Lymphology 5 (1972) 137-148

Inception and Manner of Development of the Lymph Vessels in the Chick Embryo Heart

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Summary

The initial stages of development of the cardiac lymph vessels were studied in 30 chick embryos from the 9th to 14th day of incubation. The microinjection technique in correspondance to light and electronmicroscopical analysis was used.

As a rule, mesenchymal cells form part of the wall of the primitive lymph vessels and capillary. There is practically no difference between these cells and the primitive endothelium. The vascular wall is connected to the surrounding mesenchyme by numerous processes and in places it communicates freely with the mesenchymal intercellular space. With advancing development, communications with the intercellular spaces disappear in the larger lymphatics. The development of the lymph capillary in the periphery is promoted by mitoses of primitive endothelial cells and incorporation of mesenchymal cells into the lining of the lymph bed. The differentiation of mesenchymal cells to endothelial cells occurs. During the period in question the lymph capillary wall has no basal lamina. The incorporation of mesenchymal cells into the lymph capillary bed, is followed by transformation of the simple mesenchymal cell contacts into the complex interdigitations typical for the lymph capillary endothelium. Differentiation of the lymph vessels and capillaries does not progress beyond the simple endothelial tube stage during the period in which these observations were carried out.

Introduction

Despite repeated attempts, nobody has yet found a reliable way of differentiating the lymph capillary from the blood capillary, or of determining exactly what the origin of the lymph bed looks like, even with the aid of the electron microscope.

Leak and Burke (1, 2) believed that differences between the lymph and the blood capillary could be summed up under four points: the lymph capillary has a wider lumen, a discontinuous basal lamina and numerous intercellular open junctions and is anchored in the surrounding connective tissue by means of large numbers of fine fibres. According to *Lauweryns and Boussouw* (3), however, none of these criteria allows either easy or absolute differentiation of the lymph capillary from the blood capillary. Histological analysis of the enzymes of the wall of the vessels is likewise not conclusive (4), while the difference between them in contracting muscle (5) is smallest of all.

It is even harder to distinguish between a lymph capillary and a simple tissue (intercellular) space. *Czillik* (6) therefore suggested that the formation should be described as a lymph capillary only if it was clearly lined with endothelium and otherwise recommended that such canalicular slits should be termed "tissue spaces". *Pfleger* et al. (7) found such small differences between the flat connective tissue cells lining tissue spaces and the true endothelial cells of the lymph capillaries, however, that they regarded control of the exact spatial relationship of the two structures by means of serial sections as the sole conclusive way of differentiating between them.

In various attempts to abolish the above difficulties, nobody has so far taken into account the oft-proven principle of *Descartes* (8) that it is much easier to comprehend the nature of things if we watch them developing than if we study them after they are complete. In other words, we failed to find a single report dealing with the given problems from the developmental aspect. We therefore made use of the fact that the lymph vessels grow towards the heart of the chick embryo by a topographically very confined route, i.e. in the grooves between the large blood vessels of the heart (in particular in the aortopulmonary sulcus), that they are easy to inject with a contrast substance and that they first spread over the surface of the heart in the loose subepicardial mesenchyme, which is transparent when fixed, so that information on their course can be obtained simply by examining the fixed heart wall (9). Taking advantage of this, we investigated the structure of the developing lymph vessels, especially the capillaries, and the relationship of the origin of the developing lymph capillary to the mesenchymal connective tissue.

Material and Methods

The outset of development of the cardiac lymph vessels was studied in chick embryos from the 9th to the 14th day of incubation. On these days the first lymph vessels reach the embryonic heart, spread over the surface of the ventricular myocardium in the subepicardial mesenchyme and acquire their first values (see 9 on a microinjection analysis of this process). The retrograde injection of yellow latex particles (the size of the particles varies with the colour) in excess in 1.5% gelatine into the efferent lymphatic trunk prevented the developing cardiac lymph bed from collapsing and visualized the large vessels of the lymph bed for such a distance that the course of their continuation to the lymph capillaries was clearly indicated, despite the fact that the latex particles were unable to reach the capillaries themselves. The coronary bed was injected with black latex particles so as to avoid any confusion with the lymph vessels when examining thick ultramicrotome sections. The sparingness of this technique was verified in two ways: a) by continuous stereomicroscopic control of microinjection, which basically did not go all the way to the end, and b) by comparing the electron microscopical picture of the lymph vessel wall in the injected and non-injected lymph bed. We used at least two hearts for each day of incubation and on the 11th to 13th day we actually took four, so that our observations were based on 18 embryos in which both the lymph and the blood bed were injected and 12 control embryos in which only the coronary bed was injected.

