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Analysis of human omentum-associated lymphoid tissue components with S-100: an immunohistochemical study

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Abstract

Milky spots are opaque patches in the greater omentum. They were first described by von Recklinghausen (1863) in the omentum of rabbits. In man, milky spots are relatively uniform, highly vascularized accumulations of mononuclear cells. The objective of this study was to describe in human omental lymphoid tissue components with S-100. Tissue samples (greater omentum) were collected from 14 patients operated with different reasons in our Department of General Surgery, in order to histologically present the presence of S-100 in the cells making up the milky spots in human omentum tissue. Tissue samples were cut approximately 5–8 micrometer thick with frozen-sections and stained with an indirect immunoperoxidase technique, as described previously. Then milky spots were examined by light microscopy. These data indicate that unstimulated milky spots in the human greater omentum are to a great extent just a preformed specific accumulation of primarily macrophages within the stroma of the greater omentum, secondarily B- and T-lymphocytes. In addition to these cells, we observed that a few mast and reticular cells were seen in the milky spots by S-100 reactive cross-sections of greater omentum. In the human omentum tissue that was stained with indirect immunoperoxidase method using anti S-100 monoclonal antibody, an arteriole cross-section in the center, reactive nerve cross-sections in the adjacent stroma and endogenic peroxidase reactivity in a few granulocytes in omental tissue were observed.

Keywords: milky spots, omenthal lymphoid tissue, light microscopy, S-100.

Introduction

Omentum-associated lymphoid tissue (OALT) was first described by von Recklinghausen in 1863 after monitoring white spots in omentum of rabbits, as milky spots surrounding capillary network that are intense accumulation of connective tissue cells [1].

In animal studies, including rats, rabbits, swine, dogs, squirrels, cats, bats, moles, cattle, chicken, frogs, sheep and goats, milky spots have been shown to be essentially made up of lymphocytes and macrophages with or without blood vessels [2–6]. Presence of OALT in humans was first discovered by Seifert in 1921 [7]. Milky spots were very few in number and buried in adipose tissue especially in older subjects [1]. Several studies have described the function of milky spot macrophages to clean microorganisms and carcinogenic cells, especially in peritoneal cavity [3–5]. Furthermore, other than reactions against intraperitoneal stimuli, milky spots have been considered to play a role in ontogenesis of B-cells, therefore function as primary lymphoid organ similar to thymus in intestines [6, 7]. Since milky spots are a rich growth factor source, omentum is known to have an important capacity in wound healing, thus to present an advantage in abdominal surgery [8]. Omentum limits infection by adhering to perforation and inflammation regions [4]. Omentum is a region within peritoneum only, except for diaphragm pores that have the ability to absorb bacteria and foreign particles [9, 10]. Milky spots lymphocytes are able to enter and exit the peritoneal cavity via mesothelial pores. Milky spots are specialized lymphoid structures that have an important function in immune defense of the peritoneal cavity [1]. Cell aggregation in omentum, known as milky spots, is a source of polymorphonuclear leukocytes, macrophages and lymphocytes. Milky spots composed of mesenchymal cells that are located in curves surrounding the capillary, named omental glomerulus and they are observed as woolly cotton mass with the naked eye [9, 11–13].

S-100 is a calcium-binding protein primarily produced and released by astrocytes in the central nervous system (CNS). It has neurotrophic and gliotrophic actions, possibly having important roles in normal CNS development and recovery after injury [14, 15]. S-100 has been shown to be a useful neuro-biochemical marker of brain damage in cardiac arrest [16, 17] stroke [18], subarachnoid hemorrhage [19], and traumatic head injury [20]. Neuron-specific enolase (NSE) is a glycolytic enzyme that is localized primarily to the neuronal cytoplasm. Because NSE is not physiologically secreted, an increase in its serum and cerebrospinal fluid concentrations can be associated with structural damage to neuronal cells, as reported in traumatic brain injury [2], cardiac arrest [16], and Parkinson disease [15].

Purpose of this study is to analyze human omentum-associated lymphoid tissue components with S-100.

Material and Methods

Tissue samples were obtained from 14 patients operated in Department of General Surgery, Faculty of Medicine, Dicle University, with different reasons, in order to histologically present the presence of S-100 in the cells making up the milky spots in human greater omentum tissue. Most S-100 proteins are homodimeric, consisting of two identical polypeptides held together by non-covalent bonds. Although S-100 proteins are structurally similar to calmodulin, they differ in that they are cell-specific, expressed in particular cells at different levels depending on environmental factors. To contrast, calmodulin is a ubiquitous and universal intracellular Ca^{2+} receptor widely expressed in many cells. The study was approved by the Hospital Ethical Committee.

