Extracellular matrix between normality and pathology: techniques and methodologies

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Abstract

The interest in extra-cellular matrix investigation and diagnosis in the lymphedema cronic pathology is due to the fact that the lymphatics originate from here. The lymphatic system consists of lymphatic trunks that drain a network of early lymphatics located in less compact areas of peripheral tissues. Early lymphatics have a much bigger lumen than lymphatic capillaries and form a loose, low-density mesh.

The lymph flows through a system of vessels that are normally only temporarily filled; indeed, because of their function, they are designed to be empty most of the time. The lymphatic system can be classified into two sectors: the one with high absorption capacity characterized by lymphatics (ALPA) without basal lamina, in charge of collecting interstitial fluid, plasma proteins, electrolytes, and chylomicrons from the intercellular matrix; and the conduction one, formed by vessels with basal membrane which, on the outside, is strengthened by one or more layers of smooth muscle fibers (pre-collectors, pre and post lymph node collectors, lymphatic trunks). The absorption capacity of these vessels is strongly reduced and confined to a passive or active lymph permeation within the plasmatic membrane. The extra-cellular matrix consists of extremely thin structures, hence it is impossible to study it with conventional histological means on formalin fixed and paraffin embedded specimens. To overcome this difficulty, an embedding technique was employed based on synthetic resins, by which a highly detailed histomorphological and histopathological assessment was possible. Thus, under microscopic examination, the extra-cellular matrix could be distinguished into: interstitial matrix and para-lymphatic matrix, namely the matrix portion which is densely packed against the early lymphatics Our study focused on matrix analysis and its likely implications in the pathogenesis and/or the evolution of secondary lymphedema. Our diagnostic protocol was applied to 134 cases of secondary lymphedema, 100 of which were secondary post-surgery lymphedemas and 34 secondary post-inflammation lymphedemas. Out of this group of patients, our study was then confined to 25 cases for which the matrix was available in the specimen sent for histological examination. 22 of them showed significant matrix changes, which proved to be a key pathologic component in the assessment of early lymphatics failure.

Introduction

The interest in extra-cellular matrix investigation and diagnosis in the cronic lymphedema is due to the fact that the lymphatics originate from here. The lymphatic system (Mc Gee et al., 1997; Curri, 1985; Rosati et al., 1992). consists of lymphatic trunks that drain a network of early lymphatics located in less compact areas of peripheral tissues. Early lymphatics have a much bigger lumen than lymphatic capillaries and form a loose, low-density mesh. The lymph flows through a system of vessels that are normally only temporarily filled; indeed, because of their function, they are designed to be empty most of the time. The lymphatic system can be classified into two sectors (Hultgard-Ekwall et al., 2003; Stein et al.,): the one with high absorption capacity characterized by lymphatics (ALPHA) without basal lamina, in charge of collecting interstitial fluid,

plasma proteins, electrolytes, and chylomicrons from the intercellular matrix; and the conduction one, formed by vessels with basal membrane which, on the outside, is strengthened by one or more layers of smooth muscle fibers (pre-collectors, pre and post lymph node collectors, lymphatic trunks). The absorption capacity of these vessels is strongly reduced and confined to a passive or active lymph permeation within the plasmatic membrane.

The extra-cellular matrix consists of extremely thin structures, hence it is impossible to study it with conventional histological means on formalin fixed and paraffin embedded specimens (Melis et al., 1989; Fulcheri et al., 1997). To overcome this difficulty, an embedding technique was employed based on synthetic resins, by which a highly detailed histomorphological and histopathological assessment was possible.

Thus, under microscopic examination, the extra-cellular matrix could be distinguished into (Hultgard-Ekwall et al., 2003; Stein et al.,): interstitial matrix and para-lymphatic matrix, namely the matrix portion which is densely packed against the early lymphatics.

Materials and methods

• Background

Since 1985, our Institute of Pathological Anatomy and Histhology together with the Microsurgery Center for Vascular Diseases has conducted studies on chronic, both primary and secondary, lymphedemas. Our attention is currently focused on the study of secondary post-surgery lymphedema, of which several mechanisms are believed to be the cause:

1. Surgical reduction of the lymphatic network
2. Anatomic variation in the number and course of lymphatics
3. Congenitally scarce lymphatic network
4. Absorption defect
5. Reactive post-surgery inflammation with fibrous-fatty tissue involvement
6. Chronic or acute inflammation
7. Radiation therapy

Extremely important observations can be made in this field by studying the extracellular matrix.