When the lymph and the coronary bed had been injected, the hearts were dissected out and prefixed about 10 minutes in glutaraldehyde. With finely ground eye scissors we then cut from the anterior wall of the right ventricle narrow strips in which we should be most likely to detect lymph vessels in sections cut in their long axis (Fig. 1a). When preparing these strips, control injection of the lymph vessels was a valuable guide. The heart wall strips kept their typical shape, i.e. they were convex on the epicardial side and concave on the endocardial side. On the 12th day of incubation we cut the strips so as also to detect in a more advanced stage of development the relationship of the origins of the lymph vessels to the subepicardial mesenchyme (Fig. 1b, lower strip), as in this phase the usual heart wall strip (Fig. 1b, upper strip) was satisfactory only for studying differentiation of the larger lymphatics.

After two hours in glutaraldehyde, the strips were fixed two more hours in 2% osmic acid in phosphate buffer at pH 7.2. Because of their size the fixed tissue blocks were left in the dehydrating and impregnating solutions double the normal time. The material was embedded in durcupan ACM and was cut on a *Reichert* OmU2 ultramicrotome. Sections for photomicrography were 0.5-1 μ thick and were stained with toluidine blue, while for electron microscopy ultrathin sections (400-600 Å) were cut and contrasted with lead citrate. Tesla BS 242 and Tesla TS 513 electron microscopes were used for observation and for photography.

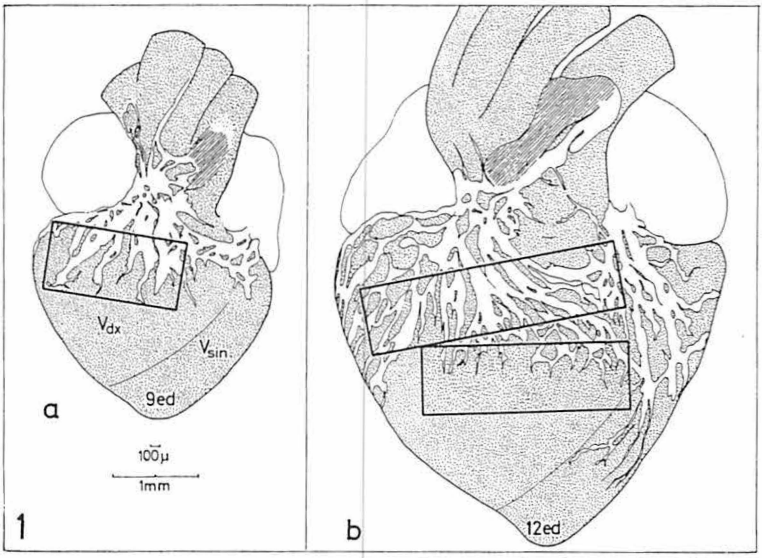

Fig. 1. Method of removing strip of heart wall in young (a) and older (b) chick embryos. In older embryos the usual strip generally detected only the lymphatic "trunks" and so another strip, which also showed the origins of the lymph capillaries, was removed below it.

Results

At the outset we should like to mention one important fact, the utilization of which allowed continuous, as well as detailed, observation. This is that any differentiation of the lymph vessel wall which can be observed in the various stages of development from the 9th to the 13th day of incubation (Fig. 2a, b, c) can also be roughly observed in more advanced stages of development of the lymph bed (starting from the 11th or 12th day of incubation), following a course from the apex to the base of the heart, i.e. we find a more primitive lymph vessel wall structure near the apex of the heart (corresponding to development in the younger stages) and a more advanced structure near the base (Fig. 2c) (corresponding to development in the older stages). We combined the two possible forms of observation as required. The results are divided into two parts. In the first, we describe the structure of the wall of the first lymphatic trunks and large blood vessels and in the second, the structure of the "peripherally" differentiating lymph capillaries, together with the structure of their origins and their relationship to the surrounding subepicardial mesenchyme.

Structure of the large lymphatics and their branches

The efferent lymphatic "trunks" and large blood vessels have an extremely wide lumen (Fig. 3, longitudinal section) lined throughout with very flat endothelium. The lymphatic endothelial cells have an oval nucleus with a pronounced nucleolus. At the site of the nucleus they project well into the markedly irregular lumen of the lymph vessel and are interconnected by their exceedingly long, attenuated cytoplasm processes. At the outset of development the large lymphatics have the structure and lumen of wide capillaries and on their outer surface are in close contact with the surrounding mesen-

