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EARLY POSTNATAL GROWTH OF THE INITIAL LYMPHATICS IN THE VENTRAL STRIPE OF SPINOTRAPEZIUS MUSCLE OF THE RAT

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ABSTRACT

The aim of this study was the morphological description of the early postnatal growth of initial lymphatics in the ventral stripe of the spinotrapezius muscle of the rat. Electron-microscopically it was found that in the muscles of newborn rats no well-developed lymphatics were apparent, but the presence of specifically polarized mesenchymal cells in the close vicinity of central blood vessels was evident. In animals aged one-day through two-weeks-old, those modified mesenchymal cells continuously joined with one another, to form simple intercellular contacts and incomplete lymphatic lumina. Morphologically, they were well demarcated relative to the surrounding muscular interstitium. In three-week-old rats, all intramuscular lymphatics were well developed, including fine intraluminal valves, and their endothelial cells presented slight pinocytotic activity and a complete absence of a basal lamina. In growing lymphatic endothelial cells, no mitoses or signs of sprouting, typical for the growth of blood capillaries, were found. In conclusion, a possible morphological mechanism enabling the expansion of the growing lymphatic endothelial cells could be the specific remodeling of cytoplasmic vacuoles accumulated in the peripheral cellular processes.

The lymphatic vessels of the skeletal muscles play a very important role in the drainage of the extracellular fluid from the muscular interstitium during both normal as well as pathological conditions (1,2). While their basic structure was described in adult muscles on the level of both the light and electron microscope many years ago (1,3-5), only limited attention was paid to their pre- and postnatal development. Aminova (6) and Poggi et al (7) have found well developed lymphatics in the diaphragms of newborn rabbits and rats and described their subsequent maturation during the early postnatal period. Similarly, the presence of fully developed lymphatics was described in the hilus of soleus and gastrocnemius muscles of newborn rats, but in the same animals the lymphatic vessels in the ventral stripe of spinotrapezius muscle were first detected at the beginning of the second postnatal week (8,9).

None of these studies were able to describe the morphological details of the early postnatal formation of intramuscular lymphatics, even on the ultrastructural level. From this point of view, the questions of origin and of the earliest postnatal growth of intramuscular lymphatics still belong to the unsolved general problems of embryology and histology, e.g., whether the *initial lymphatics* start to develop either as real processes of the peripheral venous system or from the

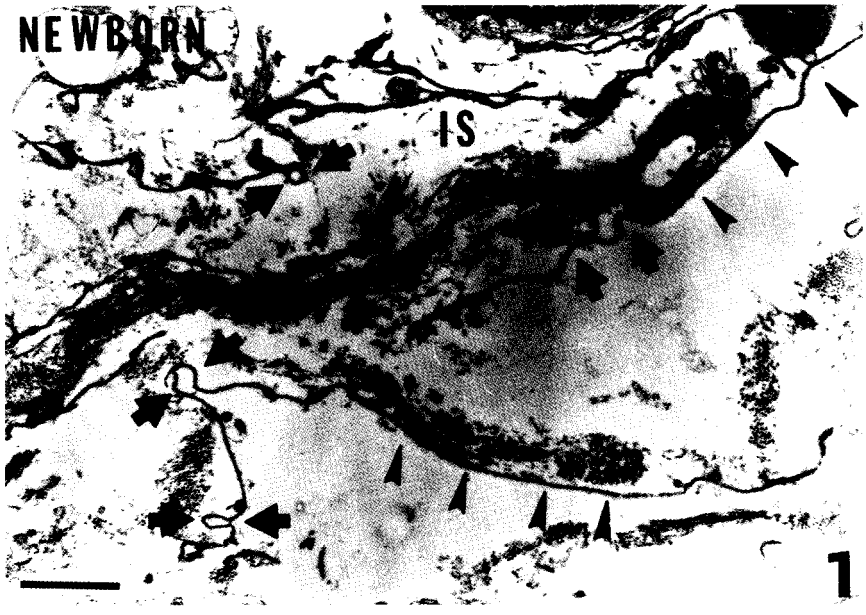

Fig. 1. The interstitial space in the close vicinity of the central blood vessels of the ventral stripe of spinotrapezius muscle of the newborn rat. Note the smooth and free surfaces (arrowheads) as well as a striking undulation (full arrows) of some mesenchymal cells. Bar: 2 μm; x 6800. IS=perivascular interstitial space of the muscular stripe.

mesenchymal cells *"in situ"* in particular regions and organs (10-12).