• Materials

Our Institute of Pathologic Anatomy receives the material necessary for lymphedema studies from the Lymphatic Surgery and Microsurgery Division of the Surgery Department of the University of Genoa (Fulcheri et al.,; Campisi et al., 2002; Campisi et al., 2004) obtained from lymphatic-venous anastomoses in patients with chronic secondary lymphedema.

Small biopsyes of subcutaneous tissue are sent in test-tubes which contain ether alcohol and 1 g of silica gel, in order to ensure maximum dehydration.

The tissues, of at least 3-4 mm in diameter, are carefully harvested to avoid any damage.

Alcohol is used to fix the tissues so as not to change their morpho-structural features (Melis et al., 1989; Fulcheri et al., 1997). It is a rather quick fixation, since ether alcohol is a rapid fixation agent. Just like with absolute alcohol fixation, all tissue enzymatic activities are preserved. Less tissue coarctation and hardening is another advantage, when compared to the more conventional alcohol fixatives. Actually, a fast fixative like ether alcohol can be employed here, because the fragments to be examined are really small. Hence, no deep penetration is required, which cannot be obtained with alcohol and with fast-acting gelling fixatives in general.

At this point, biopyes processing begins, which will be completed when the matrix is embedded in the resin according to the methods reported below.

Technovit 8100 resin by Kulzer S.p.a (Germany) was employed for resin embedding.

The material to be embedded was first fixed for at least 4 hours in ether alcohol. The sample then must be washed overnight in a saline 7.4 pH buffer, with the addition of 6.8% sucrose. The specimen is vacuum kept at 4°C in a refrigerator.

Following the washing, the tissue is then dehydrated in 100% acetone for 60 minutes. During the first 5 minutes of dehydration, acetone should be changed until the solution is clear.

After the above washing steps, the tissue is put in contact with the resin proper by means of an infiltration process. This is obtained by dipping the specimen in the infiltrating solution, which consists of 100 ml Technovit 8100 (basic resin) and 1 gram of hardener, for 6-10 hours. For proper penetration, the infiltration must be constantly kept at 4°C for maximum 2 days.

As soon as the tissue is well impregnated with the infiltrating solution, embedding is performed.

This is the step when monomers polymerize and the resin hardens. First of all, the tissue has to be cleaned of any excessive infiltrating solution by carefully drying it. At this point, the embedding solution is prepared, consisting of 15 ml of the same infiltrating solution and 0.5 ml of hardener 2 (obviously enough, the amounts of reagents may change depending on the number of specimens to be analyzed, provided the right proportions are always maintained). The tissue is then put in the recesses of teflon embedding forms which are filled up with embedding solution.

Recesses must then be sealed with a plastic film after removing any gas bubbles that could interfere with polymerization. During embedding, they must be placed on a thin ice layer to keep the temperature low. Indeed, polymerization is a strongly exothermic reaction.

According to our method, polymerization does not take more than 3 hours.

To complete the embedding process, the obtained resin must be fixed in a proper block to allow for subsequent cutting of the sections. This is done with Technovit 3040 (Kulzer S.p.a,. Germany).

When melting the plastic in the solvent, the resulting mixture should be rather dense so that it will harden quickly. This mixture must be poured into embedding blocks that must be placed on top of the recesses of the

forms after having removed the plastic film. After a few minutes, the mixture is fully hard, thus providing a rigid base for our resin.

• **Methods**

Our study is based on the analysis of a sample of 134 patients with secondary lymphedemas (Curri, 1980; Stein et al.; Fulcheri et al.; Campisi et Boccardo, 2002; Campisi et al., 2002; Dellacha et al., 1999) who were treated with microsurgery. Out of this series, 100 of them had secondary post-surgery lymphedema and 34 post-inflammation lymphedema.

In a total of 25 patients with secondary post-surgery lymphedema, also the extracellular matrix was examined. Therefore, our analysis is confined to those patients about whom we had detailed information on the following parameters: age, gender, site and type of surgery. The data we have obtained refer to the following cases:

25 cases in total, 21 females and 4 males, aged from 30 to 72 years. 15 cases of lymphedemas are secondary to breast cancer mastectomy, 6 to uterine cancer hysterectomy, 1 to resection of the ischio-pubic branch because of sarcoma, 1 to axillary lymphadenectomy for Hodgkin lymphoma, 1 to aortic by-pass, and 1 to orchiectomy for embryonal carcinoma of the testis. Post-surgery lymphedema was localized as follows: 3 cases were in the left lower limb, 5 in the right lower limb, 6 in the upper left limb, and 11 in the upper right limb.

