

Chapter 45

Application of Laser Light on the Development of Equipment for the Study of Proteins

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Abstract The use of the laser technology in medicine has allowed us to expand the knowledge of the cell and its functions. In particular, flow cytometry is a sophisticated equipment, which can do a simultaneous multi-parametric analysis of the physical, and chemical properties of thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, particularly blood cancers, but it has many other applications in basic research, clinical practice, and clinical trials. Given the extensive available information, flow cytometry was used to study the route by which transduce PKC ζ leads the activation of GSK-3 β , and the physiological significance of its accumulation in the nucleus.

Keywords Flow cytometry • Wnt signaling • GSK-3 β • PKC ζ • Colon cancer

45.1 Introduction

Since the advent of the laser (1960), humanity has seen the unstoppable growing and diversifying the development of all types of lasers, capable of operating over a wide range of wavelengths and increasing high peak powers; the laser systems have led to a broad set of applications that seems to be the key element in present and future investigation.

Lasers have been used in medicine almost from its inception; it is so, that we can now count on new technological processes in the field of surgery, especially handling delicate and dangerous areas, such as the brain or the spinal cord or even the laser systems can be used to correct visual defects, and so many other applications can be stated.

Flow cytometry is a rapid quantitative and objective method of analysis of cells, nuclei, chromosomes, mitochondria or other particles in suspension.

The principle behind this technology consists of passing cells or other particles in suspension, and align one by one in front of a light beam.

The information produced can be grouped into two basic types: the one generated by the light scattering, and the related emission of light by the fluorochromes present in the cell or particle to be excited by the light beam. The detected light signals are transformed into electrical impulses that are amplified, and converted into digital signals that are processed by a computer. It is our interest to compare this technique with biochemical methods of cell analysis, in which an average result for the entire sample is obtained; the flow cytometry is able to provide quantitative information on each particular cell and to identify different subpopulations of cells in the sample, even when they are poorly represented [1, 2].

Because our interest in GSK-3 β protein involved in multiple functions (the Wnt signaling pathway, in protein synthesis, cell proliferation, differentiation, metabolism, cell cycle, apoptosis, and embryonic development), regulating their activity is critical to ensure specificity of signaling pathways [3–5]. To accomplish this, GSK-3 β is subjected to multiple levels of regulation by phosphorylation and other post- translational modifications, their subcellular localization regulation and protein-protein interactions.

The activity of GSK-3 β is significantly inhibited by phosphorylation at Ser 9 its N-terminal; several kinases can phosphorylate that residue as the Akt/PKB protein, PKA, PKC and p90 RSK among others. It is known that this inhibitory phosphorylation of GSK-3 β plays an important role in signaling induced by insulin, and growth factors, but exactly how Wnt ligands suppress the activity of GSK-3 β remains unclear, although an essential step in canonical Wnt pathway is the inhibition of GSK-3 β activity, evidenced by the use of enzyme inhibitor drugs, which mimic the activation of the Wnt pathway. Wnts are known not to induce phosphorylation at Ser 9, but cause the recruitment of GSK-3 β and Axin to the cytosolic fraction of the co-receptor LRP5 that it phosphorylates GSK-3 β , and phosphorylated sites and inhibit GSK-3 β

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acting as the competitive inhibitors, although with low affinity (K_i of 1.3×10^{-5} M). However, this inhibition is temporary and does not explain how it is maintained for more than 1 h. In this regard, recently Taelman et al. [6] reported that the Wnt ligands, from 15 min post-treatment, cause that the GSK-3 β is internalized in multivesicular bodies “sequestering” the kinase and preventing the phosphorylation of its substrates. Thus, upon binding of the ligand, GSK-3 β accumulates in signalosomes of LRP5 and therefore endocytosis is required for Wnt signaling.

Recently in the laboratory, a novel mechanism of regulation of GSK-3 β in the Wnt signaling pathway through PKC ζ using as model for studying colon cancer was demonstrated. However, it is not clear under what way, Wnt transduces the signal in addition to the functions performed in the nucleus, and the mechanism of translocation. Then, in this paper we focus on studying the technique of flow cytometry.

45.2 Experimental Procedures

45.2.1 Reagents and Antibodies

Antibodies against GSK-3 β were from the following sources: rabbit polyclonal and mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Sta. Cruz, CA, USA) and from Millipore.

The rabbit monoclonal antibody against Phospho-(Ser) PKC substrate was purchased from Cell Signaling Technology.

