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ORIGINAL ARTICLE

Experimental Rodent and Human Models for Balanced Activity of Oncogenes and Tumor-Suppressor Genes and Their Protein Products

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ABSTRACT
The analogy of malignant rat insulinoma cells RIN-5F with human cervical carcinoma cells HeLa, both containing additional copy of tumor-suppressor gene SCGN and HACE1, respectively, was investigated. This necessity was connected with the observed in previous our studies lower phagocyte differentiation potential in vitro on the influence of both cell types, containing such a copy, in comparison with non-containing additional tumor-suppressor gene malignant cells. Similar analogy has been demonstrated in previous our investigations with normal mouse embryonic stem cells (mESCs), containing and non-containing additionally-inserted oncogene copy, with immortalized and non-immortalized normal human trophoblasts. For identification and quantification of proteins, able to connect specifically or non-specifically with peptide SCGN, protein material from E. coli bacteria strains, containing copy of gene for this peptide, inserted by transfection, was isolated and incubated in protein lysates from rat brain and pancreas, known as organs with the most active expression of this peptide. The so prepared protein fractions were subjected on label-free tandem mass-spectrometry (LC-MS/MS) assay with subsequent computer processing. Suggested in our previous studies abilities of protein SCGN to connect with cytoskeleton elements were confirmed. On this base, a mechanism for indirect influence of this peptide on the control of cell growth and proliferation, has been proposed. Future studies are necessary in this direction.
Keywords: *Normal cells, malignant cells, oncogenes, tumor-suppressor genes, cell transfection*

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INTRODUCTION

Peptide Secretagogin (SCGN) has recently been cloned and characterized as beta-cell-expressed EF-hand Ca^{2+}-binding protein [1-3]. Functional analysis of transfected cell lines, expressing SCGN, has revealed an influence on calcium flux and cell proliferation [1]. In a number of studies it has been shown that SCGN has been found to be involved in coordination of the action of oncogenes and tumor-suppressor genes, and, in this way, in the regulation and prevention of malignant transformation [1-9]. Such regulation would be achieved via direct protein-protein interactions. In this way, an evidence about the eventual involvement of this molecule in cancer development has been proposed. As an example, in RIN-5F cells (rat Langerhans islet malignant cell line), an anti-proliferative effect has been identified, which is probably due to Secretagogin-triggered down-regulation of substance P transcription [3]. At the same time, a calcium-dependent SCGN-TAU interaction, as well as co-induction of TAU, has been found in the islets of Langerhans and beta-cell-derived cell lines with high expression of the neuroendocrine-specific protein SCGN [7]. In that direction, investigations about eventual direct and/or indirect influence of protein SCGN on nuclear proteins functions by cascade regulatory mechanisms have been proposed [10]. In this aspect, signaling strength, kinetics and specificity of this pathway have been shown to be modulated at many levels by distinct regulatory proteins. As a novel method for identification of target proteins, protein-protein interactions and regulation pathways, label-free mass-spectrometry has been developed and applied in the last years [11-20].

In this connection, the main goal was connected with investigation on the similarity of RIN-5F malignant rat insulinoma cells, containing additional copy of *SCGN* gene with HeLa malignant human cells, containing additional copy of tumor-suppressor gene *HACE1*.

MATERIALS AND METHODS

Malignant cells RIN-5F and HeLa from rat insulinoma and human cervical carcinoma, containing additional copy of tumor-suppressor genes *SCGN* and *HACE1*, respectively, inserted by their transfection with appropriate recombinant gene constructs, were used. Cells from both malignant types, as well as normal cells from mouse embryos (mESCs) with similarly inserted in them additional copy of oncogene *Dcn1*, but also combinations of co-cultivated normal and malignant cells, were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification, in Dulbecco's Modified Minimal Essential Medium (DMEM) (Sigma-Aldrich), supplemented with 10% Fetal Calf Serum (FCS) (Sigma-Aldrich), antibiotic mixture (100 U/ml penicillin and 100 μg/ml streptomycin - (Sigma-Aldrich)), as well as 2 μg/ml Doxicyclin (Sigma-Aldrich) for suppression of cell proliferation and eventual stimulation of myeloid cell differentiation. The so prepared cell cultures were observed by inverted light microscope (Leica).