Fresh tissue samples were immediately frozen in liquid nitrogen and kept at -70^0C until sectioned. Using the immunoperoxidase procedure described in detail previously by Kishimoto T *et al.* [21], the sections were fixed in acetone for 10 minutes and air-dried for at least 30 minutes. They were then incubated for 60 minutes in 1:100 dilutions of the primary antibody for S-100. After washing with a 0.01 M phosphate-buffered saline (PBS) at pH 7.4, the sections were covered with 1:200 dilution of anti-mouse Ig peroxidase (Sigma, Catalogue No. A–0168, St. Louis, MO), in PBS containing 0.2% bovine serum albumin (Sigma, Catalogue No. A–7034, St. Louis, MO) and 1% normal human serum. After washing the sections in PBS, they were stained for peroxidase activity with 3,3'-diaminobenzidine-tetrahydrochloride (Sigma, Catalogue No. D–5637, St. Louis, MO) in 0.5 mg/mL Tris-HCL buffer, pH 7.6, containing 0.01% H_2O_2. Counterstaining with Hematoxylin was then carried out. A double control staining was performed by omitting the primary antibody step and using a nonspecific control mouse IgG. The stained sections were then examined and photographed using an Olympus BH2 conventional light microscope.

Results

The compact structure of the milky spots was characterized by a cellular reticulum sustained by adjoining fine bundles of collagenous fibers, but this reticulum was populated by macrophages, B- and T-lymphocytes, a few mast and reticular cells. Further, endothelial cells formed fenestrated and discontinuous capillaries to which nerve fibers were seen in close proximity (Figures 1 and 2).

Cell clusters

In the non-vascularized and vascularized clusters of cells were seen in sections. A very small number of clusters of cells of up to approximately 80 cells were present around arterioles (Figure 3). And also, a very small number of clusters more than one hundred cells were present around venules (Figure 2). When we compared the sizes cells around arterioles (Figure 3) to venules (Figure 2), sizes cells around arterioles were larger than venules. The cells sizes around arterioles and adipose cells were almost the same (Figure 3). However, adipose cells usually were seen in clusters, alone adipose cell were also seen seldom (Figures 1 and 4). The distribution of cells around adipose cells were irregular (Figures 1, 2 and 4), although those of cells were regular around arterioles (Figure 3) and venules (Figure 2).

Macrophages

In the milky spots, macrophages were found dispersed within the meshes of the reticulum cells, as well as on the surface of the mesothelial cell layer (Figure 3). Occasionally, a macrophage was spotted. Unlike the mesothelial cells of the greater omentum, the mesothelial cells of the milky spots had openings between them through which cells passed (Figure 2). Very small number of macrophages was found randomly dispersed over the entire greater omentum. Shape of macrophages had a round, oval, or elongated cell-shape (Figure 5). The relatively immature macrophages were found mainly perivascularly inside the milky spots (Figures 2 and 3), whereas the more mature forms were present in high numbers on the mesothelial cell-layer (Figure 1).

Lymphocytes

B-lymphocytes with a round nucleus and a small rim of cytoplasm could be seen throughout the milky spots, but no plasma cells were present. Specific orientation or clustering of lymphocytes was noted. B-lymphocytes were localized near reticulum cells of the milky spots (Figure 1). T-lymphocytes were stained more darker than the B-cells have a distribution between neighboring adipose cells (Figures 1, 2 and 5). No cells in B-cell follicles and T-cell areas were observed in lymphoid organs with the immunoperoxidase method of S-100.

Mast cells

Mast cells had a small round nucleus and an abundance of cytoplasm filled in the milky spots. The mast cells had no specific location within the milky spots (Figure 1).