45.2.2 Cell Culture

RKO (human colon carcinoma), or SW480 (human colorectal adenocarcinoma) malignant cells, and non-malignant 112CoN (human colon) cells, were all obtained from the American Type Culture Collection (Manassas, VA). RKO and 112CoN cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), antibiotics (120 mg/mL penicillin and 200 mg/mL streptomycin) and 2 mM L-glutamine. SW480 cells were maintained in DMEM F-12 supplemented with 5 % FBS, antibiotics and 2 mM glutamine. All cells were cultured in a humidified 5 % CO₂ incubator at 37 °C. The human cell lines were authenticated by DNA profiling using short tandem repeat (STR) analysis on an AmpFISTR[®] Identifiler™ PCR Amplification System at “Instituto Nacional de Medicina Genómica” (INMEGEN), México, D.F.

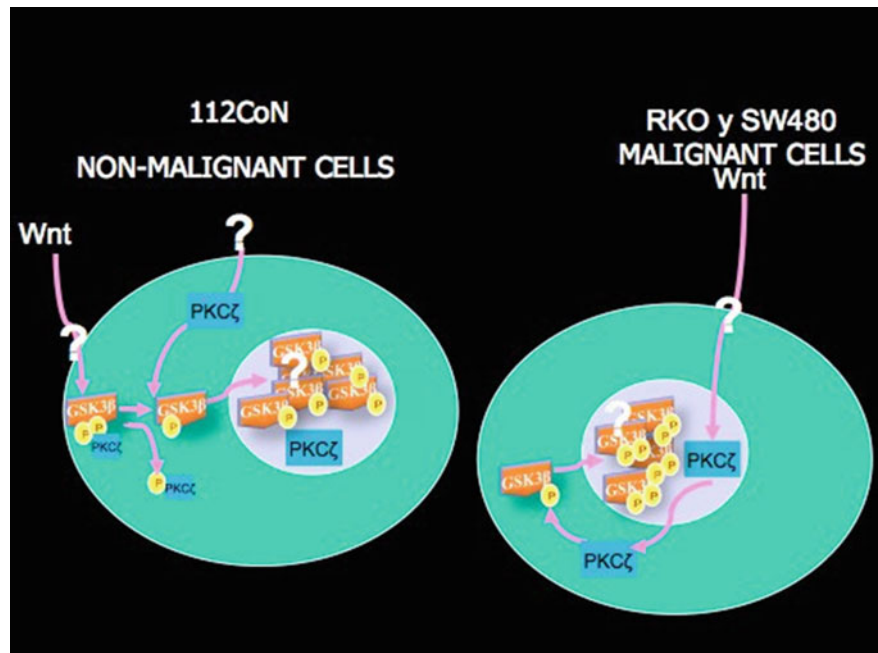
45.2.3 Incubation with Wnt Ligands and Pharmacological Inhibition of PKC ζ

For the pharmacological PKC ζ inhibition in RKO and SW480 cells, serum-starved cells (2 % serum instead of 10 %) were incubated in the absence or presence of the myristoylated PKC ζ -selective inhibitor (20 μ M) for 1 h. Then, cells were incubated in the absence or presence of Wnt3a or Wnt5a ligand (100 ng/mL) for 5 min and cells were then washed and lysed.

45.2.4 Flow Cytometry

Cells were fixed with 2 % paraformaldehyde in FACS buffer (PBS with 4 % fetal bovine serum) and incubated 10 min at 37 °C. The cells were centrifuged at 3000 rpm for 2 min and incubated with absolute methanol for 30 min at 4 °C. After removing the methanol and washing with FACS buffer 1:50 dilution of the primary antibody anti-GSK-3 β , GSK-3 β anti-p-serine 9 1/2/3 anti-AKT and anti-AKT was added p-threonine 308 for 15 min at 4 °C. FACS buffer the cells were washed and incubated in a 1:100 dilution of anti-goat secondary antibody coupled to FITC or anti-rabbit coupled to FITC for 15 min at 4 °C and then washed with FACS buffer. Cells were maintained in buffer for analysis by FACS (FACSCalibur, BD).

Fig. 45.1 Using human colon cell lines, in the laboratory a new mechanism GSK-3 β through PKC ζ was identified. This regulatory mechanism is different in normal and tumor cell lines (this will be published elsewhere), however, is not clear the way by which PKC ζ is activated and the nuclear functions of GSK-3 β



45.3 Results

The effect of stimulation with Wnt ligands revealed a rapid redistribution of GSK-3 β from the cytoplasm to the nucleus in malignant cells, and this correlation between GSK-3 β nuclear and Wnt signaling had not been shown before.

