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Flow Cytometry – the Sensitive Method for Detection of Molecular Markers in Cancer

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Abstract: Carbonic anhydrase IX (CA IX) is a superior tumor hypoxia marker, which is used in diagnosis, prognosis and treatment of several types of cancer and is also useful as a surrogate indicator of hypoxia in cultured cancer cells in experimental settings. Detection of CA IX is usually based on classical techniques such as immunohistochemistry, immunoblotting or ELISA. Here we demonstrate usefulness of flow cytometry (FCM) for evaluation of CA IX expression on the surface of cancer cells grown in monolayer under normoxic and hypoxic conditions. Advantage of FCM relies in its relatively rapid accomplishment in comparison to the other techniques as well as on its information power related to single cell recording and analysis.

Keywords: Tumor markers, Carbonic anhydrase IX, Flow cytometry, Hypoxia, Cell surface

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1. Introduction

Carbonic anhydrase IX is a cell surface protein abnormally expressed in many types of cancer. This cancer-associated expression pattern is related to tight regulation of CA IX transcription and activity by insufficient oxygenation (i.e. hypoxia) in tumor microenvironment. Squamous head, neck, colorectal and papillary renal tumors, breast, bladder and ovarian cancers show hypoxia-linked perinecrotic expression of CA IX, while clear renal cell carcinomas (ccRCC) usually show diffuse staining pattern of CA IX due to VHL mutations and constitutive activation of hypoxic response pathway [2 4]. Because tumor hypoxia is correlated with tumor progression and worse treatment outcome, expression of CA IX in tumor tissue and/or in plasma is a predictor of survival and an independent prognostic marker [5, 6]. Two CA IX-specific monoclonal antibodies (mAb) G250 [7] and M75 [8] are predominantly used for diagnostic purposes in vivo and in vitro, respectively. Moreover, a chimeric variant of G250 (cG250) directed against a conformational epitope on native CA IX protein allows for multiple administrations of the mAb into human body for immunotherapeutic purposes [9]. M75 is well characterized murine mAb isotype IgG2b [10] which binds both native and denatured CA IX and thus can be employed in many diagnostic applications. Indeed, M75 is frequently utilized in immunoblotting, ELISA, immunohistochemistry, immunoprecipitation, and fluorescence microscopy. Previous studies have shown that mAb M75 binds CA IX antigen at extracellular proteoglycan-like domain (PG) [11]. Here we evaluated FCM detection of CA IX by M75 in several cancer cell lines.

Flow cytometry is an elegant method of single cell analysis, introduced in 1954, using an instrument in which electronic measurement for cell counting and sizing was made on cells flowing in conductive liquid with one cell at time passing a measuring point [12]. Nowadays, highly sophisticated machines equipped with 4 lasers capable of detecting 20 parameters simultaneously are available. FCM is a powerful tool for studying the phenotype and other characteristics of the cells. A typical flow cytometer consists of three basic units: (1) one or more laser light sources and a system of sensors equipped with optical filters, (2) hydraulic system comprising flow chamber and (3) a computer system collecting the measured data with the analysis software [13]. The relative light-scattering and color-discriminated fluorescence of the cells is measured in FCM. Analysis and differentiation of the cells is based on size, granularity and fluorescence becoming from fluorescently labeled antibodies or dyes attached on or incorporated into the cells. The cells have to be suspended in fluid, which is sucked by a fluidic system into the flow chamber where they are forced to form laminar flow stream of single cells. As each cell passes the laser beam, the light is scattered in all directions. The light scattered in the forward direction at low angles (0.5 10) is proportional to the square of the radius of a sphere and so to the size of the cell [14]. Cells are translucent, so the light passes through them and is refracted and reflected by intracellular compartments (nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, lysosomes, etc.) in all directions and the light scattered in 90°direction is considered to be proportional to complexity or granularity of the cells. Cells can be labeled by antibodies conjugated with fluorochromes, or stained with fluorescent dyes with affinity to membranes, cytosol or nucleus. As antibodies to many cell antigens become available and specific fluorescent dyes were developed, differentiation of cell types, the presence of membrane receptors and antigens, membrane potential, pH, enzyme activity and DNA content can be studied by FCM.

2. Experimental

Cell lines and culture

The HEK 293, HeLa, SiHa and A549 cells were used in our experiments. For the experiment, 5 10^5 cells were plated at 3.5 cm dishes and cultivated for 48 hours. The cells were cultured in growth media (DMEM with 10% FCS, BioWhittaker, Verviers, Belgium) in CO_2 humidified thermostat (5% CO_2, 37 C). Hypoxic conditions (2% O_2, 5% CO_2, 10% H_2, and 83% N_2 at 37 C) in the experiments were ensured in an anaerobic workstation (Ruskin Technologies, Bridgend, UK).

Immunoblotting analysis

The cells were lysed in RIPA buffer supplemented with protease inhibitors (Roche, Basel, Switzerland). PAGE was performed where 30 μg of total proteins were load per line. mAb M75 was used for detection of CA IX as the primary Ab and goat anti-mouse-HRP as the secondary Ab.

Flow cytometry

The cells were harvested after trypsin treatment and diluted with PBS-1% FCS for the final concentration 1 10^6 cells/ml. Five hundred μl of hybridoma medium containing

M75 Ab were added to 500 µl aliquot of cells and incubated on a rotary mixer at 4 °C for 30 min. Alexa488 conjugated goat anti-mouse Ab was diluted in PBS-10%FCS and added to the cells after two washings with PBS-1% FCS. After 30 min incubation cell pellets were two times washed in PBS and immediately measured at flow cytometer (Guava EasyCyte-6HT, Millipore, Billerica, MA, USA).