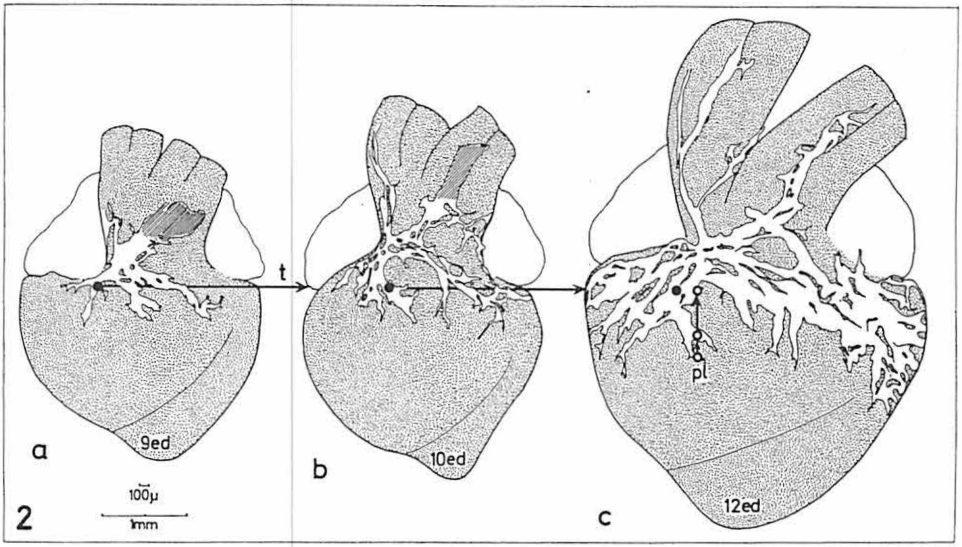

Fig. 2. Two methods, with corresponding results, of studying the development of the lymph vessel wall in chick embryos from the 9th to the beginning of the 14th day of incubation: t - in time, at the same sites of the heart wall in embryos of different ages (black circles, a-c); pl - according to place, i.e. at different sites in a basoapical direction (white circles, c) in embryos of the same age.

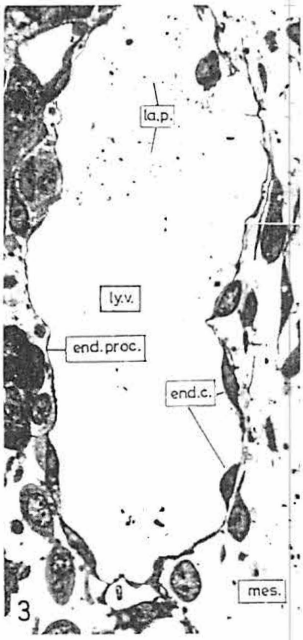

Fig. 3. Longitudinal section of lymph vessel (ly.v.) in wall of chick embryo heart (11th day of incubation). The endothelium (end.c. = endothelial cell), barely visible in places (end. proc. = process of endothelial cell), is continuous; latex particles (la.p.) can be seen inside the vessel and epicardial mesenchymal tissue (mes.) around it. x 700.

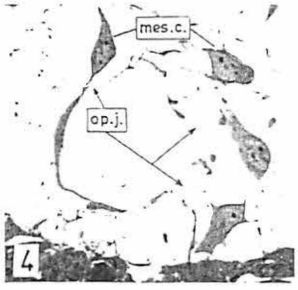

Fig. 4. Cross section of chick embryo lymph vessel (11th day of incubation). There are open junctions (op.j.) between the endothelial cells, on the outer aspect of which mesenchymal cells (mes.c.) are anchored by numerous processes. x 700.

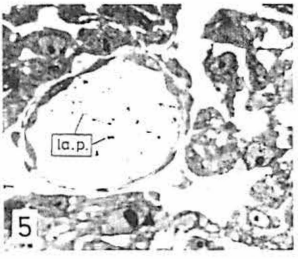

Fig. 5. Cross section of chick embryo myocardial blood vessel (11th day of incubation). Latex particles (la.p.) inside vessel. x 700.

chymal connective tissue (Fig. 4). The above picture is in marked contrast to the appearance of blood vessels with approximately the same lumen in embryos of the same age, irrespective of whether they are localized in the myocardium (Fig. 5, cross section) or in the subepicardial mesenchyme, as the blood vessels have a regular lumen and their endothelial cells have short cytoplasm processes, with the result that their wall, in general, is thicker.

The wall of the large lymph vessels also has numerous contacts with the surrounding mesenchyme. This intimate relationship is manifested in two ways, especially in the case of the smaller lymph vessels. Firstly , there is practically no difference between the structure of the endothelial and the mesenchymal cell (Fig. 6, 7, 9, 11). Secondly, in places the wall has a wide opening communicating directly with the intercellular space of the mesenchyme (Fig. 4, 7, 9) and it is even evident that the mesenchymal cells can participate directly in demarcation of the lumen of the lymph vessels (Fig. 6, 7, 8, 9, 11). As we approach the origin of the lymph bed, i.e. the thin lymphatics and capillaries which do not fill with contrast material (Fig. 8), this becomes increasingly manifest and shows the way in which the lymph bed actually spreads out over the surface of the heart.