The aim of this study was to contribute to the solution of the above mentioned problem regarding the exact detection of the initial lymphatic vessels in the ventral stripe of the spinotrapezius muscle of the rat during their earliest postnatal growth. This muscle has—besides its superficial location and small size, and unlike the diaphragm used in above mentioned studies—a very simple arrangement of both blood and lymphatic vessels, enabling an exact morphological and space orientation through the study of all segments of the vascular bed (3,13). Another reason for the use of this muscular model was based on the previously described fact that its lymphatics evidently arise and begin to grow and fully develop during the first postnatal week (9) and not prenatally as it was found until now in all other muscles studied to date. Two important advantages of this model are also substantially valuable for any experimental use: an easy surgical approach to the muscular stripe, which is well defined by two vascular hili; and the possibility of exact timing for the removal of the samples from the postnatally growing animals.

MATERIAL AND METHODS

Wistar rats of both sexes (BIOTEST s.r.o., Konárovice, Czech Republic) were used, reared under optimal laboratory conditions and without any previous experimental interference. Thirty animals were used, divided into six groups (5 animals in each group) at ages newborn, 1 and 2 days and 1, 2 and 3 weeks. Before removal of muscles all rats were anesthetized intramuscularly with Ketamine (Narcamon 5%, Spofa, Czech Republic) 100 $mg.kg^{-1}$ i.m. In all animals the central portions of the ventral stripes of the spinotrapezius muscles (precisely limited by the anterior and posterior vascular hilus) were bilaterally fixed *in situ* by immersion in Dalton's fixative (14). After dissection and dehydration in graded alcohols, the muscles

