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Angiogenesis - a crucial step in breast cancer growth, progression and dissemination by Raman imaging

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ABSTRACT

Combined micro-Raman imaging and AFM imaging are efficient methods for analyzing human tissue due to their high spatial and spectral resolution as well as sensitivity to subtle chemical, structural and topographical changes. The aim of this study was to determine biochemical composition and mechanical topography around blood vessels in the tumor mass of human breast tissue. Significant alterations of the chemical composition and structural architecture around the blood vessel were found compared to the normal breast tissue. A pronounced increase of collagen-fibroblast-glycocalyx network, as well as enhanced lactic acid, and glycogen activity in patients affected by breast cancer were reported.

1. Introduction

The development of a variety of cancers is linked to changes in the biochemical properties of living cells. Oncogenically transformed cells are expected to have differences in all cell and tissue layers, but it still remains unclear whether biochemical alterations are a cause or a consequence of cancer development. The study of biochemistry of cancer cells by Raman and AFM imaging is presumably one of the most promising directions of cancer research, because the acquired knowledge will help design new treatments for cancer. It has been reported that the processes occurring in tumor microenvironment composed of non-cellular (i.e. vascular and interstitial) and cellular compartments are as important as the processes in epithelial cells where most cancers including the ductal breast cancer develop [1]. These processes must be coupled because the epithelial cells are avascular, and the blood supply from the microenvironment to the tumor mass is one of the most important factors of cancer development.

In this paper we will concentrate on biochemical signatures of tumor mass around vascular compartments and interstitial environment.

There is a great number of abnormalities in the tumor vessels comparing with normal ones. Among them the most important are: increased vessel tortuosity, deficient pericytes, increased numbers of proliferating endothelial cells and abnormalities in the basement membrane [2,3]. Biochemical changes (upregulation of growth factors, bradykinin, prostaglandins, nitric oxide) are responsible for hyperpermeability what is revealed in high interstitial pressure and absence of functioning lymphatic network in tumor interstitium. This good vascularization of tumor helps with delivery of drugs, however because of high heterogeneity this effect is not effective in poorly vascularized regions [1,4,5].

The development of new blood vessels is a crucial step in breast cancer growth, progression and metastasis, because like healthy cells, cancer cells cannot live without oxygen and nutrients. The diversity of responsible angiogenic pathways that encourage new blood vessels to grow into the tumor in breast cancer for different tumors has been studied for many years [6–15].

The most important angiogenic factor is VEGF (Vascular Endothelial Growth Factor) [16]. When VEGF is overexpressed, cancer cells encourage the growth of blood vessels to feed a tumor by producing the hormone-like protein, vascular endothelial growth factor. This guarantee a good blood supplies which are needed for growth and in consequences for metastases [17]. Large number of researches suggest that angiogenesis (new blood vessel formation process) promote transformation of mammary hyperplasia to malignancy [10]. Clinical data show that microvessel density (MVD) is highest in aggressive breast tumors, and is associated with increased VEGF expression [13]. It seems appropriate to design drugs, which interrupt and inhibit angiogenesis in breast. The most commonly used drug is bevacizumab, which is a humanized monoclonal antibody directed against the VEGF-A ligand, but there is not yet definitive evidence for the efficacy of agents that specifically target angiogenesis [6].

Understanding mechanisms of angiogenesis as well as biological predictive markers is crucial in development of antiangiogenic therapies in clinical practice. One route to optimize the efficacy of these targeted agents is to better understand biology and biochemistry

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around the vessels in the tumor environment. Highly advisable is therefore seeking new methods breaking the previous limitations and with potential clinical applications in order to satisfy the increasing demand of molecular biology. Reports of world literature in recent years clearly indicate that the particular role in the development of innovative techniques are played by multimodal imaging technologies, among which the dominant role belongs to Raman-AFM-fluorescence imaging, which allow simultaneous monitoring of morphological, biochemical properties with very high spectral and spatial resolution [18]. The Raman imaging is capable of simultaneously recording vibrational spectra from multiple regions and thereby map out the spatial distribution of proteins, lipids, nucleic acids, and metabolites in surrounding of the vessel supplying blood to tumors in contrast to the classical methods LC/MS, NMR, HPLC, based on the analysis of samples in the mass subjected to homogenization that prevents spatial characteristics of the systems investigated [19–24].

We will show that Raman spectroscopy and imaging are capable of monitoring biochemical composition of tumor vascularity, which is markedly heterogeneous with densely vascularized areas supplying oxygen and nutrients to rapidly growing parts of the tumor. Raman imaging can monitor also areas where there is a decreasing amount of oxygen available to tumor cells which are further away from blood vessels. This information will help understand how new blood vessels are synthesized by tumors in a process known as angiogenesis.

The aim of this study is to use micro-Raman and AFM imaging to analyze biochemical composition around blood vessels in cancerous and normal human breast tissue. The biochemical composition of human normal and cancerous breast tissues will be analysed by Raman and AFM imaging.