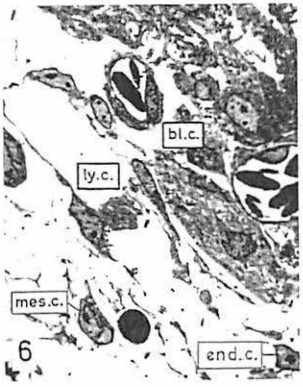

Fig. 6. Comparison of lymph capillary (ly.c. - longitudinal section) with blood capillary (bl.c. - cross section) in chick embryo (11th day of incubation). There is no visible difference in the structure of the endothelial (end.c.) and mesenchymal (mes.c.) cells, which are attached by numerous processes to the outer surface of the lymph capillary. x 700.

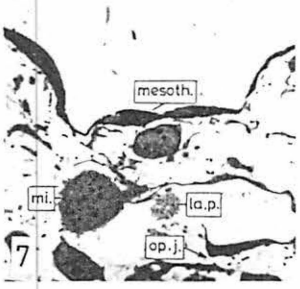

Fig. 7. Lymph capillary in superficial mesenchyme layer (covered with mesothelium - mesoth.) of chick embryo heart wall (11th day of incubation). Mitosis (mi.) of endothelial cell; latex particles (la.p.) escaping through open junctions (op.j.) into pericapillary space. x 700.

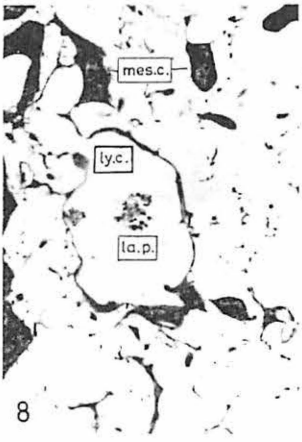

Fig. 8. Transition of lymph vessel (still containing latex particles - la.p.) of chick embryo heart wall (11th day of incubation) to attenuated lymph capillary (ly.c.), whose wall includes a typically branched mesenchymal cell (mes.c.). x 700.

Formation of lymph capillary bed

With reference to the above findings, the way in which the lymph capillary bed is formed can be unequivocally described as follows: Firstly, mesenchymal cells not dif-

fering in any way from the other cells of the mesenchyme directly circumscribe the lumen of the lymph capillary and form an integral part of its wall (Fig. 6, 7, 8, 9, 11). Secondly, at the sites of origination of the lymph bed, the mesenchymal layer of the wall of the heart is so thin, that in places to which the lymph bed has not yet spread it is virtually non-existent (cf. Fig. 10 and 11). Thirdly, at the sites of origination of the lymph capillary bed, the distribution and density of the mesenchymal cells is not in any way influenced by the presence of the lymph capillaries (Fig. 10, 11), this is indirectly due to incorporation of the intercellular spaces into the lymph bed system without an immediate change occurring in their size. The above picture contrasts

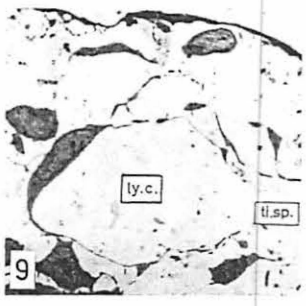

Fig. 9. Lymph capillary (ly.c.) of chick embryo heart wall (11th day of incubation) undergoing differentiation from tissue space. Incipient differentiation of mesenchymal cells to cells of capillary wall, which communicates with tissue space (ti.sp.) through wide opening. x 700.

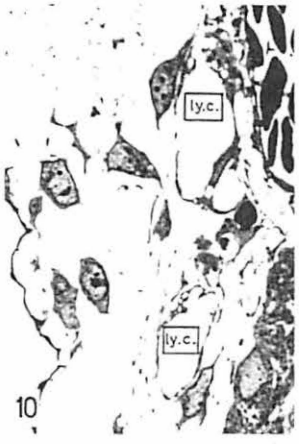

Fig. 10. Relationship of initial lymph capillaries (ly.c.) to basoapically attenuating epicardial mesenchyme layer of thick embryo heart wall (11th day of incubation). Segment of heart wall near base. x 700.