Other structures

In all specimens the greater omentum was characterized by a continuous lining of mesothelial cells on both sides (Figure 3), and a widely meshed cellular reticulum sustained by fine bundles of collagenous fibers in which blood vessels and nerve fibers were sporadic (Figures 1–6). The endothelial cells of the blood vessels in the greater omentum and in the milky spots were the same with the following exceptions. The endothelial cells of the greater omentum rested on a continuous basal lamina whereas the endothelial lining of the blood vessels in the milky spots was discontinuous (Figure 3). Observing human omentum tissue that is stained with indirect immunoperoxidase method using anti S-100 monoclonal antibody, adipose cells, omentum-associated lymphoid tissue showing minimal infiltration and cross-sections of two nerves showing strong S-100 reactivity are visible (Figure 4). In the human omentum tissue that

is stained with indirect immunoperoxidase method using anti S-100 monoclonal antibody, an arteriole cross-section in the center, reactive nerve cross-sections in the adjacent stroma and endogenic peroxidase reactivity in a few granulocytes in omental tissue are observed (Figure 3). In low magnification of omentum cross-section, omentum-associated lymphoid tissue (milky spot), stromal region richer than adjacent adipocyte and S-100 reactive cross section of a small nerve fiber within a smaller lymphoid region adjacent the venule in the upper left corner were seen (Figure 1). In a similar region, adipocytes, two venules and omentum-associated lymphoid tissue around the lower venule are visible. Cross sections of periphery nerves are seen to show strong S-100 immunoreactivity (Figure 2). In higher magnification, cross-section of nerve fiber in another region, adipocytes, lymphoid tissue cells and fibroblasts can be differentiated (Figure 6).

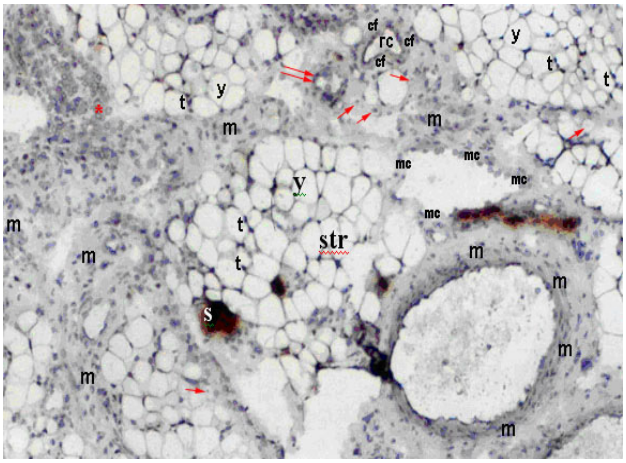

Figure 1 – ***One cross-section of a milky spot demonstrating the lining of mesothelial cells (mc), the reticulum cells of the greater omentum (rc), macrophages (m), B-lymphocytes (arrows), T-lymphocytes (t) were stained more darker than the B-lymphocytes (arrows) have a distribution between neighboring adipose cells (t), mast cells (double arrows) which were triangularly shaped and formed adherent junctions in contact areas with collagen fibers (cf), adipose cell (y), omentum-associated lymphoid tissue (*), lipid-cell-rich stroma (str) and nerve fiber (s). (Indirect immunoperoxidase technique – immunoperoxidase-counterstained with Hematoxylin staining, original magnification, ob. ×10).***

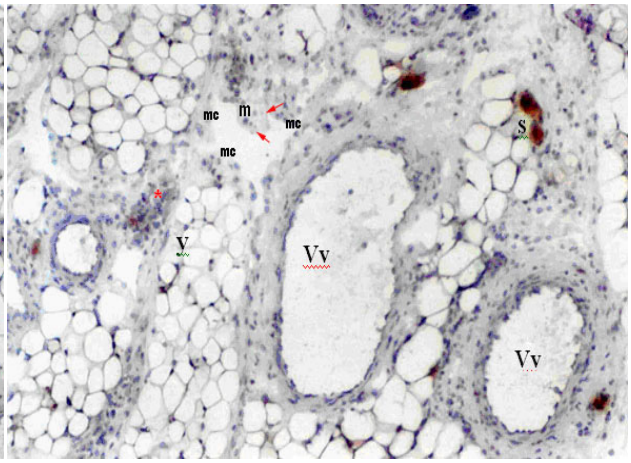

Figure 2 – ***One cross-section of a milky spot demonstrating the lining of mesothelial cells (mc) interrupted by macrophages (m) and B-lymphocytes (arrows), arteriole (Aa), venule (Vv), adipose cell (y), omentum-associated lymphoid tissue (*), S-100 immunoreactivity in nerve cross-section (s). (Indirect immunoperoxidase technique – immunoperoxidase-counterstained with Hematoxylin staining, original magnification, ob. ×10).***

