Comparative proteomic analysis of secreted proteins from nasopharyngeal carcinoma-associated stromal fibroblasts and normal fibroblasts

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Abstract. Since the concept of the secretome (ensemble of proteins secreted and/or shed from cells) was proposed, it has become an attractive and challenging proteomic technology in recent years. However, secretome analysis still faces some difficulties mainly related to sample collection and preparation. In the present study, we established a reliable method for extracting secreted proteins by ultrafiltration centrifugation and conducting secretomic analysis. Accumulating evidence suggests that carcinoma-associated stromal fibroblasts (CAFs) play an important role in cancer initiation and progression. To investigate the expression patterns of secreted proteins from fibroblasts and to identify the secreted proteins involved in nasopharyngeal carcinoma (NPC) carcinogenesis, we conducted comparative secretome analysis between CAFs and normal fibroblasts. After two-dimensional gel electrophoresis (2-D PAGE), 11 significant spots were differentially expressed and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). These proteins may take part in the regulation of the tumor microenvironment through different processes. The expression level of galectin-1 in the CAF supernatant was also determined by ELISA. This study provides useful information and new clues for the further understanding of the regulatory mechanisms of CAFs in the NPC microenvironment.

Introduction

As the most abundant cells in tumor stroma, tumor-associated fibroblasts or carcinoma-associated stromal fibroblasts (CAFs) have distinctly different morphological and biological characteristics compared with normal fibroblasts (NFs). CAFs may promote the malignant transformation of epithelial cells by secreting a variety of growth factors, cytokines, chemoattractants and enzymes. Scatter factor/hepatocyte growth factor, insulin growth factor, matrix metalloproteinases and fibroblast activation protein have been confirmed to play an important role in tumor-host crosstalk (1-3). Accumulating evidence suggests that CAFs play an important role in cancer initiation and progression (4). Co-culture system or three-dimensional (3D) culture system has been used to study the interaction between CAFs and cancer cells in recent years. However, the study of cells in 3D cultures has been hampered by the lack of simple methods to analyze them. Current techniques are time-consuming, require expensive equipment such as confocal microscopy for optimal results, and are poorly adapted for study (5,6). Moreover, these tumor-promoting properties of CAFs appear to be partially independent of the presence of tumor cells and are maintained *in vitro* even in the absence of epithelial cells (1,7). Therefore, it is feasible to isolate fibroblasts from carcinoma specimen and to culture *in vitro* for understanding the role of CAFs in the tumor microenvironment. Since the concept of the secretome (ensemble of proteins secreted and/or shed from cells) was proposed (8), it has become an attractive and challenging proteomic technology in recent years. However, secretome analysis still faces some difficulties mainly related to sample collection and preparation. In the present study, we explored one method of sample preparation, and we conducted secretome analysis of CAFs and NFs to evaluate the expression patterns of secreted proteins from fibroblasts in NPC carcinogenesis. The experimental results will provide a basis for the further understanding of the regulatory mechanisms of CAFs in the NPC microenvironment.

Materials and methods

Nasopharyngeal mucosal samples. All experimental procedures in this study were approved by the Ethics Committee of the Central South University School of Medicine. In total, 8 nasopharyngeal mucosal samples from patients with poorly differentiated nasopharyngeal squamous cell carcinoma and

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8 from patients with sinusitis were obtained with informed consent at the Second Xiangya Hospital of Central South University. Diagnoses were pathologically confirmed, and none of the patients had received prior chemotherapy or radiation therapy.

