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Signatures of protein expression revealed by secretome analyses of cancer associated fibroblasts and melanoma cell lines

Tarcísio Liberato[a], Dayelle S. Pessotti[a], Isabella Fukushima[a], Eduardo S. Kitano[b], Solange M.T. Serrano[b], André Zelanis[a,*]

[a] Functional Proteomics Laboratory, Department of Science and Technology, Federal University of São Paulo (ICT-UNIFESP), São José dos Campos, SP, Brazil
[b] Laboratório Especial de Toxinologia Aplicada, Center of Toxins, Immune-Response and Cell Signaling (CeTICS), Instituto Butantan, São Paulo, Brazil

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ABSTRACT

The imbalance of cellular homeostasis during oncogenesis together with the high heterogeneity of tumor-associated stromal cells have a marked effect on the repertoire of the proteins secreted by malignant cells (the secretome). Hence, the study of tumoral secretomes provides insights for understanding the cross-talk between cells within the tumor microenvironment as well as the key effectors for the establishment of the pre-metastatic niche in distant tumor sites. In this context, we performed a proteomic analysis of the secretomes derived from four cell lines: a paired set of fibroblasts - Hs 895. T, a cell line obtained from a lung node metastatic site from a patient who had melanoma and Hs 895.Sk, a skin fibroblast cell line (derived from the same patient); two malignant metastatic melanoma cell lines - A375, a malignant melanoma cell line from primary source and SH-4, a cell line derived from pleural effusion of a patient with metastatic melanoma. Clustering of expression profiles together with functional enrichment analysis resulted in patterns that mirrored each cell type. In addition, these patterns might be the result of cell-specific protein expression programs and reveal the emergence of trends in the co-expression of functionally related proteins in cellular melanoma models.

Significance: Melanoma is an aggressive skin cancer and a lethal melanocytic neoplasm with increasing annual number of cases, faster than any other solid tumor. In this context, the imbalance of cellular homeostasis during oncogenesis together with the high heterogeneity of tumor-associated stromal cells have a marked effect on the repertoire of the proteins secreted by malignant cells (the secretome). Therefore, the identification of protein expression patterns in malignant cells together with functional enrichment analysis provide insights into cell-specific protein expression programs and may reveal the emergence of trends in the co-expression of functionally related proteins regardless of cell type. Moreover, the identification of networks of protein interactions together with their expression profiles can be used for the targeted analysis of co-expressed proteins, allowing the identification of regulatory motifs in melanoma protein-protein interaction networks.

1. Introduction

Melanoma is an aggressive skin cancer and a lethal melanocytic neoplasm with increasing annual number of cases (faster than any other solid tumor) [1,2]. Despite of accounting for about 1% of all skin cancer cases, invasive melanoma is responsible for the vast majority of skin cancer deaths [2]. In this context, both tissue samples and cellular models have been used to identify patterns of gene expression and biological pathways relevant to oncogenesis aiming to provide insights for personalized therapeutic interventions and address distinct outcomes of melanoma development [3–5]. Although the analysis of tissue samples plays a vital role in oncology research it does not allow the reliable detection of secreted proteins or those released into the extracelular environment. Actually, the contribution of neighboring cells (i.e. stromal cells, such as neutrophils, macrophages and fibroblasts) is also of paramount importance for a number of biological signaling events related to oncogenesis such as tumor invasion and migration. By secreting bioactive molecules such as growth factors and proteases, stromal cells are often recruited by tumoral cells to participate in the oncogenesis, which eventually leads to tumor progression and dissemination. The imbalance of cellular homeostasis during oncogenesis together with the high heterogeneity of tumor-associated stromal cells have a marked effect on the repertoire of the proteins secreted by malignant cells (the secretome). Hence, the study of the secretomes from tumoral and stromal cells, such as cancer associated fibroblasts (CAFs) provides insights for understanding the cross-talk

* Corresponding author at: Department of Science and Technology, Federal University of São Paulo (ICT-UNIFESP), Rua Talim, 330, 12231-280, São José dos Campos, SP, Brazil.
E-mail address: andre.zelanis@unifesp.br (A. Zelanis).

