A STUDY OF THE DYNAMICS OF CELL REPRODUCTION DURING DIFFERENTIATION OF THE CONNECTIVE TISSUE IN THE SKIN OF WHITE RAT EMBRYOS

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Numerous publications have dealt with morphological and biochemical changes of skin connective tissue during the course of development [1-3, 5-7]. However the problem of kinetics of cell reproduction during histogenesis of connective tissue has not been given much attention. The use of autoradiography with thymidine-H^3 is very promising for the study of this problem.

The purpose of this work was to investigate the proliferative activity of cells in different regions of skin connective tissue at consecutive stages of development of rat embryos by means of the autoradiographic method.

METHODS

This work was done on white rat embryos at 15, 18 and 20 days of development. Connective tissue in the skin of a well-defined area on the dorsal side of the thorax of the embryos was studied. Morphological changes in the connective tissue in the course of development were studied in usual histological sections stained with hematoxylin eosin, Mallory's stain, van Gieson's stain, and with Heidenhein's iron hematoxylin. The proliferative activity was determined through analysis of the related number of proliferating cells and the duration of their interphase. The results of our second series of experiments with thymidine-H^3 were used to establish these indices of proliferative activity.

In the first series of experiments pregnant rats at 15th, 18th and 20th days of pregnancy were injected subcutaneously with thymidine-H^3; 18 and 20 day old embryos were fixed after 2, 4, 8, 9, 12, 15, 18, 20, 24 and 28 h while 15 day old embryos were fixed after 4 h following the injection of thymidine-H^3. In the second series of experiments thymidine-H^3 was injected three times into the placenta of embryos at the 15th and the 18th day of development, and 4 times subcutaneously into embryos on the 20th day of development. The injections of thymidine-H^3 were done with 6 h intervals and the embryos were fixed 1 h after the last injection. The material was fixed in Carnoy's fluid for 2 h and embedded in paraffin. Autoradiographs were made on emulsion type R (NIKFI, Moscow).*

Autoradiographs obtained in experiments in which thymidine-H^3 was injected only once were used to determine the index of labelled nuclei. The duration of the entire mitotic cycle and of different interphase periods were calculated by the method of Quastler and Shurman [7] by means of graphic analysis of changes in the percentage of "labelled" mitoses in time. The relative number of proliferating cells in cell populations (proliferative pool) was determined in autoradiographs obtained in experiments in which thymidine-H^3 was injected several times.

RESULTS

Two layers could be distinguished in the skin connective tissue of embryos at the 15th day of development: an outer layer immediately below the epidermis with cells more closely packed, and an inner layer in which the cells

*The experimental work and the preparation of autoradiographs were conducted by the staff of Laboratory of Cell Morphology of the Institute of Cytology of Academy of Sciences of USSR. I would like to express my thanks for Professor L. N. Zhinkin and to the entire staff of the laboratory for the preparations and for making it possible for this study to be carried out.

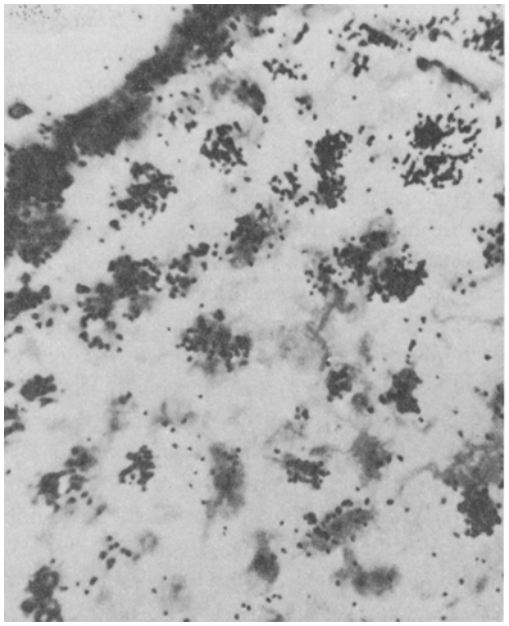

Fig. 1. Autoradiograph of section of skin of a 15 day old embryo. Three injections of thymidine-H^3 with 6 h intervals. Fixation 1 h after the last injection. Hemallum-eosin Obj. 60×, Oc. 10×.