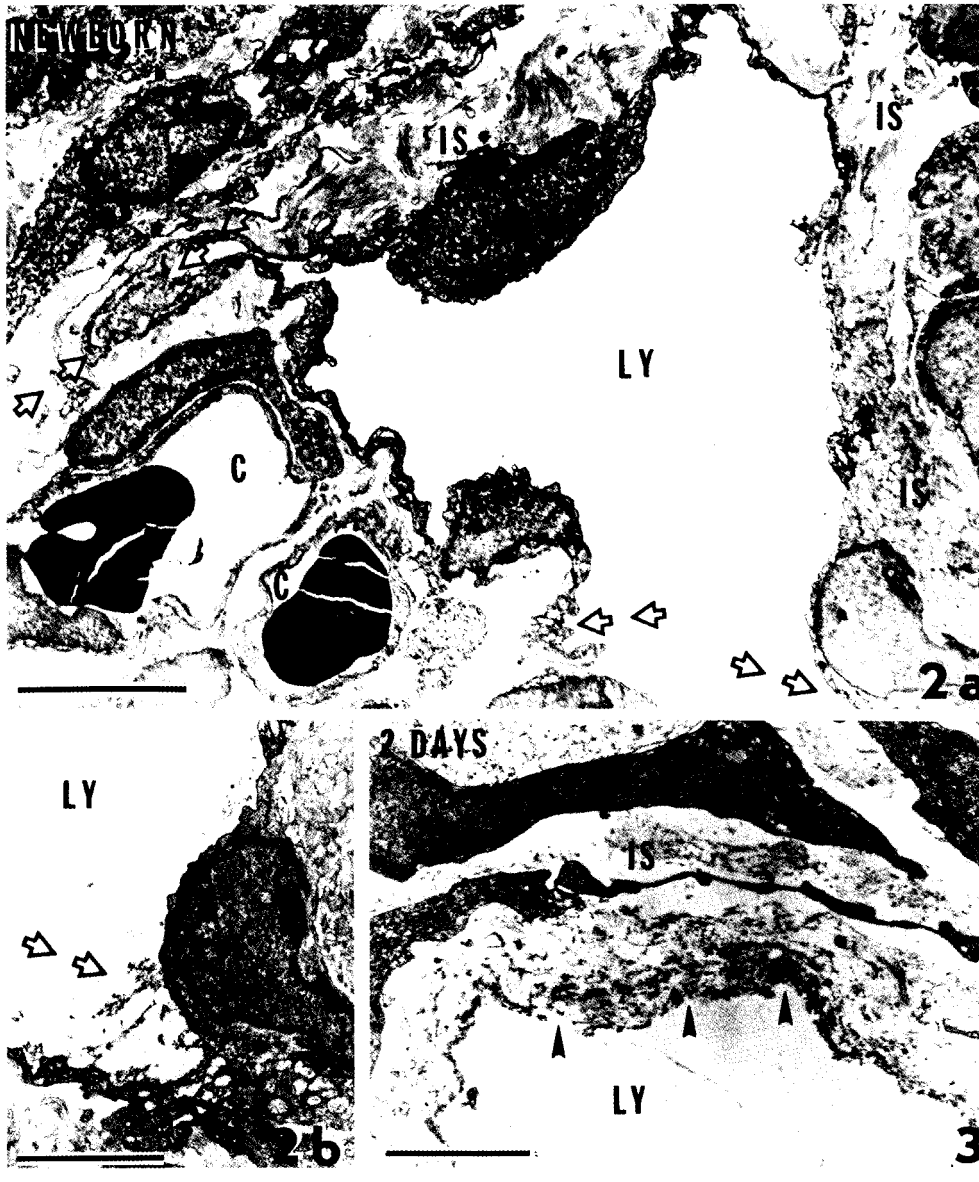

Fig. 2. a) Well-developed initial lymphatic in the muscle of newborn rat. Double empty arrows designate the parts of lymphatic endothelial cells filled with numerous small vacuoles. Bar: 5.4 µm; x 5200. b) Detail of the right lower corner of a. Bar: 4 µm; x 6200. C=blood capillaries, IS=perivascular interstitial space of the muscular stripe, LY=lymphatic lumen.

Fig. 3. The wall of the initial lymphatic in the muscle of 2-day-old rat. Arrowheads designate the absence of the endothelium. Bar: 4.7 µm; x 5100. IS=perivascular interstitial space of the muscular stripe, LY=lymphatic lumen.

were embedded in Epon. The semi-thin sections (1 µm) were sectioned on the ultramicrotome (Reichert) and stained with toluidine blue. The ultrathin sections were contrasted with uranyl acetate and lead citrate, and observed in an electron microscope (TESLA BS 500) at 60 kV.