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between cells within the tumor microenvironment as well as the key effectors for the establishment of the pre-metastatic niche in distant tumor sites [6–8]. In addition, secreted proteins are important sources for biomarker discovery or druggable targets for therapy [7,9]. It was recently shown that the therapy-induced secretome of human malignant melanoma cells treated with kinase inhibitor vemurafenib stimulated dissemination and metastasis of drug-sensitive cancer cells [10]. The authors demonstrated that targeted inhibition of a cancer driver pathway can paradoxically promote drug resistance via induction of a complex reactive secretome [10]. Moreover, Lunavat and coworkers [11] showed that serine/threonine-protein kinase B-Raf (BRAF) inhibitors altered the microRNA cargo in the vesicular secretome of malignant melanoma cells. The identification of significantly mutated genes by whole exome sequencing led to the genomic classification in cutaneous melanoma [4]. However, while there are some driver mutations that are involved in tumor progression, a number of passenger mutations turn complex the reliable identification of the driver ones [9]. Therefore, the analysis of the set of secreted proteins derived from tumoral cells as well as the pattern of protein expression in different cancer associated cell types have allowed a systemic (and functional) overview of the repertoire of the molecules potentially involved in the physiological mechanisms related to oncogenesis.

In this context, we performed a proteomic analysis of the secretomes derived from four cell lines: (i) a paired set of fibroblasts - Hs 895. T, a cell line obtained from a lung node metastatic site from a patient who had melanoma and Hs 895.Sk, a skin fibroblast cell line (obtained from the same patient); (ii) two malignant metastatic melanoma cell lines - A375, a malignant melanoma cell line from primary source and SH-4, a cell line derived from pleural effusion of a patient with metastatic melanoma. The analysis of the secretome from this panel of cells, representing distinct human melanoma biological contexts (i.e. melanoma cells from primary source, stromal cells and metastatic cells), revealed the occurrence of co-expression programs, as illustrated by the identified signatures of protein expression/function related to each cell type. Our findings represent important features for further proteomic studies on human melanoma cellular models and are of potential use in the proteomic analysis of tissue samples.

2. Material and methods

2.1. Cell culture and secretome harvesting

Cell lines Hs 895.T (CRL-7636™), Hs 895.Sk (CRL-7637™), A375 (CRL-1619™) and SH-4 (CRL-7724™) were obtained from the American Type Culture Collection (ATCC, USA). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1.5 g/L of sodium bicarbonate, 100 mg/L streptomycin, 25 mg/L ampicillin, 4 mM Glutamine and 10% of Fetal Bovine Serum (FBS). Cells were grown in a humidified incubator at 37 °C, with 5% CO_2. Subconfluent cell cultures were washed three-times with warm buffered phosphate-saline (without Ca^{2+} and Mg^{2+}) and, after culturing the cells in T175 cm^2 flasks for 24 h under phenol red-free, FBS-free DMEM medium, the conditioned media was harvested and centrifuged (2200 × *g*, 10 min, 4 °C) for removing any remaining cell. Protease inhibitor cocktail (SIGMA*FAST*™, Sigma, USA) was added to each sample and the secretomes were filtered (0.22 μm; Millipore, USA) and concentrated (5000 × *g*; 4 °C) using ultrafiltration devices (Vivaspin 20, 3 kDa cutoff, GE Healthcare, USA). Protein contents were measured by the Bradford method [12]. Secretomes were independently obtained from three biological replicates derived from each cell line.

2.2. Cell viability analysis

In order to measure cell viability, dye-exclusion analysis (trypan blue) was performed in cells submitted to secretome harvesting. After secretome collection cell cultures were detached from culture flasks with trypsin-EDTA solution (0,25% trypsin, 0,53 mM EDTA) and counted using a haemocytometer. The results were expressed in percentage as the number of viable cells over the total counted cells and multiplied by 100.