Recombinant rat protein SCGN, isolated from transfected with recombinant DNA-plasmids *E. coli* bacteria strains, was incubated in previously prepared rat pancreatic and brain lysates, respectively, as anatomic organs, known as the most actively expression of *SCGN* gene in building cells. Because of the revealed very near protein profiles in both organs, different concentrations of the isolated from them proteins were also used in the current study – protein lysate from rat pancreas was diluted 10 folds. Bacteria cells and both organs were treated with lysis buffer and put on 4^0C for 2 hours. After addition of specific anti-rat SCGN antibody to the so obtained bacteria cell protein lysate and subsequent centrifugation, the pellet was resuspended in PBS. The so prepared protein suspension was mixed with the obtained protein lysates from rat brain and rat pancreas, respectively, and both mixtures were subjected on GST-Agarose columns separation for 2 hours with intensive shaking for connection of proteins with affinity to the recombinant rat peptide SCGN of both organs. After elution of protein materials from both columns with SDS (Sodium dodecylsulphate) and LDS (Lithium dodecylsulphate) buffers, the so prepared probes were put at 70^0C for 10 minutes, and subsequent addition of LDS-leading buffer and Reducing agent, they were separated by LDS-Polyacrylamide Gel Electrophorhesis (PAGE). As a control comparison base, proteins from nuclear extracts (NE) of non-transfected human malignant cells HeLa and from human knee joint synovial fluid (SF) were simultaneously used. The use of these probes also aimed for confirmation the power of label-free tandem mass-spectrometry (LC-MS/MS) assay system as a novel technique. The isolated protein fractions were precipitated in cold 100% EtOH overnight, after which the so formed precipitate were washed with cold 80% EtOH and after centrifugation, the supernatants were turned off and the pellets were diluted in LDS buffer. After SDS-PAGE and consequent Comasie-blue staining, the gel was washed and sliced with an in-house tool. Gel lanes were fractioned into 23 slices and trypsin in-gel digested. The so obtained gel slices were then washed with water and acetonitrile, followed by reduction and alkylation of cysteine residues by DTT and iodoacetamide. Following overnight trypsin digestion, peptides were extracted by acetonitrile and 5% formic acid, and subsequently concentrated in a speed-vacuum centrifuge In this way, all probes were prepared for LC-MS/MS.

RESULTS AND DISCUSSIONS

In cultivation of cells in the presence of Doxicyclin, inhibited cell proliferation and active myeloid differentiation was established **(Fig. 1)**.

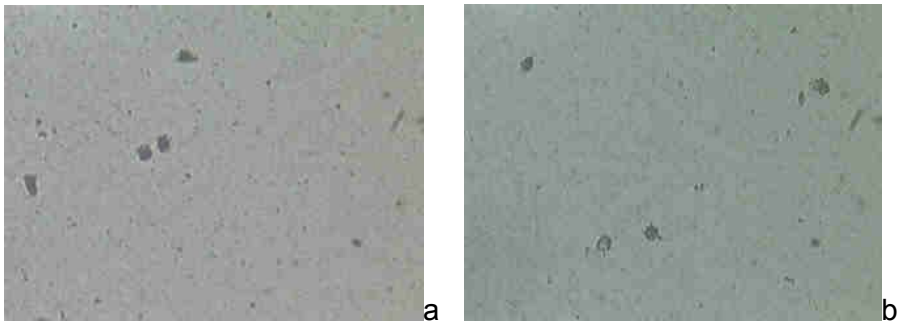

Figure 1: Co-cultivation of normal stem cells from mouse embryos, containing additional oncogene copy, with malignant cells, containing additional copy of tumor-suppressor gene, in the presence of Doxicyclin: With malignant RIN-5F rat insulinoma cells, containing additional copy of tumor-suppressor gene *SCGN* (a); With human cervical carcinoma HeLa cells, containing additional copy of tumor-suppressor gene *HACE1* (b)