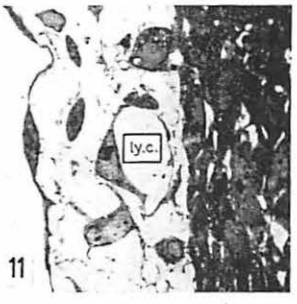

Fig. 11. Relationship of initial lymph capillaries (ly.c.) to basoapically attenuating epicardial mesenchyme layer of thick embryo heart wall (11th day of incubation). Segment of heart wall near apex. x 700.

sharply with the appearance and structure of the blood capillaries (Fig. 5, 6). The presence of mitoses in the lymph vessel wall (Fig. 7) demonstrates that the media of propagation of the lymph bed in the subepicardial mesenchyme over the surface of the myocarcium combine. They include proliferation of the elements of the lymph vessel wall as well as communication of the lymph bed with the tissue spaces and conversion and modification of the latter to the lymph bed. This developmental process of "invasion" of the lymph bed follows a smooth, unbroken course (9).

Ultrastructure of the lymph capillary wall

Since many details of the structure of the lymph capillary were beyond the discriminating power of the light microscope, we concentrated on these in our electron microscopy analysis of the lymph capillary wall. We found that the inner and outer surface of the lymph capillary wall was uneven and highly broken up (Fig. 12). Numerous endothelial cell processes projected far into the capillary lumen and also outwards, where they

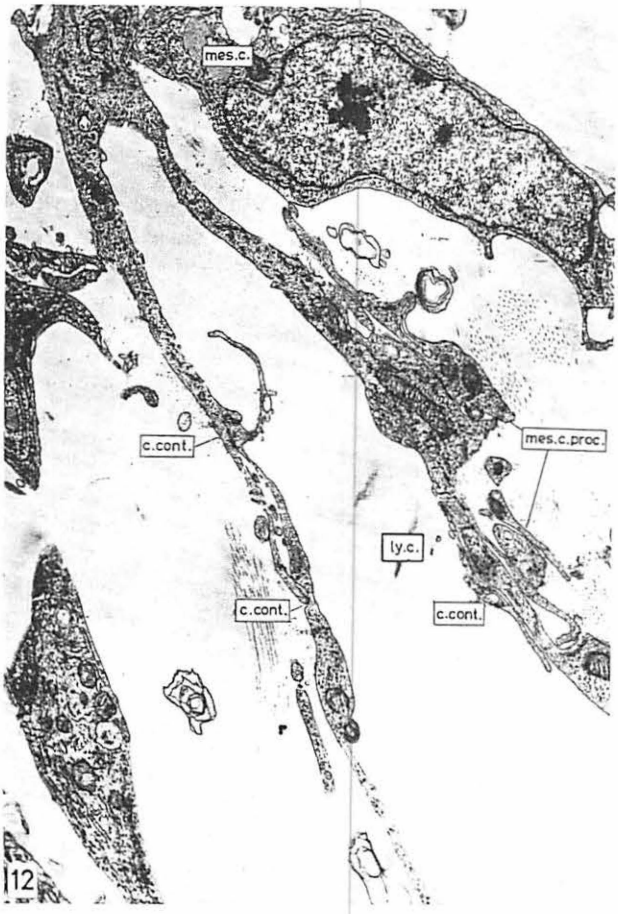

Fig. 12. Part of wall of lymph capillary (ly.c.) of chick embryo heart wall (11th day of incubation), whose endothelial cells are joined by varyingly formed interdigital contacts (c.cont.). Numerous mesenchymal cell (mes.c.) processes (mes.c.proc.) are attached to the outer surface of the wall. x 10,000.

came into multiple contact with the mesenchymal cells of the pericapillary space. It should be emphasized that in the phase of development studied, the basic picture of the cytoplasm (the shape, size, distribution and number of the individual organelles) of the primitive undifferentiated endothelial and mesenchymal cells displayed no significant differences (Fig. 13, 14). The endoplasmic reticulum was rather more developed in the mesenchymal cells, while in differentiating lymphatic endothelial cells it decreased, although the latter still contained clearly discernible cisternae of granular endoplasmic reticulum, a Golgi apparatus and numerous free ribosomes. Between differentiating lymph capillary endothelial cells we found variable intercellular contacts (Fig. 12, 16, 17, 18); among interdigital contacts of varying complexity there were "tight junctions" (Fig. 16, 17) and "zonulae et fasciae occludentes" (Fig. 15, 18), while in some places all the possible types of intercellular contacts seemed to be present together (Fig. 18). These intercellular contacts of the endothelial cells differentiate the lymph capillary from the blood capillary, in which intercellular contacts between endothelial cells are effected on the luminar side by means of "zonulae occludentes", the course of which is practically always perpendicular to the surface of the vessel (Fig. 19). On the outer