2.3. In-solution trypsin digestion and reductive isotopic dimethylation labeling

The *in-solution* trypsin digestion was performed according to the protocol described by Kleifeld et al. [13] with slight modifications. Briefly, a solution of 6 M guanidine hydrochloride (GuHCl) was added to a sample of 100 μg of protein from each secretome to a final concentration of 3 M GuHCl, followed by the addition of 5 mM dithiothreitol (DTT) (final concentration). The mixture was incubated at 65 °C for 60 min. Iodoacetamide (IAA) was then added to a final concentration of 15 mM and the samples were incubated for 60 min at room temperature, in the dark. To quench the excess of IAA, DTT was added to a final concentration of 15 mM. Clean-up of samples was performed by the addition of ice cold acetone (8 volumes) and methanol (1 volume), followed by the incubation of samples for 3 h at − 80 °C. After centrifugation at 14,000 × *g* for 10 min, protein pellets were washed twice with one volume of ice cold methanol and then resolubilized with NaOH solution (final concentration of 2.5 mM), followed by the addition of 50 mM HEPES buffer, pH 7.5, to a final volume of 100 μL. Trypsin (Proteomics grade; Sigma, USA) was added at 1:100 ratio (enzyme/substrate) and protein samples were incubated at 37 °C for 18 h. Tryptic peptides were differentially labeled via stable-isotope dimethyl labeling, as previously described [14]. In brief, tryptic peptides were submitted to reductive dimethylation with either light or heavy formaldehyde/cyanoborohydride solutions, as follows: Hs 895 Sk (light) vs. Hs 895 T peptides (heavy); A375 peptides (light) vs. SH-4 peptides (heavy). Tryptic peptides (pH 7.5) from each sample were incubated overnight at 37 °C with either light or heavy sodium cyanoborohydride ($NaBH_3CN$, light, or $NaBD_3CN$, heavy) to a final concentration of 20 mM followed by the addition of formaldehyde $^{12}CH_2O$ (light) or $^{13}CD_2O$ (heavy) to a final concentration of 40 mM, resulting in mass differences of + 28.031300 Da and + 36.075670 Da for the light and heavy-labeled samples, respectively. The reaction was terminated by adding 1 M Tris (pH 6.8; to a final concentration of 200 mM) to each sample and the mixture was incubated for 2 h at 37 °C. Samples were then combined at 1:1 ratio into two pools: (i) Hs 895 Sk + Hs 895 T and (ii) A375 + SH-4. After desalting using C-18 cartridges (3 M Empore™ SPE Extraction disks, USA) peptide samples were dried in a SpeedVac and redissolved in 50 μL of 0.1% formic acid prior to nanoflow liquid chromatography/tandem mass spectrometry (LC–MS/MS) analysis.

2.4. Mass spectrometric analysis

An aliquot (5 μL) of the resulting peptide mixture was injected into a trap column packed with C18 (100 μm i.d. × 2 cm) for desalting with 100% solvent A (0.1% formic acid). Peptides were then eluted onto an analytical column (75 μm i.d. × 100 mm) packed *in house* with Aqua® C-18 5 μm beads (Phenomenex, USA). Nanoflow liquid chromatography was performed on an Easy nanoLC system (Thermo Fisher Scientific, USA) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, USA). Peptides were loaded onto the column with solvent A (0.1% formic acid) and eluted with a 150 min linear gradient from 3 to 30% of solvent B (acetonitrile in 0.1% formic acid) at a flow rate of 200 nL/min. Spray voltage was set at 2.1 kV, 200 °C and the mass spectrometer was operated in data dependent mode, in which one full MS scan was acquired in the *m/z* range of 300–1650 followed by MS/MS acquisition using Collisional Induced Dissociation (CID) of the fifteen most intense ions from the MS scan. MS spectra were acquired in the Orbitrap analyzer at 60,000 resolution (at 400 *m/z*). Dynamic exclusion was defined by a list size of 500 features and exclusion duration of 60 s. For the survey (MS) scan AGC target value of 1,000,000 was set