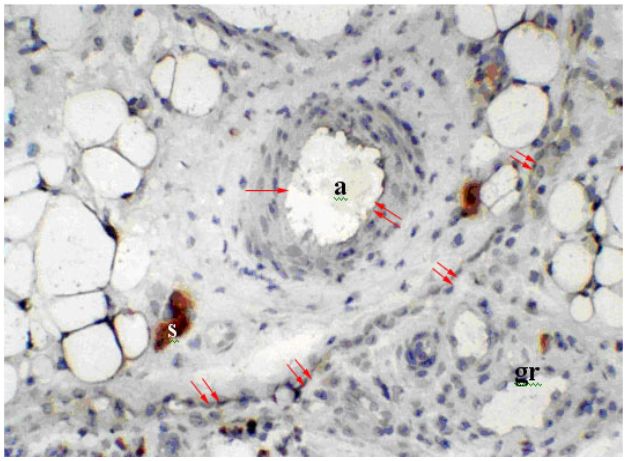

Figure 3 – ***One cross-section of a milky spot demonstrating arteriole (a), nerve cross-section (s), S-100 reactivity in granulocytes (gr) and the endothelial cells of the greater omentum rested on a continuous basal lamina (double arrows), whereas the endothelial lining of the blood vessel (a) in the milky spots was discontinuous (arrow). (Indirect immunoperoxidase technique – immunoperoxidase-counterstained with Hematoxylin staining, original magnification, ob. ×10).***

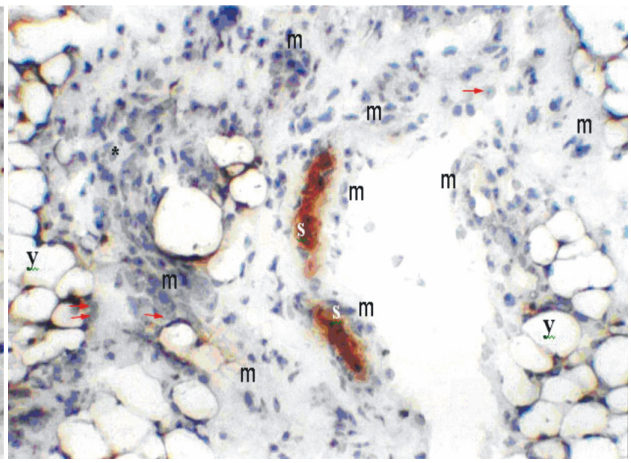

Figure 4 – ***One cross-section of a milky spot demonstrating mainly macrophages (m), adipose cell (y), omentum-associated lymphoid tissue (consist of mainly macrophages) (*), nerve cross-section (s), B-lymphocytes (arrows), which are dispersed throughout the milky spots. (Indirect immunoperoxidase technique – immunoperoxidase counterstained with Hematoxylin staining, original magnification, ob. ×10).***

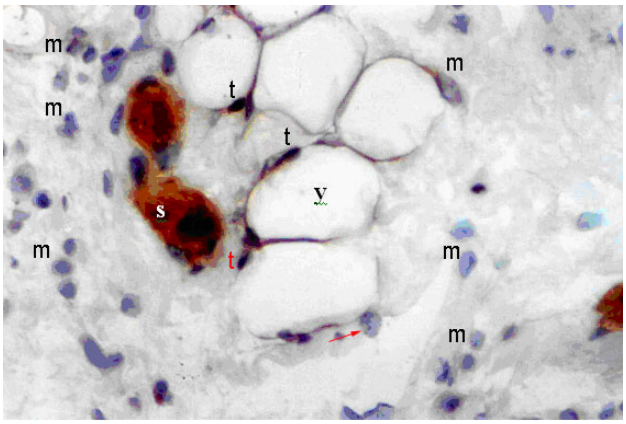

Figure 5 – ***One cross-section of a milky spot demonstrating B- (arrows) and T-lymphocytes (t), nerve cross-section (s) and adipose cell (y) with S-100, which are dispersed throughout the milky spots. (Indirect immunoperoxidase technique – immunoperoxidase-counterstained with Hematoxylin staining, original magnification, ob. ×40).***