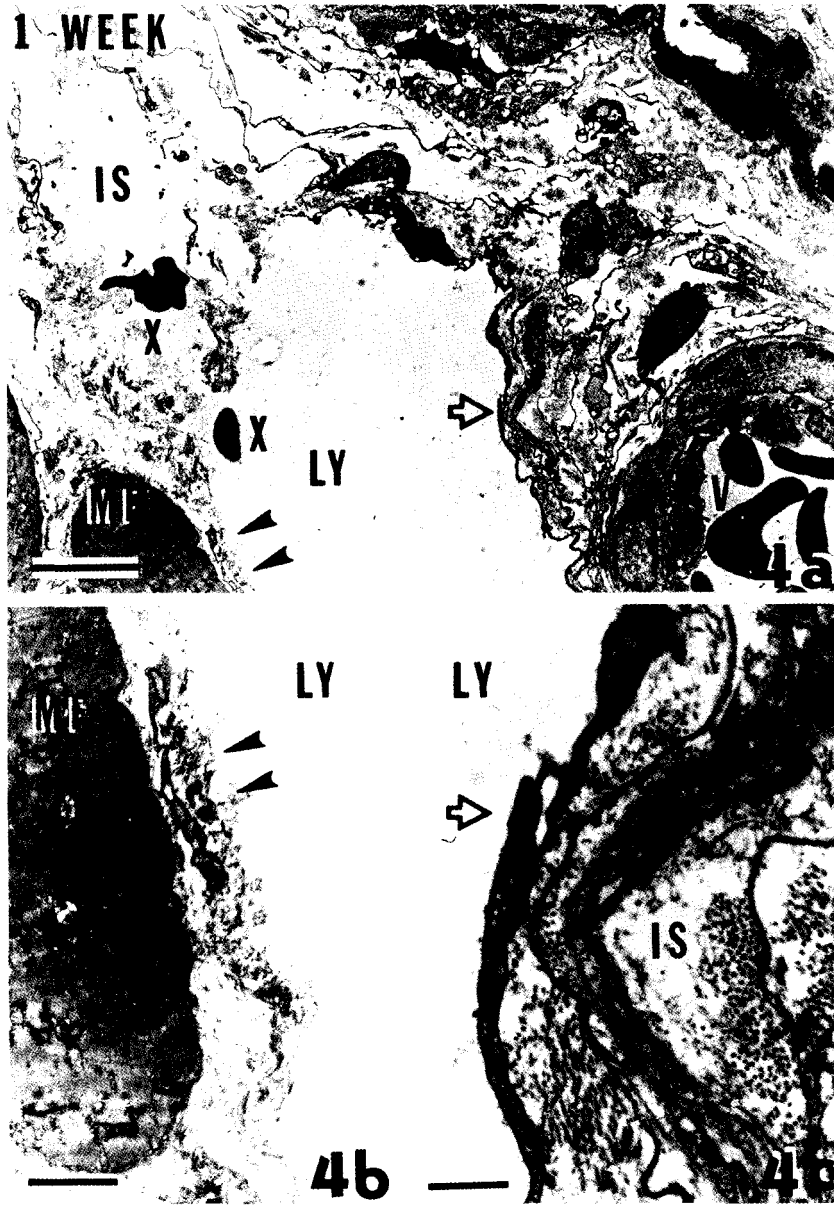

Fig. 4. a) Growing initial lymphatic in the muscle of one-week-old rat. Empty arrow – simple contact of two neighboring endothelial cells, arrowheads – free mesenchymal cell on the opposite, still uncomplete wall of the lymphatic. Bar: 5.2 µm; x 2700. b) Detail from the left part of a. Bar: 2 µm; x 5700. c) Detail from the right part of a. Bar: 1 µm; x 11000. IS=perivascular interstitial space of the muscular stripe, LY=lymphatic lumen, MF=skeletal muscle fiber, X=free extravasal erythrocytes.

RESULTS

The light and electron-microscopic investigations brought the following results:

1. In the muscular stripes of *newborn rats* the entire perivascular interstitial space of the central blood vessels was filled with irregularly oriented flat mesenchymal cells,

Fig. 5. Three lumina of the initial lymphatics in the close vicinity of the central vein in the muscular stripe of three-week-old rat. Bar: 2 µm; x 12000. IS=perivascular interstitial space of the muscular stripe, LY=lymphatic lumen, M=smooth muscle cells of media in the central vein, V=central vein of the muscle stripe.

and no fully developed lymphatic lumina were apparent in the semi-thin sections using light microscopy.