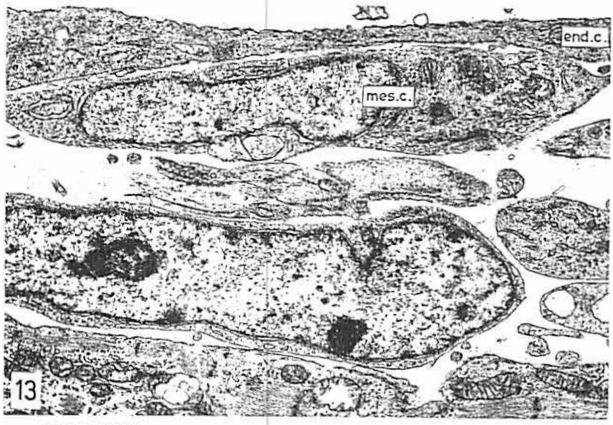

Fig. 13. The structure of the endothelial cell (end.c.) process of the lymph capillary of the chick embryo heart wall (11th day of incubation) is in principle the same as that of the adjacent mesenchymal cell (mes.c.). x 10,000.

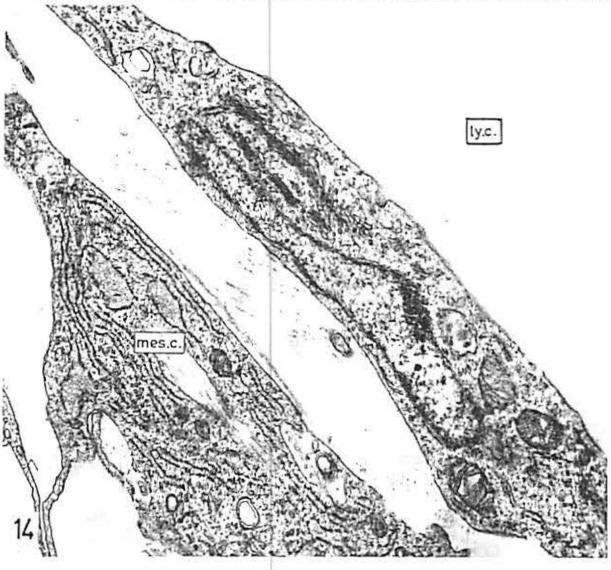

Fig. 14. Section of endothelial cell of lymph capillary (ly.c.) of chick embryo heart wall (11th day of incubation) at site of cell nucleus. No basal membrane on outer surface of endothelial cell (end.c.); mesenchymal cell (mes.c.) in vicinity. x 10,000.

surface of the blood capillary we find a continuous basal lamina (Fig. 19), while there is no trace of a basal lamina on the surface of the lymph capillaries of embryos of the same age (Fig. 15, 17). We find one on the surface of some already differentiated larger lymph vessels, but in these it is porous and intermittently discontinuous (Fig. 18).

The intercellular contacts of the mesenchymal cells depict the process of incorporation of the intercellular spaces into the lymph capillary bed system. Reciprocal contact with the mesenchymal cells is usually of the short "tight junction" type, thus shutting off the intercellular space (now a lymph bed lumen) from the surrounding mesenchyme, with which abundant contact is still maintained by means of the many cell processes. The system of point contacts is progressively transformed to forked interdigitations interspersed by tight junctions, until deep interdigitations and extensive "fasciae occludentes" finally complete the process of formation of the intercellular contacts

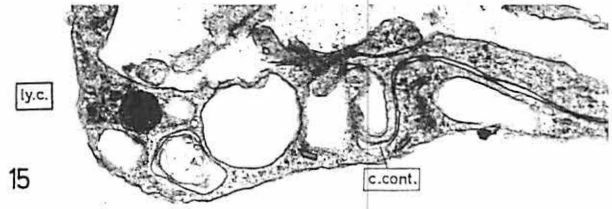

Fig. 15. Deep interdigital contacts (c.cont.) of two endothelial cells of lymph capillary (ly.c.) of chick embryo heart wall (11th day of incubation), with clearly differentiated zonulae et fasciae occludentes. x 20,000.

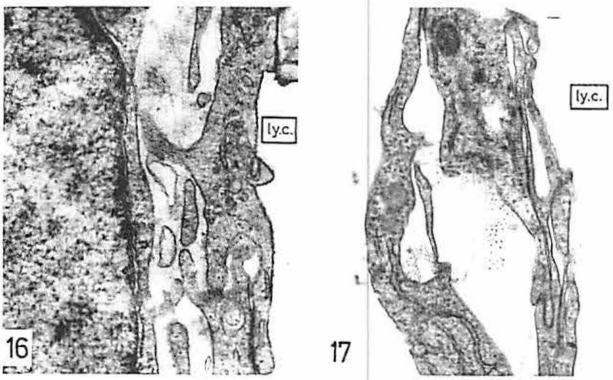

Fig. 16, 17. Simple (Fig. 16) and complicated (Fig. 17) interdigital contacts of lymph capillary (ly.c.) endothelial cells of chick embryo heart wall (11th day of incubation). x 10,000.