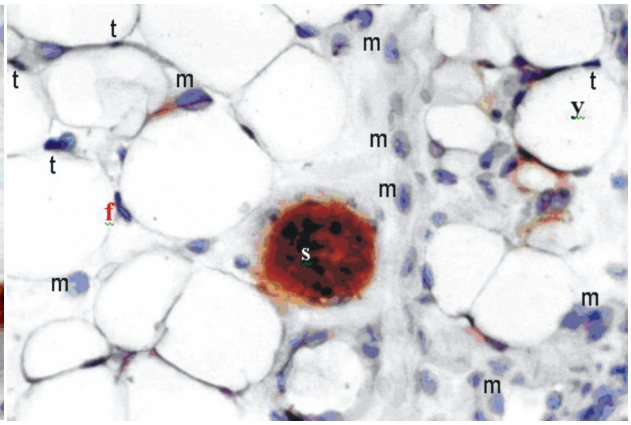

Figure 6 – ***One cross-section of a milky spot demonstrating macrophages (m), T-lymphocytes (t), adipose cell (y), nerve cross-section (s) and fibroblast cells (f) with S-100. (Indirect immunoperoxidase technique – immunoperoxidase-counterstained with Hematoxylin staining, original magnification, ob. ×40).***

Discussion

The objective of the present immunochemical study was to show unstimulated milky spots in the human greater omentum, and to determine whether these milky spots should be considered as secondary lymphoid organs. Second lymphoid organs are specialized structures in which antigen is presented to a population of recirculating lymphocytes. Our data show that unstimulated milky spots in the human greater omentum are uniform, the milky spots are consist of mainly vascularrized accumulations of macrophages, a few lymphocytes and mast cells within the stroma of the greater omentum. These cell types were localized in the milky spots. Cell clusters of macrophages were localized vascularized and non-vascularized areas. Macrophages situated around vessels were immature, but not in the milky spots. Specific B-cell areas or T-cell areas could be distinguished in the milky spots. While B-lymphocytes were localized near reticulum cells of the milky spots, T-lymphocytes stained more darker were localized between neighboring adipose cells.

Milky spots are opaque patches in the greater omentum. They were first described by von Recklinghausen (1863) as white spots in the omentum of rabbits and were named "taches laiteuses" by Ranvier (1874). Milky spots have been described in a variety of mammals [22]. Different studies have described the involvement of milky spots in the clearance of particles, tumor cells and bacteria from peritoneal cavity [8, 23]. Cellular elements of human milky spots are clearly observed. They can easily be counted, as complete peripheral smears preparates of the large omentum are transparent. Milky spots are elliptic with a diameter of 756±22 µm. Diameters of cells in vary between 15 and 20 µm. Macrophages are the cellular components of milky spots that are the largest in number (47.54±7.5%) and they exhibit a special arrangement within the milky spots. Second common cellular components are B-lymphocytes (29.1±5.2%) and T-lymphocytes (11.7±2.4%). Diameters of T- and B-lymphocytes vary between 7 and 10 µm. Mast cells that are the cells lowest in number (6.1±2.6%) in the human milky spots can easily be observed due to being very well stained with Toluidine Blue. Their diameters are 10–15 µm and are placed along capillary vessels [1]. Our quantification results differ from those described by Shimotsuma M *et al.* [1]. They found a lower percentage of macrophages (47.5±7.5%, mean ± standard error), a higher percentage of B-lymphocytes (29.1±5.2%) but almost the same percentage of T-lymphocytes (11.7±2.4%) in human milky spots. However, our results compatible with those described by Krist LF *et al.* [22]. Krist LF *et al.* showed that macrophages in fully-grown milky spots form about 70% of the cell population [22]. They were reported that based on immunohistochemical and ultrastructural criteria, milky spots in the human greater omentum are to a great extent just a preformed specific accumulation of primarily macrophages and cannot be classified as true secondary lymphoid tissue. In according to our result, we also observed that cell clusters were consist of mainly macrophages, a few lymphocytes and mast cells. In a different immunoperoxidase study, they examined specimens of human greater omentum were obtained from fetuses of 20 to 40 weeks gestation and one newborn 3-day-old. As in their result, small accumulations of cells with about 50% monocytes/macrophages were presented at 20 weeks of gestation. With increasing gestational age the number of clusters of cells were increased significantly ($p<0.01$) as well as their size ($p<0.01$). Starting at 29 weeks, vascularized clusters of cells were seen; true milky spots were presented at 35 weeks. A significant ($p<0.05$) increase in the percentage of mature macrophages was found in developing milky spots, whereas no activated macrophages were seen. The percentage of B-lymphocytes and T-lymphocytes were found in the clusters of cells and milky spots increased significantly ($p<0.05$) but did not exceed 10% of the total number of cells [24]. When we compared our findings with this study, the percentage of B-lymphocytes and T-lymphocytes was not exceed 10%