Fibroblast culture. CAFs and NFs were obtained by tissue culture as described in detail elsewhere (9). Nasopharyngeal mucosa tissue was washed extensively with sterile PBS to remove contaminating debris and red blood cells, and cut into small pieces. Then cells were dissociated by 0.25% trypsin (Sigma, St. Louis, MO, USA) and purified using a curettage method combined with trypsinization. The outgrowing fibroblasts were cultivated with RPMI-1640 medium (Gibco, Life Technologies Inc, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma) in an incubator set at 37˚C and 5% CO_2. Cells were transferred and plated into T-75 flasks. While cells were grown to 70% confluence, the culture medium was removed and cells were washed more than 3 times with Hank's solution (Sigma), and then serum-free RPMI-1640 medium supplemented with 5 μg/ml transferrin and 5 μg/ml insulin were added to the culture for 72 h. For all experiments, cells used were passaged no more than three times.

Preparation of secreted protein samples. Culture medium was collected and centrifuged for 10 min at 1,000 x g, 10 min at 3,000 x g at 4˚C to remove cell debris or dead cells. The supernatant was harvested and filtered through a membrane filter (nominal pore size 0.22 μm), and then secreted proteins were enriched and concentrated by ultrafiltration centrifugation (Millipore, Bedford, MA, USA). Finally, approximately 250 μl of the supernatant containing secreted proteins was obtained every time according to the CentriPlus Centrifugal Filter Device User Guide. Protein lysis buffer (2 mol/l thiourea, 7 mol/l urea, 40 mmol/l Tris, 4% CHAPS, 65 mol/l DTT) was added to the concentrated supernatant. The mixture was centrifuged for 30 min at 15,000 x g at 4˚C and the final supernatant (ie. secreted proteins) was obtained. The protein concentration was measured using a 2D Quantitative kit (Amersham Biosciences, Piscataway, NJ, USA).

2-D PAGE. Aliquots containing approximately 600 μg of secreted protein extract were diluted in rehydration buffer for separation by isoelectrical focusing followed by SDS-PAGE (4-15% gradient gels) exactly in the same manner as previously described elsewhere (10). After SDS-PAGE, the gels were then stained with Coomassie blue staining solution overnight followed by destaining with 18% methanol and 5% acetic acid, Triplicate gels were made for each group.

Image analysis. 2-D PAGE images were digitized with an Image scanner (Amersham Biosciences, Piscataway, NJ, USA) and image analysis was conducted using the PDQuest software (Bio-Rad, V7.1) as previously described in detail elsewhere (10).

Identification of proteins by MALDI-TOF MS. Protein spots showing temporal changes in expression in each group were excised and digested in-gel with sequencing-grade modified trypsin (Promega, Madison, WI, USA). Tryptic peptides were separated and analyzed using MALDI-TOF MS. The differentially expressed proteins were identified by peptide mass fingerprint (PMF), SWISS-PROT and NCBI protein databases searching using Mascot Distiller software exactly in the same manner as previously described (10).

Galectin-1 ELISA assay. CAF and NF culture media were collected, respectively, and applied to ELISA assay of galectin-1 in the supernatant.

Results

2-D PAGE. A reliable method for extracting secreted protein from conditioned medium was established. We isolated CAFs from cancer specimens and cultivated *ex vivo* for up to 3 passages. Secreted proteins were enriched from the culture supernatant by ultrafiltration centrifugation, and approximately 250 μl of the supernatant containing secreted proteins was finally obtained. The 2-D PAGE reference map of the secreted proteins from the fibroblasts was constructed. The differentially expressed proteins were selected and numbered by PDQuestTM (Fig. 1).

Protein identification. Eleven significant spots were identified through MALDI-TOF MS and SWISS-PROT databases (Table I). These secreted protein were consistently expressed in both the CAF and NF supernatants, while 8 were differentially up-regulated in the CAF supernatant when compared with the NF supernatant. The other proteins were down-regulated including cystatin C, complement component C1s precursor and heterogeneous nuclear ribonucleoprotein A1. The concentration of galectin-1 in the supernatant was determined by ELISA. We detected a marked difference between the two groups indicating that CAFs may secrete more galectin-1 than NFs.

