, made the same observation. He claimed that the intensely proliferating marginal cells occupied the place of the necrosed central hepatic cells. It is not easy to decide this problem : OAKLEY's assumption may be correct, but it is also possible that what is observed is just a temporary, and thus reversible, degeneration of the central cells. Although necrosis would preclude such a possibility, it should be understood that what we encountered in embryonic liver transplants was invariably degeneration and in no case necrosis.

That the *new-born* heterologous liver displays a behaviour similar to that of the embryonic one, is by no means surprising, as this phenomenon occurs in the case of other organs just as well (RADITZ, TÖRŐ 1954).

That the *adult hen liver* shows a similar behaviour may be explained by the conditions of homologous transplantation which are of course very favourable to the survival of the transplanted tissue. OAKLEY thinks that hen liver has a poorer growth than embryonic chick liver : we have not been able to observe a difference in this respect. *Adult rat liver* perishes very soon after being transferred onto the chorio-allantoic membrane. Literary data are conflicting in this respect. Many authors failed to effect successful grafts of heterologous tissues on chorio-allantois. (SANDSTROM 1932, SACCOMANNO 1951, etc.), while successful grafts of this kind were reported by others (GOODPASTURE 1938, KIRBER 1950, etc.).

It is clear that, like those with tissue cultures, also the present experiments revealed a pronounced interdependence between the transplant's power of growth and its capacity to synthetize RNA. A pyroninophilic marginal zone is developed in the beginning both in livers transplanted on the chorio-allantoic membrane and those transferred to cultures. Yet, while RNA disappeared from the central zone of the cultures in an irreversible manner, the liver tissues grafted onto chorio-allantois presented, with the exception of the adult rat liver, quite a different picture: any difference between central and marginal zone disappeared after the 8th day and an uniformly intense pyroninophilia characterized the entire transplant, i. e. it contained the same amount of RNA as at the time of explantation. This phenomenon admits various explanations, viz.

1. As it is impossible to subject to examination the same piece of explanted liver first on the 2nd and then again on the 8th day, it is conceivable that the RNA content of some transplants suffered no decrease during this time. Although we are not in a position to prove the fallacy of this supposition beyond any doubt, we feel nevertheless justified in not accepting it because we invariably observed the development of a separate central and a separate marginal zone in the first days in every one of our cases, and, further, because in each experimental group the liver of one and only one animal was kept under observation.

2. It is also possible that the phenomenon as suggested by OAKLEY lies at the back of the changes occurring in the RNA and DRNA (desoxyribonucleic acid) content of the transplant, although OAKLEY did not keep the behaviour of the nucleic acids under observation. According to his concept, it is only the peripheral cells which are capable of proliferation, be they in chorio-allantois or in tissue culture: the growth and multiplication of the marginal cells is, however, directed not only outward but also towards the central part of the transplant, so that — after some time — these cells come to take the place of the necrotized tissue. Were this theory correct we ought to have encountered rests of the dead tissue in some cases at least, for it is quite inconceivable that it should have been completely adsorbed in the space of a few days. Besides, in no other case than in that of adult heterologous transplantation did we ever observe necrosis. We therefore believe that we must rule out the hypothesis in question.

3. The most obvious explanation seems to us to be offered by our assumption that the disappearance of the nucleic acids as observed in our experiments was but a transitory process, and that the cells were able to *recover* their nucleic acid content during the very course of the experiments. Literary data add weight to this hypothesis. DAVIDSON (1947), STOWEL (1948) and others observed an increased synthesis of nucleic acids in regenerating livers. It is known from the communications of DAVIDSON (1947), LAGERSTEDT (1949), etc. that the nucleic acid content of hepatic cells diminishes or even disappears during fasting, to be restored after a protein diet. STENRAM (1954), who encountered basophilic