of the total number of cells. In addition, rest of the cells of the milky spots was consisting of immature and mature macrophages. However, we also determined that activated macrophages area was seen in our section (Figures 1 and 4). In according to the percentage of B-lymphocytes and T-lymphocytes in Krist LF *et al.* [24], we understand that the milky spots is not considered as secondary lymphoid organs even at 20 weeks of gestation. Previous work suggested that the omentum had an immunological function and showed that plasma cells could be found in the omentum after intraperitoneal immunization with T-independent antigens [25, 26] as well as classic T-dependent antigens [3, 27, 28]. Nevertheless, some authors have concluded that the milky spot should not be classified as secondary lymphoid organ [3, 29, 30]. Solvason N and Kearney JF [7] demonstrated that the human greater omentum is a primary site of B-cell development. They found a decrease of pre-B-cells in the investigated period up to 23 weeks and suggested that development of B-cells in the fetal omentum is a transitory process. Beelen RH studied unstimulated milky spots in adults and found the cellular quality to be approximately 70% macrophages, 10% B-lymphocytes and 10% T-lymphocytes and mast cells. B- and T-lymphocytes are aggregations close to peritoneal surface of milky spots. They are directed towards peritoneal cavity and stay ready for migration [13]. In our study, despite the percentage of B- and T-lymphocytes were lower than those of Beelen's study [13], the same findings were found in an adult milky spots.

In animal studies, too, it has been shown that milky spots develop in the fetus [31, 32]. Moreover, in pigs [31] and sheep [32] the same pattern of development is seen as in humans concerning the monocyte/macrophage population in milky spots. However, the presence of B-cells and T-cells varies considerably. In developing milky spots, B-cells appeared late in pigs and were absent in sheep, whereas T-cells were present in sheep and rarely present in pigs. It has been shown that the intra-abdominal status, like infection or tumor growth, has a profound influence on the type and percentage of cells present in milky spots [13, 33, 34]. The presence of disease in the abdominal cavity could have influenced the data presented by Shimotsuma M *et al.* [1]. Our results are largely in accordance with the results of animal studies; macrophages are the predominant cells, with lymphocytes and mast cells also present [13, 33]. Granulocytes were absent in our study in contrast with animal studies [12, 35]. In animals and humans, milky spots are primarily an accumulation of macrophages; B-cells and T-cells form but a small portion of the cells are present. This indicates that the development of milky spots in humans and animals as a macrophage organ is comparable. However, in different species the milky spots might have a different function in respect to the lymphocyte populations. As we used S-100 in this study, it was also used for different organs. S-100 is released into the peripheral blood because of an impaired blood–brain barrier injury due to ischemia and edema with high intracranial pressure [36]. In some cases, even a short period of anoxia was followed by an immediate leakage of S-100 into the serum. This initial rise may reflect reversible brain edema combined with a disturbance of astroglial cell membrane integrity and blood–brain barrier function [16].

Earlier researchers that studied omentum-associated lymphoid tissue made various comparisons in order to classify milky spots with different components. For instance, Renault J divided milky spots in their studies into two as primary milky spots containing blood vessels and secondary milky spots not containing blood vessels and stated that primary milky spots lost their association with blood vessels in time and were converted into secondary milky spots [37]. Similarly, Imai Y *et al.* [38] divided milky spots into two according to presence of lymphoid follicles. Type 1 milky spots do not contain lymphoid follicles, are not associated with blood vessels or adipose tissues and are only located in connective tissues of serous membranes. On the other hand, type 2 milky spots are stated to be associated with lymphoid follicles and located in adipose tissues near capillary networks. Furthermore, Borisov AV examined milky spots in human omentum in autopsy tissue samples and showed presence of milky spots in fetus omentum and that number of these decrease with increasing age [39].

Conclusions

Our data show that unstimulated milky spots in the human greater omentum are consist of mainly macrophages, B- and T-lymphocytes, a few mast cells, highly vascularized adipose tissue, omental lymphoid tissue, granulocytes, and nerve fiber within the stroma of greater omentum. Various cells were randomly localized in the human greater although milky spots in the human greater omentum are well vascularized, they do not show characteristics of peripheral lymphoid organs and cannot be classified as true lymphoid tissue or secondary lymphoid organs. In result, we think that milky spots may only play a role in bactericidal and tumoricidal functions due to accumulation of mainly macrophages.

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