2. Experimental

2.1. Patients

We have employed Raman spectroscopy, Raman imaging, AFM topography imaging to measure tissue biochemical composition around a vessel supplying blood to duct tumor in human breast tissue. These techniques have been applied for ex vivo fresh breast tissue samples without any fixation. The ex vivo samples were obtained during the resection surgery from the tumor mass (cancerous tissue) and the safety margin (normal tissue). All tissue samples were frozen and stored at −80 °C. Before the measurements the frozen samples were cryosectioned at −20 °C with a microtome (Microm HM 550, Sermed) into a few 6 μm-thick slices. Some of them were used for Raman and AFM analysis without typical for histology examination paraffin embedding procedure. The thin sections were placed onto calcium fluoride windows (CaF_2, 25 × 1 mm) and examined by Raman and AFM imaging. After the Raman and AFM measurements these sections were stained and histologically examined. The samples were stained in hematoxylin for 3 min, rinsed in water and stained in eosin for 2 min. The adjacent tissue was analysed by histopathologists.

All methods were performed in accordance with relevant guidelines and regulations. All tissue procedures were conducted under a protocol approved by the institutional Bioethical Committee at the Medical University of Lodz, Poland (RNN/323/17/KE/17/10/2017). Written informed consent was obtained from patients.

2.2. Chemicals

All chemicals have been purchased from Sigma-Aldrich: palmitic acid (P0500; Sigma Aldrich), stearic acid (S4751; Sigma Aldrich), arachidic acid (A3631; Sigma Aldrich), oleic acid (O1008; Sigma Aldrich), linoleic acid (L1376; Sigma Aldrich), γ-linolenic acid (L2378; Sigma Aldrich), arachidonic acid (10,931; Sigma Aldrich), docosapentaenoic acid (D-120; Sigma Aldrich), eicosapentaenoic acid (E2011; Sigma Aldrich), α-linolenic acid (L-039; Sigma Aldrich), eicosatetraenoic acid (H7768; Sigma Aldrich), tetracosahexaenoic acid (153,745; Sigma Aldrich), docosahexaenoic acid (D2534; Sigma Aldrich).

2.3. Raman Imaging

Raman spectra and images were obtained with an alpha 300 RSA+ (WITec, Ulm, Germany), and preprocessed with WITec Control/Project Plus 2.1. Detailed description of equipment and methodology on data pre-processing and multivariate data analysis used in the paper is available elsewhere [19,21,25–27].

The 2D array images of tens of thousands of individual Raman spectra were evaluated by the basis analysis method (BAM). In BAM method, each measured spectrum of the 2D spectral array is compared to basis spectra using a least squares fit. Such basis spectra are created as the average spectra from different areas in the sample. The weight factor at each point is represented as a 2D image of the corresponding color and mixed coloring component. The color code of Raman maps were based on the integrated Raman intensities in specific regions (sum option in the filter manager in the WITec project 2.1). Using a lookup table, bright colors indicate the highest intensities, whereas dark colors indicate the lowest intensities of the chosen region.

2.4. AFM Measurements

To obtain the topography of the samples AFM measurements were performed on an alpha 300 RSA+ (WITec, Ulm, Germany) based on an Olympus microscope. The AFM module was equipped with a 25 mm x- and y-range linearized piezoelectric scanner and 980 nm laser. The AFM was operating in the constant force mode, in which the feedback loop controls the z-height of the piezo table to keep the cantilever deflection constant. To generate AFM images we used the pyramidal cantilevers, with the spring constant of 0.2 N/m.

3. Results and Discussion

3.1. Raman Imaging

Raman images allow looking inside the biochemical composition of cancerous cells around the lumen of the vessels supplying blood to the tumor mass and the extracellular matrix surrounding the tumor. To understand information that is provided from Raman images and vibrational spectra around the blood vessels in normal and cancerous breast tissues, we need to associate these features with the vessel morphology. Briefly, the walls around the lumen of artery are lined by an exceedingly thin single sheet of endothelial cells, the *endothelium*, separated from the surrounding outer layers by a basal lamina followed by many layers of smooth muscle cells and a thick, tough wall of connective tissue. Endothelial lining is present regardless amounts of connective tissue and smooth muscle in the vessel wall.

Fig. 1 shows a cross section through a small artery supplying blood to a tumor mass in human breast tissue (G3, ductal cancer, P149) obtained by Raman imaging, compared with the H&*E*-stained histological image, microscopy image, AFM topography image and the characteristic vibrational Raman spectra for different areas of the cancerous tissue. One can see that there is an almost perfect match between the morphological features obtained from the histological, microscopy, AFM and Raman images. Raman imaging is a powerful technique which has many advantages over other imaging techniques, because it offers not only morphological image, but also a very detailed biochemical characterization by probing individual chemical bond vibrations. As a result, Raman spectra and images are information rich, and contain data related to the specific chemical structure and biocomposition of the biological material being analysed. The Raman spectra presented in Fig. 1g show the biochemical composition of the various substructures around a small artery supplying blood to a tumor mass in human breast tissue. The inside wall of the

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