The observed decreased proliferation of mESCs, co-cultivated with containing additional *SCGN* gene insulinoma cells RIN-5F cells **(Fig. 1 - a)** and additional *HACE1* gene HeLa carcinoma cells **(Fig. 1 - b)**

from rat and human origin, respectively, in both cases in the presence of Doxicyclin, and their differentiation in myeloid cell progenitors could be explained with activation of tumor-suppressor genes of *STAT*-family in these conditions [21]. These data also confirmed the results of our previous studies [10], including about the preserved normal/non-malignant cell characteristics of the normal cells, containing additional oncogene copy, on the one hand, as well as the eventually decreased malignancy of both cell types, containing additional copy of tumor-suppressor gene, on the other. In this connection, for understanding the mechanisms of normal cell differentiation and prevention of malignant cell transformation, the first task was directed to reveal of proteins, common for both malignant and normal differentiating cells, as well as of specific protein molecules for each one of them. For this goal, HeLa NE was mixed with SF protein lysate (lanes 3, 5 and 7), but also HeLa NE protein extract alone (lanes 2, 4, 6, 9 and 10) and SF protein extract alone (lane 8), respectively, were tested **(Fig. 2)**. According to the results of the performed LC-MS/MS assay, almost 700 proteins were identified in HeLa NE and 130 proteins - in SF, with 48 proteins being observed in common for both samples. The protein identifications not identified in the HeLa NE sample were termed as unique for the SF and the other 44 protein identifications were manually evaluated for number of peptides, associated with each sample and their intensity. 33 of these 48 common proteins were characterized as specific for SF extract and the other 15 - either for HeLa NE or for both samples. That was also the other main reason we chose HeLa cell NE and SF - two protein mixtures that should have few proteins in common: HeLa NE should contain proteins, originating from the cell nucleus and associated with chromatin maintenance, transcription and other biochemical processes. SF, on the other hand, is a bio-fluid, involved in lubricating the knee and nurturing chondrocytes in the cartilage tissue.

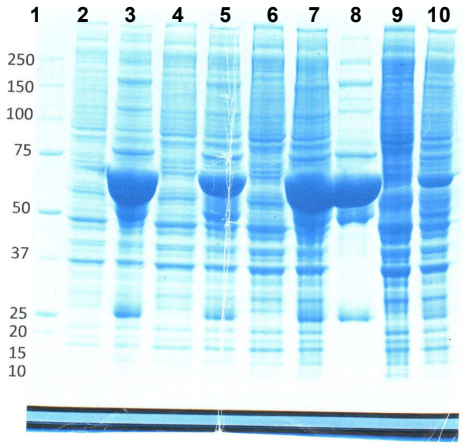

Figure 2: Optimization of proteins from HeLa NE (lanes 2, 4, 6, 9 and 10); SF (lane 8) and mixture from both protein extracts (lanes 3, 5 and 7)

Similarly, for test the analogy of rat RIN-5F malignant experimental model with HeLa malignant human cells, protein material from *E. coli* bacteria cells with inserted copy of rat *SCGN* gene, (additional copy of which was also inserted in rat RIN-5F malignant cells), used in their role as analogist of HeLa human cervical carcinoma cells with similarly inserted additional copy of tumor-suppressor gene *HACE1*, was isolated and characterized.

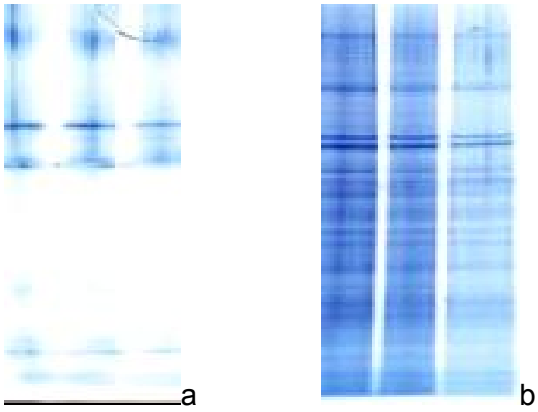

Figure 3: LDS-PAGE of proteins, incubated recombinant rat peptide SCGN: from rat pancreas (a); from rat brain (b)

The data obtained supported the usefulness of the used method for precise establishment of novel protein-protein molecular interactions, but also confirmed the results of our previous studies about the affinity of peptide to cytoskeleton components [10], which could be additionally confirmed by the observed molecular weights of the respective proteins (Fig. 3), as well as by the established intensity of both absorbance and fluorescence spectra of these molecules (Fig. 4). These data were also supported by the electrophorhetic profiles of protein lysates different dilutions from two anatomic organs because of the established very near protein composition of them [22-29]. On the other hand, the current study also confirms ones more the power of label-free LC-MS/MS assay as a novel method about protein distinguish from different samples, which was in agreement with many literature data [11-20]. Furthermore, the current study indicates some advantages of the applied technique about protein separation by GST-Agarose (Fig. 3; Fig. 4), in comparison with CNBr-Sepharose, which was probably connected with the escaped protein degradation on the influence of CNBr [30].

