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Confocal Laser Scanning Microscopy in Translational Research of Molecular Tumour Markers

L. Csáderová, A. Hulíková, E. Švastová, S. Pastoreková*

Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovakia

Abstract: Confocal laser scanning microscopy (CLSM) has become an established method for biological applications employing fluorescence. It creates high contrast images representing a thin cross-section of a specimen, allowing analysis of subcellular localisation of selected molecules and their interactions. Molecules involved in the process of tumour metastasis represent new anti-cancer therapy targets. Carbonic anhydrase IX is an important tumour hypoxia marker and an indicator of tumour aggressiveness. It contributes to maintenance of cancer cell pH profile supporting processes involved in cell migration and acquisition of invasive phenotype. CLSM proved to be an excellent method for investigation of functional properties of CA IX, which could lead to a better understanding of its role in metastasis and development of new anti-cancer strategies.

Keywods: Confocal laser scanning microscopy, Fluorescence, Cancer markers, Carbonic anhydrase IX

***Corresponding author:** *virusipa@savba.sk* (S. Pastoreková)

1. Introduction

In recent years, confocal laser scanning microscopy has become an established research instrument, often employing fluorescence in its biological and medical applications. Compared with conventional microscope, the confocal microscope creates images with less haze and better contrast, representing a thin cross-section of a specimen [1, 2]. This is achieved by point-by-point illumination of a sample and rejection of out-of-focus light. The laser beam is guided across the specimen in point-wise fashion, minimizing the part of the sample which is illuminated, thus reducing the fluorescence from other regions obscuring the analysed area. The most important feature of confocal LSM is the confocal aperture (often called pinhole) which is positioned in front of the detector. The pinhole is arranged in a plane conjugated to the focal plane. This arrangement results in the rejection of the light emitted from out-of-focus regions, elimination of background caused by light scattering in a specimen, improvement of the resolution, optimization of signal-to-noise and signal-to-background ratio and allows quantitative approach to imaging optical probes [3]. The pinhole diameter is variable, so the degree of confocality can be adapted to practical requirements. When the pinhole is fully open, the image is non-confocal as the signal is detected from the whole sample. Closing the pinhole to ever smaller diameter provides the image of a correspondingly thin slice of a sample, but at the same time, the intensity of detected light is reduced. This possibility to exclusively imagine only a thin optical slice of

the thick sample allows the building of z-stacks in a method called optical sectioning. When number of optical slices is recorded at different planes of a sample by moving the focal plane in z direction in set implements, 3D data usable for 3D software reconstruction and creation of sections of various spatial orientations is obtained. Biological samples, such as cells or tissues, are generally three-dimensional objects, so acquiring 3D data in series of images taken at different depths in the object gives a possibility of gathering new and more precise biological information.

Metastasis is the most frequent cause of death in cancer patients. It is accompanied by the transition from non-invasive tumour cell phenotype to the invasive one, leading to a more malignant form of cancer. Majority of anti-cancer drugs used today are pro-apoptotic and the fact that migratory cancer cells are resistant to apoptosis may partly explain bad prognosis of patients with dramatic tissue invasion (such as glioblastoma). Therefore, new treatment strategies are needed, such as those targeting molecules involved in cancer cell migration and invasion.

Carbonic anhydrase IX (CA IX) is a glycoprotein associated with tumours derived from various organs, whilst it is not present in corresponding healthy tissues. Its physiological expression in a human body is linked to some regions of gastrointestinal tract, mainly epithelium of stomach, gall bladder and intestine [4]. Increased expression of CA IX is switched on and maintained by hypoxia, often occurring in solid tumours and by high cell density. CA IX is used as a hypoxic marker associated with bad prognosis and resistance to chemotherapy [5].

CA IX is a transmembrane protein calatalysing the reversible hydration of CO_2 and H_2O to H^+ a HCO_3 [6]. It is considered to be a protein with dual function. Being one of the most active isoforms among carbonic anhydrases, CA IX could play a pivotal role in pH regulation of tumour microenvironment and in maintaining a stable pH of intracellular space. In hypoxic tumours, metabolism changes to anaerobic glycolysis, when lactate and protons are extruded from cells and accumulated in extracellular space, leading to local acidosis. Cellular production of CO_2 increases and it diffuses across the cell membrane to pericellular space, where it is used as a substrate for transmembrane CAs, becoming the second main source of tumour acidity. Svastova et al. (2004) showed that CA IX is able to acidify extracellular environment in vitro and its activity is increased by hypoxia.