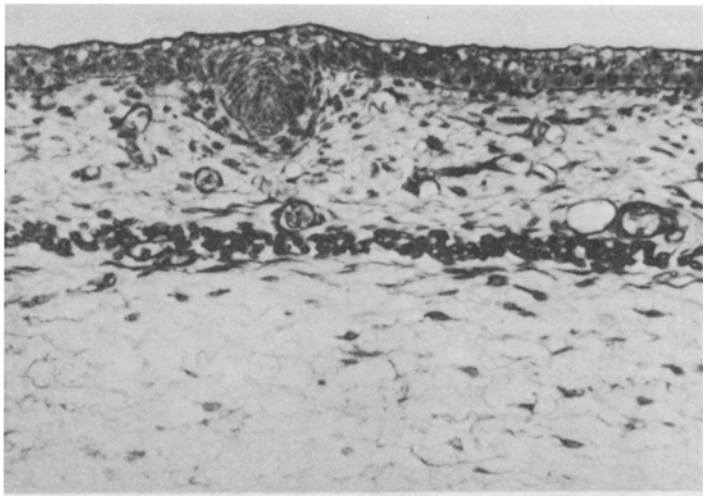

Fig. 2. Skin of an 18 day old embryo. Van Gieson's stain. Obj. 10×, Oc. 10×.

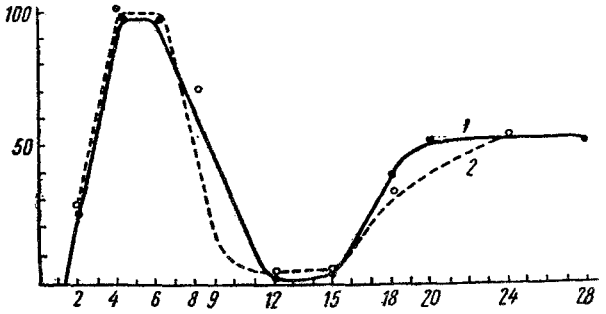

Fig. 3. Change in the percentage of "labelled" mitoses in time. Number of "labelled" mitoses on the ordinate; duration of the mitotic cycle (in hours) on the abscissa. 1) 18 day old embryos; 2) 2 day old embryos.

were less closely packed. Cells with outgrowths in both the layers appeared to be only slightly differentiated. In the outer layer these cells were mainly found distributed parallel to the surface of the epidermis, while in the inner layer they were oriented in different directions. A beginning of formation of a vascular network could be seen at the border between the two layers of the connective tissue.

Following a single injection of thymidine-H^3 the number of cells with labelled nuclei in the outer layer of the connective tissue constituted 40%, and in the inner layer 27%.

Similar differences between the outer and inner layers of skin connective tissue were noted also in experiments in which thymidine-H^3 was injected three times (Fig. 1). In the outer layer 72% of the cells had labelled nuclei, while in the inner layer such cells constituted only 56%. It follows from this that at a relatively early stage of skin

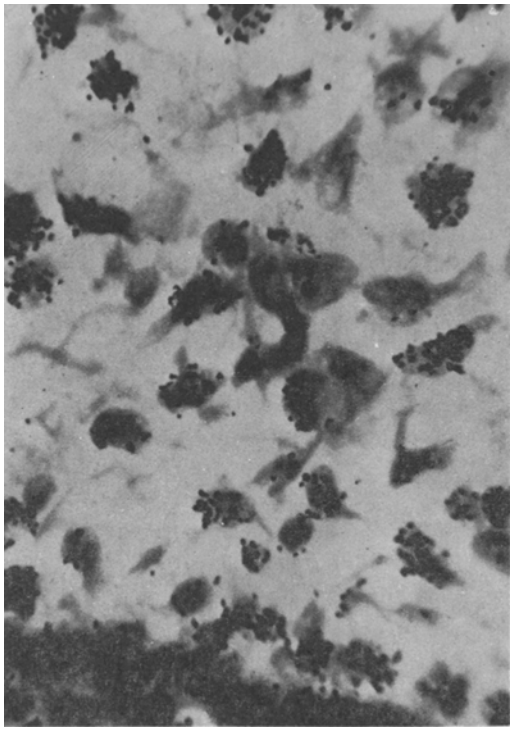

Fig. 4. Autoradiograph of section of skin of an 18 day old embryo. The legend is the same as in Fig. 1.

histogenesis, definite differences in the intensity of cell reproduction and in the ratio of proliferating and differentiating cells correspond to the furture topographical peculiarities of connective tissue.