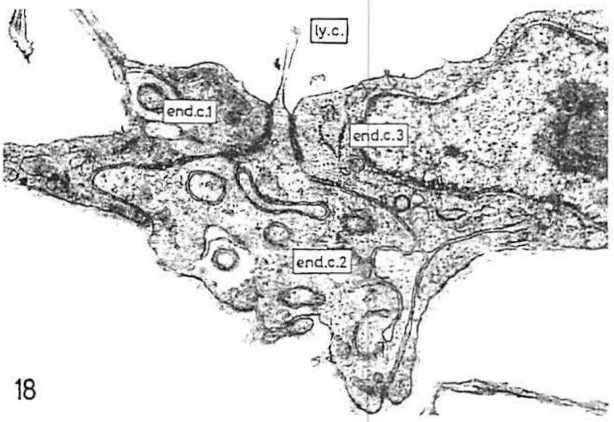

Fig. 18. Different types of cell contacts occurring simultaneously between three endothelial cells (end.c.1,2,3) at same spot in lymph capillary (ly.c.) of chick embryo heart wall (11th day of incubation). x 10,000.

of the differentiating lymph capillary endothelium (the whole process is illustrated topographically and morphologically in Fig. 20a, b, c, d and Fig. 21a,b).

Discussion

Since the development of the blood supply of the myocardium precedes the development of its lymph bed both in time and place, and since differentiation of the endothelial cells of the blood and lymph capillary are two separate processes, differences in the structure of the blood and lymph vessel endothelial cells are clearly evident in both the light and the electron microscope – more so than in the adult organism (see the reports 1, 2 and 3, mentioned in the introduction). The endothelial cell of the

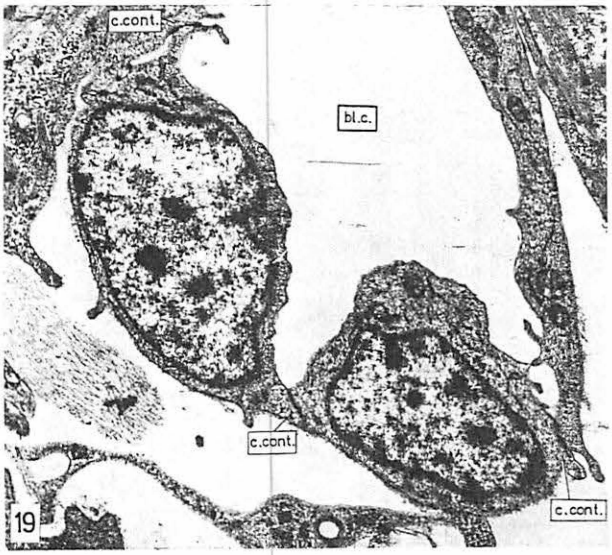

Fig. 19. Developing blood capillary (bl.c.) in chick embryo myocardium (11th day of incubation), characterized by cell contacts (c.cont.) lying roughly perpendicularly to the vascular wall. x 10,000.

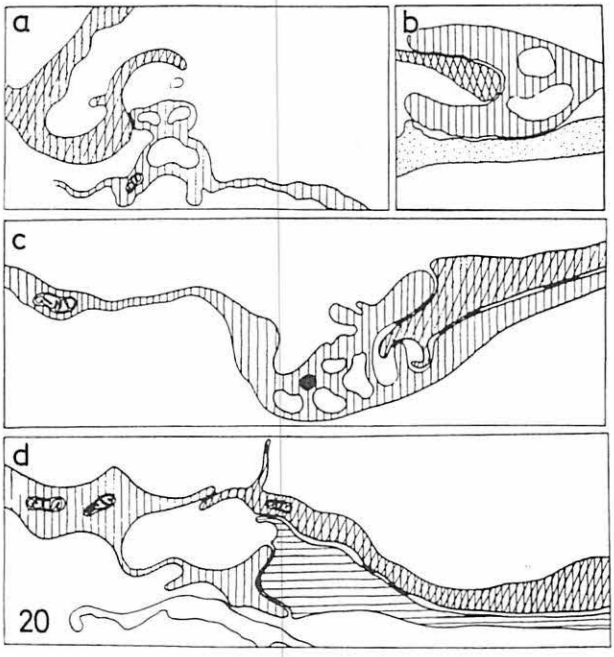

Fig. 20a,b,c,d. Diagram of different types of cell contacts between endothelial cells of lymph capillaries of chick embryo heart wall.

primitive lymph capillary is at first almost identical with the mesenchymal cell, while the blood capillary endothelial cell is already highly differentiated and thus differs from the lymph capillary endothelial cell. In future, this could be utilized for explanation of the fundamentals of the process of differentiation of the endothelial cell and for determining whether the course of differentiation of the two types of endothelial cells is the same.