The second function of CA IX is linked to its ability to influence the strength of intercellular contacts [8]. Ectopic expression of CA IX weakens the intercellular adhesion and leads to destabilisation of intercellular contacts and increased cell dissociation by reducing E-cadherin bond to -catenin. E-cadherin suppresses the processes of cell invasion and metastasis and its reduced expression or dysfunction caused by uncoupling to actin cytoskeleton is often observed in the invasive front of malignant tumours [9]. Migration of tumour cells is the necessary step in the metastatic process which requires cell dissociation, dynamic cytoskeleton reorganisation and extracellular matrix degradation [10]. All these processes depend on appropriate extracellular and intracellular pH microenvironment, determined by local ion and water transport across the membrane. Due to its role in cell dishesion and pH regulation, which are key processes leading to metastasis, CA IX could play an important role in potentiating tumour malignancy. For investigation of this role, precise techniques allowing the localization of proteins in various cell regions and determination of their interplay are needed. Determination of functional aspects of CA IX

could lead to development of new therapeutic strategies based on targeting CA IX function in processes linked to metastasis.

2. Experimental

2.1. Cell culture

MDCK cells stably transfected with CA IX [8] were cultured in DMEM containing 10% FCS. To maintain standard conditions cells were plated at coverslips in 3.5 cm Petri dishes for 24 hours before their transfer to hypoxia (2% O_2 and 5% CO_2 balanced with N_2 at 37 C) generated in hypoxic station (InVivo2, Trigon). Parallel normoxic dishes were incubated in air with 5% CO_2 at 37 C. After 48 hours of culture cells were fixed for immunofluorescence.

2.2. Indirect immunofluorescence

Cells grown on glass coverslips were fixed in ice-cold methanol at 20 C for 5 min. Non-specific binding was blocked by incubation with PBS containing 1% BSA for 30 min at 37 C. Then, cells were incubated with primary antibody polyclonal rabbit anti-CA II solution (1 : 500, Exbio) for 1 h at 37 C, washed four times with PBS containing 0.02% Tween 20 for 10 min, incubated with secondary antibody anti-rabbit Alexa Fluor 594 (1 : 2 000, Invitrogen), and washed four times with PBS for 10 min. Finally, the cells were incubated with monoclonal anti-CA IX antibody M75 FITC-conjugated (1 : 300) for 1h 37 C [6]. All antibodies were diluted in 1% BSA in PBS. In the end, cells were stained in DAPI solution (1 : 36 000, Sigma) for 3 min and washed four times in PBS for 10 min. Finally, the cells were mounted onto slides in fluorescence mounting medium (Mounting medium, Calbiochem).

2.3. Confocal scanning microscopy

Samples were viewed at scanning confocal microscopy LSM 510 Meta mounted on Axiovert 200M (Zeiss) in scanning multitrack mode. Samples were excited with lasers at 405 nm, 488 nm, 543 nm wavelengths. Band-pass filters BP 420-480 (DAPI), BP 505-550 (FITC), BP 560-615 (AF594) were used for collection of emitted light. Deconvolution software Huygens (Scientific Volume Imaging SVI) was used to remove the background fluorescence and unwanted effects of scanning. 3D colocalisation maps (Pearson's coefficient) were calculated by colocalisation analyser (SVI).

3. Results and discussion

Transport of bicarbonate HCO_3 across cell membrane plays an important role in the processes leading to the creation of pH microenvironment of cells. Bicarbonate transporters, such as AE1, are closely linked with carbonic anhydrases. Sterling et al. (2002) described the formation of transport metabolon consisting of 3 parts: cytoplasmic anhydrase CA II, anionic exchanger AE1, 2 or 3 and CA IV forming its extracellular part. In this way the bicarbonate transport activity is increased.