In the skin of 18 day old embryos (Fig. 2) there was a beginning of formation of hair rudiments, a considerably more complex vascularization, a continuation of the intensive processes of growth, multiplication and differentiation of cells in both layers of connective tissue. Fibroblasts and kambial mesenchyme cells in the outer layer were distributed irregularly. They were considerably more numerous in the outer region of the inner layer of the connective tissue, adjacent to the epithelium. The thickness of this layer reached 80 microns. The processes of formation of intercellular structures were intensified. The number of precollagen fibers and of blood vessels, especially in the region of formation of the papillar layer of skin, was considerably increased. The inner layer of connective tissue reached a thickness of 130 microns. The cells in this layer were not closely packed and were characterized by their strongly elongate shape and regular orientation parallel to the body surface. A characteristic peculiarity of this layer was a large number of fibrous structures grouped in thick bundles. These distinct morphological differences of the different regions of the connective tissues were accompanied by considerably greater differences in the proliferative activity of cells than in 15 day old embryos (Fig. 4).

The labelled nuclei index and the proliferative pool in the outer layer of connective tissue increased to 45% and 82.5% respectively. In the inner layer, on the contrary, there was a considerable decrease in the values of these indices of proliferative activity—18% of cells with labelled nuclei following a single injection of thymidine-H^3 and 34% after 3 injections of thymidine-H^3 (Fig. 3).

The papillary and the reticular layers of the dermis could be clearly distinguished in the skin of embryos at the 20th day of development. In the papillary layer the cells remained closely packed but the collagen fibers were relatively weakly developed. The increase in the thickness of the reticular layer up to 180-200 microns occurred mainly as a result of development of intercellular fibrous structures. As a result of this the relative number of cells per unit area became considerably decreased as compared with the number of cells in similar regions of skin connective tissue of embryos on the 18th day of development.

The transversely striated muscle tissue in the skin could be seen in 18 day old embryos (Fig. 2), and in 20 day old embryos it attained a thickness of 40-50 microns and separated the skin from the underlying connective tissue. The latter gradually became transformed into fascia of superficial body muscles and into layers of intermuscular connective tissue. The number of fibrous structures in this connective tissue, mainly of bundles of collagen fibers, became considerably increased, while the relative number of cells became smaller than in embryos on the 18th day of development. Among the cells there was a large number of differentiated fibrocytes with pycnotic nuclei.

The number of cells in the stage of DNA synthesis at the time of injection with thymidine-H^3 (in experiments with a single injection) and the number of cells able to synthesize DNA (in experiments with 3 and 4 injections) became decreased in all the layers of connective tissue in embryos at the 20th day of development. However, in the papillary layer, the labelled nuclei index (33%) and the proliferative pool (50-60%) were found to be considerably higher than in the reticular layer in which these values were 12% and 20% respectively. The fall of the labelled nuclei index could be caused by a decrease of the relative number of proliferating cells in the cell population, as

Proliferative Activity of Cells in Skin Connective Tissue of White Rat Embryos (Percentage of labelled nuclei following injection of thymidine-H^3)

No. of injections	Stage of development of embryos						
	15-days		18-days		20-days		
	connective tissue layer						
	I	II	I	II	I		II
					1	2	
1	40	27	45	18	33	21	12
3	72	56	82,5	34	50	31	20
4	—	—	—	—	60	35	20
Mitotic index							
	2,8	2,2	3,4	1,4	1,5	1,2	0,3

well as by a change in the duration of the mitotic cycle. Consequently we determined the duration of the mitotic cycle and of its different stages at different periods following a single injection of thymidine-H^3 in the papillary layer of embryos at the 18th and the 20th days of development (Fig. 3).