For the microscopic analysis of the extracellular matrix, we have employed the following two diagnostic parameters:

1. Presence of (acute, chronic, none) inflammation infiltrates;
2. Quantitative assessment of the interstitial and/or peri-lymphatic matrix (absent, slightly visible, moderate and marked increase).

At the same time, early lymphatics and small size lymphatic can be observed, and their distribution examined (normal, numerous, scarce), lumen size (narrow, normal, ectasic), type of wall (thin, fragmented, normal, thickened, fibrotic) and intramural inflammation infiltrates (acute, chronic, absent).

Tot. cases	Inflam-mation	Increase	Inflam-mation + increase	Negative
25	2	11	9	3
	8%	44%	36%	12%
Total cases with matrix changes 22 (88%)				

Results

The following data were obtained from our study: in 2 patients, only inflammation was observed in the interstitial and/or peri-adventitial matrix, in 12 patients only morpho-structural changes were observed (increase), in 8 patients there was both inflammation and increase, and in 3 patients no significant changes could be observed.

The data obtained are schematically reported in the following table:

Discussion and conclusion

The above data show that significant matrix changes are observed in patients with secondary lymphedema. By comparing the data obtained with initial lymphedema diagnosis made without examining the matrix, it can be seen that inflammation had not been detected in one of the two identified cases. Even morpho-structural changes (increase) in 3 out of 11 patients had not been detected, just like the association of both lesions in 7 out of 9 patients.
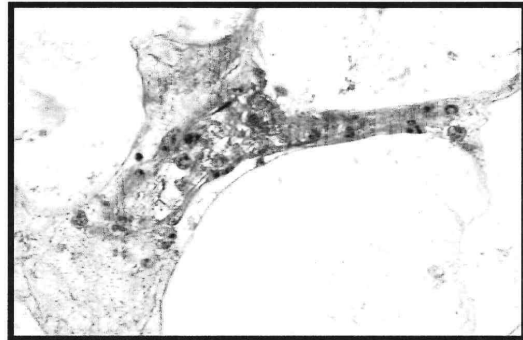

Fig. 1 - Cases have inflammatory infiltrate and increase of the matrix.
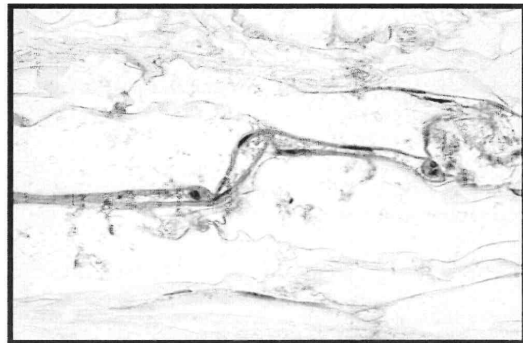

Fig. 2 - Cases have only increase of the matrix.

Therefore, the matrix must not be neglected in lymphedema diagnosis. As shown above, it provides useful and additional information on the pathologic condition of the lymphatics, for example it tells us if there is inflammation or lymphostasis, as can be inferred from a matrix increase.

It should be pointed out that system is quite difficult to

study the matrix: under the microscope this component of the lymphatics shows exceptionally fine structures, which would escape any detailed examination if studied from a standard paraffin embedded specimen. Therefore, in order to solve this problem, synthetic resins were employed in our study for specimen embedding. As previously illustrated, resins have certain features that best meet the requirements of this type of investigation.

Therefore, with our research it was possible to properly assess the matrix from a histo-morphological and pathological point of view. Further, in the light of the outcome of our microscopic investigations and clinical data available, we have been able to demonstrate that any changes in the matrix are a likely pathogenic factor or co-factor in secondary lymphedema.

From a clinical point of view, our work shows that lymphedema must be treated as early as possible in order to avoid any further progression in interstitial and peri-lymphatic matrix damage that may take place concurrently with changes in the superficial lymphatics, lymphatic collectors, and lymph nodes. Therefore, the ideal treatment of lymphedema is based on microsurgical lymphatic drainage to be performed as early as possible and in a – as much as possible – physiological manner, in order to avoid any further tissue damage and achieve a fully and final treatment.

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