We are investigating the mechanism that GSK-3 β uses to enter and exit the nucleus and the functions in the organelle (Fig. 45.1). As it can be observed in Fig. 45.2, GSK-3 β levels increased with both treatments compared to the control. Importantly in other cellular processes, there are numerous examples showing a partial accumulation of GSK-3 β in the nucleus, and apoptosis [7, 8], replicative senescence, [9] and the S phase of the cell cycle [10]. In human pancreatic tumors there is a redistribution of GSK-3 β , which is overexpressed and accumulated in the nuclei of tumor cell lines, and in most poorly differentiated adenocarcinoma [11].

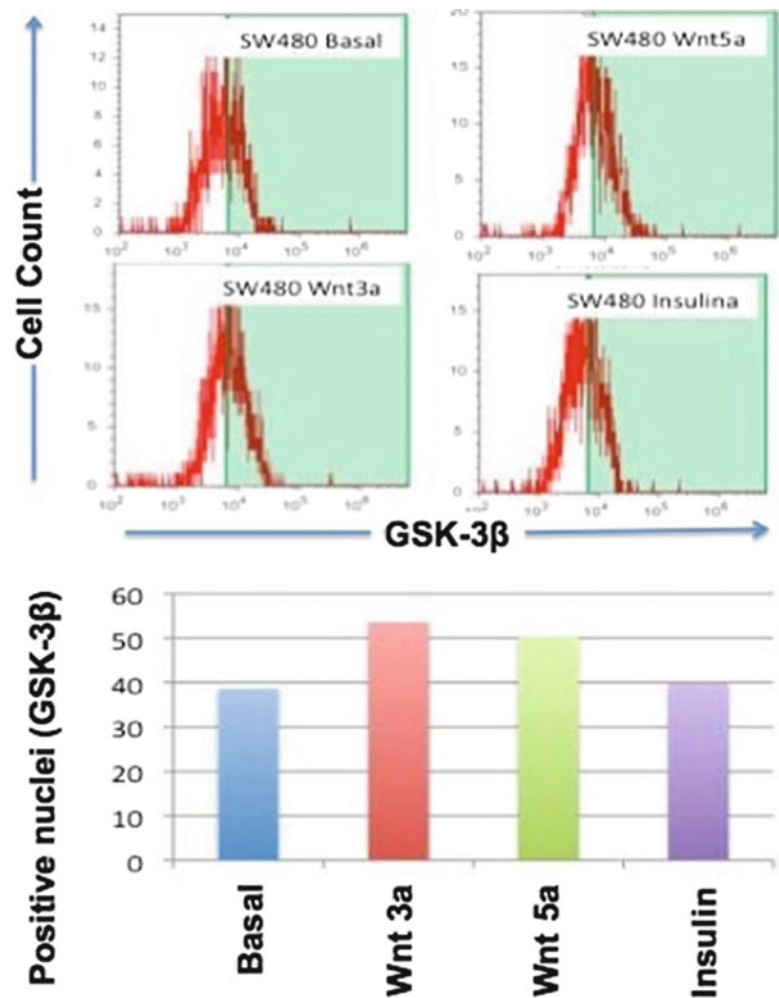
Once in the nucleus, and depending on the cell type, GSK-3 β can phosphorylate substrates such as cyclin D, NFAT, and c-myc.

The import mechanism of GSK-3 β appears to be dependent on a bipartite nuclear localization signal (NLS) [12], while exports under some circumstances require FRAT [13]. Only one report [14] has described a nuclear-cytoplasmic shuttling of GSK-3 β , and so the generality of this observation was previously clear.

45.4 Conclusions

The GSK-3 β is an important therapeutic target in colon cancer so it is fundamental to study the way in which it transduces, and its core functions; as it is reported that both positively and negatively regulates transcription factors. We present experimental evidence that Wnt ligands stimulate the accumulation of GSK-3 β in the nucleus and its functions are being studied.

Fig. 45.2 Quantification of GSK-3 β in the core by flow cytometry. (a) Graph of the amount of nuclear GSK-3 β SW480 cells at baseline, and SW480 cells stimulated with Wnt3a ligand, Wnt5a and insulin. Cells were stimulated (100 ng/mL) ligand Wnt 3a, 5a and insulin (10 mg/L) for 5 min. (b) The amount of nuclear GSK-3 β increases when stimulated with Wnt ligands and stimulate insulin the amount of GSK-3 β is similar to the baseline



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