As seen in Fig. 3 the duration of the mitotic cycles in the proliferating cells of the papillary layer in the skin of embryos at the 18th and 20th days of development were identical and equal to approximately 20 h.

In these cells the period of DNA synthesis lasted approximately 6 h, the G_2 period $2\frac{1}{2}$ h and G_1 + m period about $11\frac{1}{2}$ h. It was possible to determine the last mentioned period only in some cells in our material. Possibly towards the 20th day of development the heterogeneity of the population becomes considerably increased. However in order to answer this question it is necessary to conduct further experiments and to make observations after longer periods of time following injections of thymidine-H^3.

The results obtained by us in our study of the dynamics of cell reproduction in the course of development of connective tissue are summarized in the table.

In all the areas of connective tissue in the skin of 15 day old embryos studied the cells were young and poorly differentiated. There were no apparent topographical differences in the number of mitotically dividing cells in the outer layer (2.8%) and in the inner layer (2.2%) of the connective tissue. However the data obtained on the labelled nuclei index and the proliferative pool (the table) show that in this tissue it was possible to distinguish layers: an outer layer which in the future will form the connective tissue framwork of the skin, and an inner layer from which will be formed the subcutaneous adipose tissue and the connective tissue of intermuscular layers and fasciae.

Between the 15th and the 18th days of development the increase of proliferative activity of cells in the two layers paralleled the morphological differentiation of the dermal and the subcutaneous embryonic connective tissue.

The proliferative activity of cells in the outer layer became increased while that of cells in the inner layer became decreased. Thus the percentage of cells with labelled nuclei in the connective tissue of dermis in 18 day old embryos following 3 injections of thymidine-H^3 reached 82.5%. In the inner layer the number of such cells fell to 34%. These findings which characterize the volume of the proliferative pool are in accordance with the indices of change of the mitotic index. In the connective tissue of the dermis the mitotic index of 2.8% in 15 day old embryos rose to 3.4% in 18 day old embryos, while in the cells of subcutaneous connective tissue it fell from 2.2% to 1.4%.

In the course of further development the proliferative activity of cells in all the areas studied was considerably decreased in accordance with the progressing specialization of different layers of connective tissue. The predetermined differences in the intensity of reproduction of cells in the different regions were sufficiently distinctly retained in accordance with the functional peculiarities and the degree of morphological differentiation of the same regions of the connective tissue. A comparison of the data presented in the table and of results obtained by us in our determination of the duration of the mitotic cycle in proliferating cells in 18 and 20 day old embryos shows that the regulation of intensity of cell reproduction during this period was governed mainly by a relative decrease in the number of cells which went through the mitotic cycle.

However the data on the duration of the mitotic cycle are still insufficient to make definite conclusions on its duration and on that of its stages for all, especially for the more differentiated stages of skin connective tissue. There is some information [4] that cells in the connective tissue in the skin of adult animals have a longer mitotic cycle than those during embryonic histogenesis.

The results obtained by us show that throughout the entire period of embryonic histogenesis of skin the process of specific differentiation of cells in the inner layers of connective tissue is considerably more rapid than similar processes in the subepidermal and the papillary layers of skin.

SUMMARY

Thymidine-H^3 was injected into female rats on the 15th-20th day of pregnancy, and the isotope inclusion was investigated on radioautographs of the connective tissue of embryos 2-28 h after injection.

Thymidine-H^3 was repeatedly injected into embryos interplacentally and subcutaneously so as to detect the proliferative pool. In the period from the 15th to the 20th day of development there was differentiation of the subcutaneous connective tissue. Conformably to this the proliferative activity of the external layer of the connective tissue increased from the 15th to 18th day, which is evidenced by a change in the mitotic index and the number of labelled cells, and slightly decreased from the 18th to 20th day. At the same time, both the mitotic index and the number of labelled cells showed a marked decrease in the internal layer. The reduction of the proliferative activity takes place mainly due to the lessening of the number of cells taking part in the mitotic cycle. The duration of the mitotic cycle in embryos of an early age was identical (approximately 20 h), period S-6 h, G_2-$2\frac{1}{2}$ h.

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