substances in the cytoplasm of white rat livers even after a fasting of 5 days, refuses to believe that these structures with nucleic acid contents disappear from hepatic cells during fasting. If — so he reasons — it is true that submicroscopic microsomes of the hepatic cells are capable of developing basophilic "inclusions", it is very difficult to ascertain with the aid of light microscopy whether the nucleic acids have actually disappeared or not. Since methyl-green-pyronine was used in our experiments for the histochemical demonstration of nucleic acids, we cannot claim these acids to have disappeared from the hepatic cells of the central zone of the 2-day transplants : all we are justified to affirm is that, under the given experimental conditions, we were unable to demonstrate their presence. Which means that the nucleic acids had suffered such a far-reaching decomposition as made it impossible to make them visible by means of methyl-green-pyronine. Also our transplants showed this process to be slower in respect of RNA than in that of DRNA. Pieces of liver grafted on chorio-allantois seem to be, during the first 24 to 48 hours, under conditions that are not favourable to nutrition or metabolism. Like fasting, these conditions lead to a predominance of the processes of decomposition in the hepatic cells where the synthesis and the splitting of nucleic acids are in a state of constant equilibrium. Nutritional conditions being least satisfactory in the central part of the transplants, it is not surprising that the decomposition of nucleic acids should be more pronounced here than anywhere else. This explains why it is from the central zone that pyroninophilic substances disappear, and that even the nuclear substance suffers diminution or injury. Such a degree of depolymerization of the nucleic acids is, however, not an irreversible process. We have seen that the synthesis of nucleic acids is notably increased if, after fasting, a protein-rich diet is introduced : the very same phenomenon occurs in livers grafted upon chorio-allantois as soon as the transplant becomes vascularized. The abundance of capillaries invading the transplant creates favourable conditions for the nutrition of the liver, so that a synthesis of nucleic acids dominates in the cells. This is indicated by the appearance of numerous pyroninophilic granules in the cytoplasm, as well as the presence of an intensely pyroninophilic nucleolus in the brightly-staining nucleus which contains a loose chromatin substance. Thus, the loss of response to methyl-green-pyronine as observed in our experiments was but an indication of the fact that processes of decomposition had come to predominate in the hepatic cells. However, the process of decomposition affecting the nucleic acids stopped short of the limit beyond which it would have become irreversible even under the most favourable conditions. Such extremely favourable conditions, rendering reversibility still possible, were created in our experiments by the vascularization of the transplant.

Not even vascularization sufficed to offer adequate nutritional conditions to adult heterologous tissue, wherefore, in such cases, decomposition was free to go until the complete deterioration of the nucleic acids.

Summary

(i) Embryonic and adult chick and rat livers were transplanted to the chorio-allantoic membrane of chick embryos. The behaviour of the transplants was kept under observation for 8 days.

(ii) By the 6th hour following the operation, a marginal and a separate central zone were observed in the transplants. The marginal zone consisted of intensely pyroninophilic intact cells, while the cells of the central zone revealed symptoms of degeneration and did not stain with methyl-green-pyronine.

(iii) The degeneration of adult rat livers went on uninterruptedly, and the grafts completely underwent necrosis by the 6th to 8th day.

(iv) The process of degeneration was seen to come to a stop and regress in adult chick livers, as also in embryonic chick and rat livers, so that, by the 6th to 8th day, the entire transplant consisted of intact hepatic tissue which was well stained with methyl-green-pyronine.

(v) It is suggested that the phenomenon is one of a temporary, reversible depolymerization of the nucleic acids.

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ОБМЕН ВЕЩЕСТВ НУКЛЕИНОВОЙ КИСЛОТЫ ПЕЧЕНИ, ПЕРЕСАЖЕННОЙ НА ХОРИОАЛЛАНТОИДНУЮ ОБОЛОЧКУ

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1. Печень эмбриональных и взрослых цыплят и крыс пересаживалась на хориоаллантоидную оболочку эмбриональных цыплят. Судьба пересаженной печени наблюдалась в течение 8 дней.

2. В пересаженной печени образовались уже после 6 часов одна краевая и одна средняя зоны. Краевая зона состоит из неповрежденных клеток, показывающих оживленную пиренинофилию, в то время как клетки средней зоны при помощи M—Z—Py не окрашиваются. Эти клетки проявляют признаки дегенерации.

3. Дегенерация печени взрослой крысы усиливается и после 6—8 дней она полностью некротизируется.

4. В печени взрослых или же эмбриональных цыплят и крыс наблюдалось обратное развитие процесса дегенерации, и на 6—8 день вся область состоит из оживленно окрашивающейся посредством M—Z—Py неповрежденной печеночной ткани.

5. Авторы того мнения, что в данном случае имеет место проходящая обратимая деполимеризация нуклеиновых кислот.

NUKLEINSÄURESTOFFWECHSEL DER AUF CHORIO-ALLANTOIS TRANSPLANTIERTEN LEBER

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1. Die Leber von embryonalen und erwachsenen Hühnern und Ratten wurden auf Hühnerembryo-Chorio-Allontois transplantiert. Das Schicksal der Transplantate wurde während 8 Tage beobachtet.

2. In den Transplantaten bildete sich schon nach 6 Stunden eine Rand- und eine mittlere Zone. Die Randzone besteht aus intakten Zellen, die eine lebhafte Pyroninophylie bekunden, während die Zellen der mittleren Zone sich mit Methylgrün-Pyronin nicht färben, und Zeichen von Degeneration aufweisen.

3. Die Leber von erwachsenen Ratten weist eine sich stets verstärkende Degeneration auf bis nach 6—8 Tagen die Leber vollständig nekrotisiert.

4. In den Lebern von erwachsenen und embryonalen Hühnern, sowohl von embryonalen Ratten bildet sich der Degenerationsprozeß zurück und am 6—8. Tage besteht das ganze Transplantat aus intaktem Lebergewebe, das sich mit Methylgrün-Pyronin lebhaft färbt.

5. Verfasser sind der Meinung, dass es sich um die vorübergehende reversible Depolimerisation der Nukleinsäuren handelt.

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