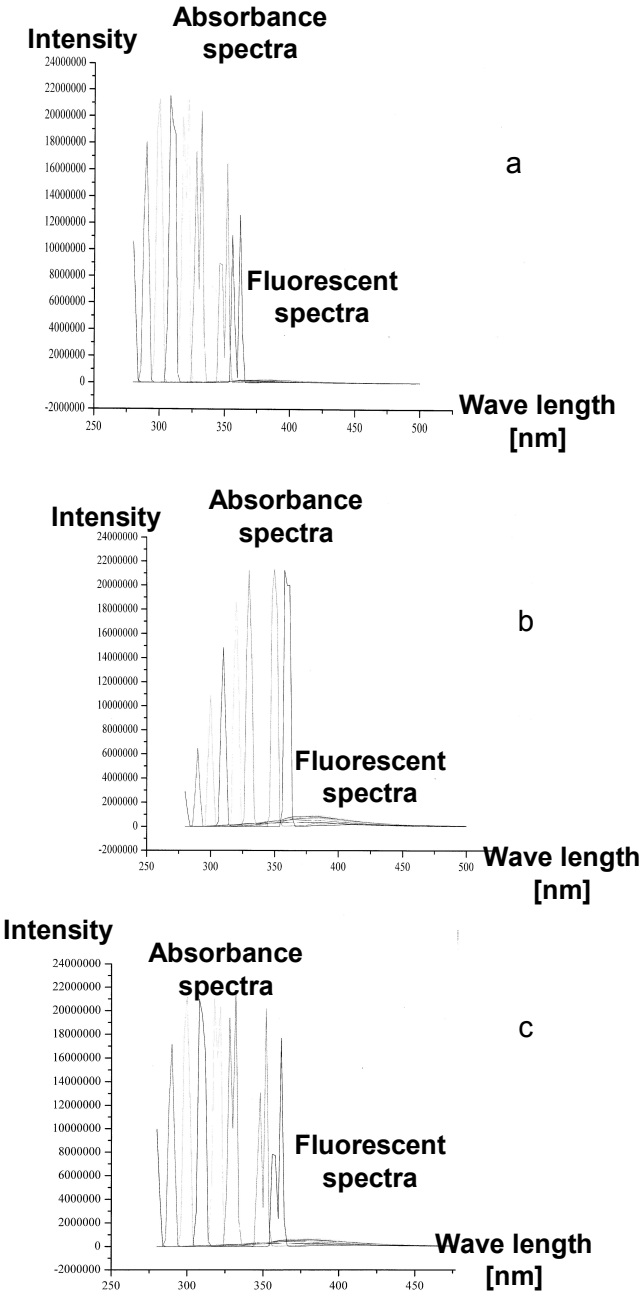

Figure 4: Intensity of absorbance and fluorescent spectra in different wave length values: Negative control of SDS in the absence of protein material (a); Proteins from rat brain, able to connect with recombinant rat peptide SCGN (b); proteins from rat pancreas, able to connect with recombinant rat peptide SCGN (c)

Both SCGN and HACE1 proteins have been proven as able to connect with cytoskeleton components as microtubule proteins [5-8, 21-35] and cyclins [36-38], known on the other hand as able to connect with histones and histone-like nuclear proteins [37, 38], by similar cascade regulatory pathways. Similar analogy has been demonstrated in our previous investigations with normal stem cells from mouse embryos (mESCs), containing and non-containing additionally-inserted oncogene copy [10], with immortalized and non-immortalized normal human trophoblasts, respectively [39, 40].

CONCLUSIONS

Specific proteins, able to connect with peptide SCGN in cascade regulatory pathways, should be revealed. For this goal, future investigations for comparison of with control protein profiles from brain and pancreas with these with SCGN peptide, connected with proteins from each one of both anatomic organs, are necessary.

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