On the other hand, in the same specimens studied electron-microscopically, many of the above mentioned mesenchymal cells were found in the close vicinity of the central blood vessels, differing substantially from the other ones *(Fig. 1)*. Above all, the peripheral parts of those cells presented an obvious morphological polarization, e.g., no apparent evidence of collagenous fibril production on one side of their surfaces while on the opposite surfaces a rich accumulations of irregularly oriented fibrils were present. As a second morphological sign, typical of those

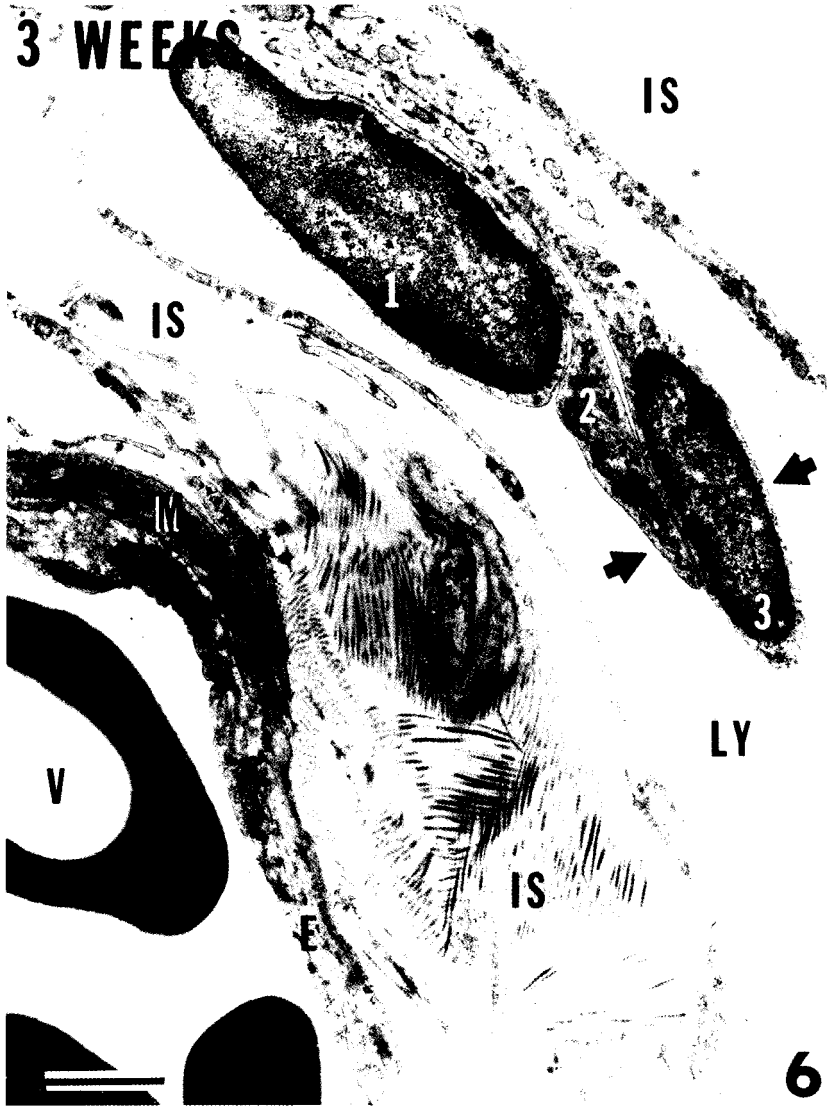

Fig. 6. Well-developed lymphatic vessel located close to the outer surface of the central vein in the muscular stripe of three-week-old rat. Numbers 1-3 and full arrows designate three endothelial cells participating on the construction of an intraluminal valve . Bar: 1 µm, x 15400. IS=perivascular interstitial space of the muscular stripe, LY=lymphatic lumen, M= smooth muscle cells of media in the central vein, V=central vein of the muscle stripe.

cells, irregular undulations and propagation of their flat and thin peripheral parts into the periphery, covered on the convex surfaces by collagenous fibrils, were detected.

There was one exception: in one sample from the group of newborn rats, we did find one case of a well developed lumen, typical for an initial lymphatic capillary (*Figs. 2a,2b*). The wall of this vessel was built by thin and flat cells with poor pinocytotic activity and without a basal lamina; only simple flat contact types of neighboring cells were found. The wall of this lymphatic was very irregularly curved and its inner diameter varied from 5 to 10 µm. In several places irregular protrusions of the peripheral parts

of lymphatic endothelial cells were found containing numerous small vacuoles but no evidence of endothelial sprouting, typical of growing blood capillaries, was detected.