CA IV is localised in membrane, such as CA IX, so it is expected that transmembrane CA IX could also form the extracellular metabolon component. CA IX structure is more

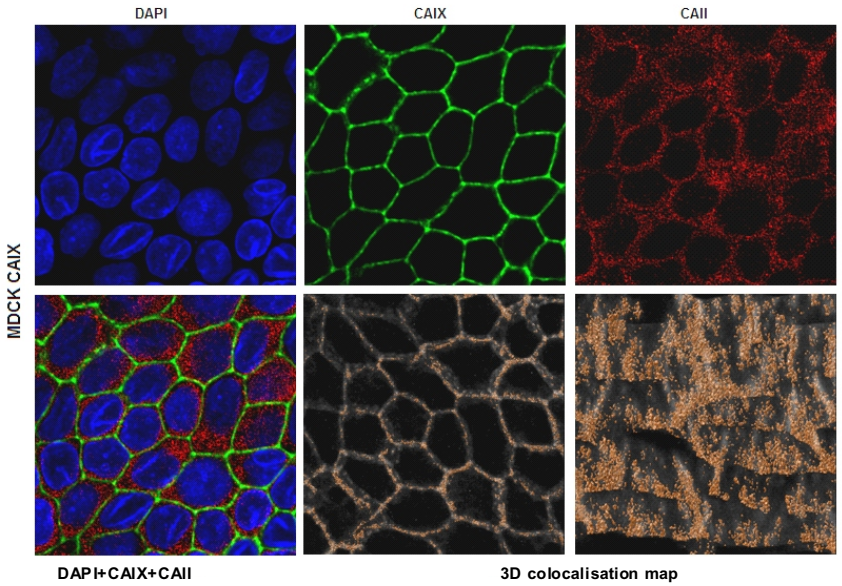

Fig. 1. Colocalisation of CA IX and CA II in CAIX overexpressing MDCK cells. Membrane-localised CAIX is stained green, predominantly cytoplasmic CA II red, cell nuclei blue. 3D colocalisation maps representing the Pearson's colocalisation coefficient were generated by colocalisation analyzer module of Huygens SVI. Despite low overall colocalisation (Pearson's coef. < 0.2) of CAIX and CAII, areas of higher colocalisation (marked orange) can be seen located close to the plasma membrane of MDCK transfectants.

complex than that of CA IV, but it was shown by GST-pull down method that CA IX connects to bicarbonate transporters via its catalytical domain [12]. The possibility of the cooperation between CA IX and AE2 is supported by the same localisation of CA IX and AE2 in stomach mucosis where CA IX is expressed under physiological conditions. However, it was not clear, whether CA IX metabolon involves CA II as an intracellular component.

MDCK cells have a high natural expression of CA II, so we could investigate the localisation of proteins CA II and CA IX under normoxic and hypoxic conditions. Confocal microscopy was used to reach a better resolution and reduce the out-of-focus signal. CA II is a protein located in the whole cytoplasm and, by the selection of appropriate pinhole diameter, the images of sections of plasmatic membranes were captured, reducing the signal coming from other cytosolic areas.

The total colocalisation of CA IX with CA II is relatively low in hypoxia and normoxia as well. Pearson's coefficient reaches the value of 0.1 in hypoxia, and 0.026 in normoxia, respectively. But if we analyse the 3D colocalisation map showing the places when fluorescent signals from CA II and CA IX molecules colocalise (marked orange, Fig. 1), we can see that a portion of CA II molecules present in the proximity of basolateral part of plasmatic cell membranes colocalises with CA IX. These findings, together with the fact that CA IX colocalises with E-cadherin and can be coprecipitated with -catenin and

E-cadherin [8], could indicate the existence of two populations of CA IX in MDCK transfectants: one cooperating with CA II and AE2 or other ion transporters in the maintenance of pH homeostasis and the second one taking part in the regulation of cell adhesion.

4. Conclusions

Metastasis of the primary tumours connected with migration and invasion of cancer cells into surrounding tissue drastically reduces the chances of survival of cancer patients. Therefore, molecules involved in these processes present potential candidates for new anti-cancer therapy targets. CLSM is an excellent method to understand functional aspects of molecular tumour markers, such as CA IX. This system provides powerful tools for analysing subcellular localisation of tumour markers and for determination of their interactions with biologically relevant molecules or therapeutical agents.

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