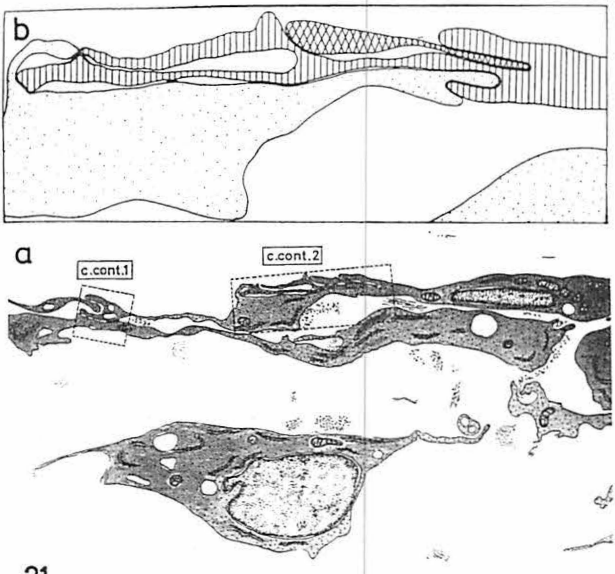

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Fig. 21a,b. Varyingly complicated interdigital contacts between endothelial cells of lymph capillaries of chick embryo heart wall. a) Diagram of part of lymph capillary, showing cell contacts (c.cont. 1,2) outlined by broken line. b) Diagram of more complicated cell contact (c.cont. 2). The simpler cell contact (c.cont. 1) is illustrated in Fig. 20b.

As far as the relationship of the origin of the lymph capillary to the mesenchymal intercellular space is concerned, the situation is somewhat simpler than *Czillik* (6) supposed. We cannot uphold his view that the lymph capillary must have a clearly discernible endothelial lining. Firstly, the plane of the section can be chosen so as to allow quite good observation of the relationship of the lymph capillary and the tissue space and secondly, the degree of difference in the appearance of the differentiated endothelial cell and the cell lining a tissue space depends on how long their contact has lasted. The difference is greatest at the moment when the capillary opens into the space; in time it diminishes and finally disappears altogether. At least, this is the case during development, but it is not clear whether the same applies during adulthood. The developmental process seems to be irreversible, as if it had a permanent direction. In adult life, however, there is evidently no increase in the capillary network. It is questionable whether *Clark*'s observation (10) – i.e. that the slender processes of the lymph capillary (in his case in the tail of the frog tadpole) were formed, regressed and reappeared elsewhere in its wall (in ultrastructural terms this means that the communications of the lymph capillary with the intercellular spaces were alternately closed and reopened at different sites) – also applies to the lymph capillary bed of the adult organism. It is not clear whether such new communication of the lymph capillary with the intercellular space would make the connective tissue cells of the adult organism undergo the same changes (differentiation to endothelial cells) as during development. If so, these changes would necessarily be reversible, as otherwise there would be a continuous increase in the population of new endothelial type cells in the connective tissue.

In conclusion, it should be borne in mind that, at the time when the lymph bed is being propagated over the surface of the heart in the subepicardial mesenchyme, no pronounced differentiation of the wall of the lymph vessels occurs and that the intracellular filaments typical for the lymphatic endothelial cell of the adult organism (1, 2) are formed much later. According to *Kuprianov* (11, in dog), the simple endothelial

wall of the lymph capillary also starts to undergo alteration, i.e. differentiation, behind the first valve in the adult organism. This, however, only confirms that the phase of embryogenesis from the 9th to 14th day of incubation, in which we studied the initial development of lymphatic drainage of the wall of the heart up to formation of the first valves, is defined entirely naturally.

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Lymphology 5 (1972) 148-152

Trapping of Calibrated Microspheres in Experimental Rat Lymph Node Metastases

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Summary

The position in rat lymph nodes of microspheres relative to embolized tumor cells and micrometastases was studied histologically to evaluate some aspects of intralymphatic cancer treatment.

Walker carcinosarcoma cells were injected into afferent lymphatic channels of rat lymph nodes. Calibrated, nonradioactive microspheres were also injected, either together with the tumor cells or after 3-day or 7-day intervals. Twenty-four lymph nodes were available for study. In 18 of the 24 positive lymph nodes, microspheres were intermingled with tumor cells or micrometastases. However, in every instance, most of the microspheres were found in the uninvolved portions of the lymph nodes, both near the tumor-cell infiltrates and micrometastases and at a distance from them. Even when microspheres were in contact with tumor cells, the number of microspheres relative to the tumor cells was very small. Lymph node metastases probably cause diversion of the lymph flow and thus prevent optimal contact of tumor cells and microspheres.