2. In the muscles of *one-day up to two-week-old rats* the lymphatic lumina were easily and regularly found also in the semi-thin sections, but their detailed analysis was possible only through electron-microscopy *(Figs. 3,4)*. All detected lymphatic vessels were typically located in the close vicinity of the central vein and had irregular light lumina. In most cases, their endothelial layers were incomplete, although the intraluminal clean contents were strictly separated from the heterogeneous abluminal space, rich above all on collagenous fibrils, even in the places where the endothelial cells were absent.

The detailed analysis of these segments of initial lymphatics has shown that the width of the intervals where the endothelial cells were absent, was usually about 1-5 μm, and the participation of free mesenchymal cells in the construction of the lymphatic wall was evident in many places *(Fig. 4b)*. In the well developed parts of the wall, simple contacts between endothelial cells were found, joined with their rich pinocytotic activity and a complete absence of basal lamina *(Fig. 4c)*.

3. In *three-week-old rats,* all detected intramuscular lymphatics, from the morphological point of view, were well and fully developed. Their very irregularly curved lumina were encircled by a complete layer of flat endothelial cells *(Fig. 5)*. In some places the nuclear parts of endothelial cells formed typical valves, partially closing the lymphatic lumina *(Fig. 6)*. The submicroscopic structure of the lymphatic endothelial cells fully corresponded with the previously described findings in adult muscles.

DISCUSSION

The results obtained have shown that, from the methodical point of view, the muscular model used is well suited for such a developmental study. In this particular muscle model, all lymphatics were located, at the very beginning of their growth, only in the close vicinity to the central blood vessels, as an integral part of the main neurovascular bundle of the stripe. Therefore their histological detection during all postnatal stages studied was reliable and easily accomplished. This morphological arrangement is also substantially simpler than that found in the diaphragm (6,7), where the situation is complicated by the presence of both intramuscular lymphatics proper and the extensive subpleural and subperitoneal lymphatic nets on both surfaces of the muscle (15).

The observed growth of the lymphatics corresponds well with similar, previously described postnatal maturation of its muscular fibers (16,17), blood vessels (8,9), and its vascular and motor activities (18). From all of these studies, it is evident that from the third postnatal week all parts of the muscle are morphologically and functionally fully mature. The fact that its lymphatics start to be apparent and grow postnatally, contrasts with the prenatally detected lymphatics in the diaphragms of rabbits and rats (6,7), and can be explained by the different sizes of the studied muscles (19).

The study has also shown that the earliest stages of the growing lymphatics in newborn rats are only detectable electron-microscopically, because the process of the transformation of the mesenchymal cells into the primitive prelymphatic lumina is very subtle. During development, the neighboring lymphatic endothelial cells start to join with one another, forming simple contacts, typical of initial muscular and intestinal lymphatics development (20). None of these cells showed evidence of mitoses or endothelial sprouting (21). Therefore it seems that the only possible morphological mechanism, enabling the growth of lymphatic endothelial cells, is the dynamic transformation of small intraplasmatic vacuoles, typically found in peripherally expanding parts of the lymphatic walls.

It is also evident that the classical electron-microscopic analysis is not able to detect the real precursors of the future lymphatic endothelial cells, e.g., exactly which family of intramuscular mesenchymal cells belongs to them. There is no doubt that this problem can be solved only by using more specific methods like electron-microscopic immunohistochemistry, molecular biological and genetic procedures (22-25).

Nevertheless, using a simple experimental muscular model, it was possible to describe some important aspects of the origin and early postnatal growth of the intramuscular lymphatics, beginning *"in situ"* with the transformation of the described specific mesenchymal cells. Our hope is that we have been able to contribute to the explanation of the classical question "where do the initial lymphatics come from and how do they develop?" (24). It is also evident that the very interesting and important problem, e.g., when and how the *"in loco"* growing initial lymphatics start to join with the central lymphatic system, remains for future thorough morphofunctional studies.

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