3. Results and discussion

CA IX expression in cell lines cultivated in normoxia and hypoxia, respectively, was first evaluated by immunoblotting. CA IX was undetectable in protein extract from HEK 293 cells either in normoxia or in hypoxia. This was in line with the known fact that HEK293 cells do not express endogenous CA IX. In all the other studied cell lines, CA IX is expressed weakly in normoxia and is increased in hypoxia (Fig.1). The most significant increase was evident in SiHa cervical carcinoma cells, in accord with the earlier data from mRNA expression studies (not shown). Less prominent increase was found in A549 and HeLa cells. Flow cytometric analysis revealed a similar induction pattern (Fig. 2). Here, CA IX detection was performed on non-fixed living cells, so that the antibody could not cross the membrane and therefore bound only to cell-surface subpopulation of CA IX molecules. HEK 293 cells were found negative both in normoxia and hypoxia (2 % of cells falling within "positive population" can be attributed to the background signal). The other cell lines showed positive staining in hypoxia but negative staining in normoxia, with the following percentage of CA IX expressing cells: 79 % in HeLa, 86 % in SiHa and 73 % A549. The apparently similar percentage in these three carcinoma cell lines detected by FCM differs from the variable induction of CA IX seen in immunoblotting. This could be related to the fact that immunoblotting detects both cell-surface and intracellular pools of CA IX molecules, suggesting that the three analyzed cell lines differ by the level of immature intracellular CA IX and possess similar levels of cell surface CA IX protein. Thus, combination of these two methods gives a better insight into expression levels of CA IX in cancer cells.

The main benefits of the FCM over immunoblotting are (1) more detailed output information as it is shown in Fig. 2 (2) less time consumption (3) opportunity to follow several parameters at once and (4) possibility to examine membrane localized and total protein

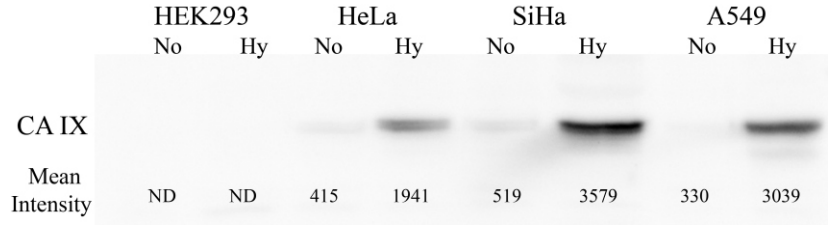

Fig. 1. Expression of CA IX in different cell lines under normoxic or hypoxic conditions estimated by immunoblotting. The cells were incubated for 32 hours in normoxia (No) and hypoxia (Hy). Cell extracts were prepared and 30 µg of total proteins were loaded per line for PAGE. CA IX immobilized at PVDF membrane was detected by the primary mAb M75 and the secondary Ab goat anti-mouse-HRP on Kodak InVivo Imaging System FX. The mean intensities of CA IX bands were calculated by Kodak Imaging Software Ver. 4.5 in grey level, ND not detectable.

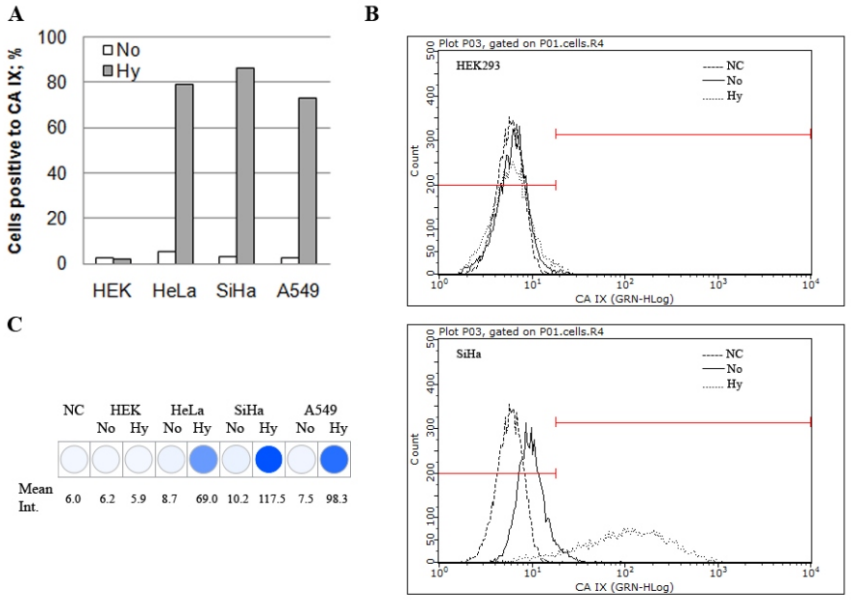

Fig. 2. Expression of CA IX in different cell lines in normoxia (No) or hypoxia (Hy) estimated by flow cytometry. The cells were harvested and labeled by the primary mAb M75 and the secondary Ab goat anti-mouse-Alexa488 and measured on Guava EasyCyte-6HT. A cells positive to CA IX in % B The mean intensities of green fluorescence, C Examples of CA IX negative (upper) and CA IX positive (lower) cells depicted in histograms.

expression (it can be achieved by fixation and permeabilization of cells before the labeling). Limitation of the method is that the cells have to be in single cells state what is hardly achievable in the case of solid tumors. Overall, FCM is a convenient supplementary method to detect CA IX as well as the other tumor cell markers by specific mAbs.

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