Discussion

As it is well known that secreted proteins play a key role in cell signaling, communication and migration, the investigation of secreted proteins has received increased attention in recent years. Secreted proteins are responsible for the cross-talk among cells and understanding this language can largely increase our knowledge concerning the molecular mechanisms of neoplasia. Significant technological advances in the field of proteomics during the last few years have provided shortcuts for the research of secreted proteins, and the concept of the secretome was correspondingly proposed. The secretome has facilitated the study of cell secreted proteins, and it may be a viable strategy for identifying candidate diagnostic and prognostic markers, potential drug and therapeutic targets (11,12). In the present experiment, we explored a set of methods with reference to other studies (13). At first, fibroblast cells were washed repeatedly more than 3 times to eliminate interference of bovine serum albumin, and then serum-free RPMI-1640 medium supplemented with transferrin and insulin as necessary nourishments was added to continue culture. Secondly, the culture medium was harvested and filtered through a 0.22-μm membrane filter. Thirdly, the culture medium was concentrated and desalinated by ultrafiltration centrifugation.

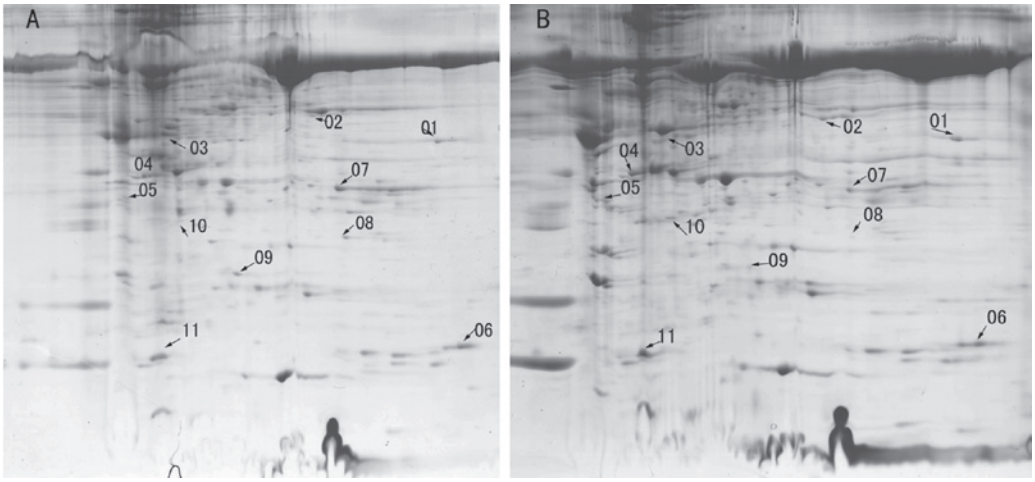

Figure 1. Differentially expressed proteins were selected and numbered. 2-D PAGE of proteins secreted from (A) NFs and (B) CAFs.

Table I. Eleven spots obtained from 2-D PAGE identified via MALDI-TOF MS.

No.	Accession no.	Protein name	Relative molecular weight (kDa)	Isoelectric point (pI)	Variable multiples[a]
1	P04075	Fructose bisphosphate aldolase A	24.7	8.47	3.10
2	P05121	Plasminogen activator inhibitor 1	42.75	6.98	3.60
3	P07711	Cathepsin L	37.9	5.32	2.50
4	P08758	Membrane annexin A5	35.3	5.15	2.90
5	P31947	14-3-3σ protein	27.7	4.69	3.20
6	P01034	Cystatin C	12.9	7.85	0.47
7	P16035	Complement component C1s precursor	78.16	4.87	0.23
8	P08294	Cu/Zn-SOD	16.2	5.85	5.20
9	P09651	Heterogeneous nuclear ribonucleoprotein A1	21.0	6.78	0.22
10	P52565	Rho-GDP dissociation inhibitor 1	2.2	6.74	3.90
11	P09382	Galectin 1	14.89	5.32	3.10

[a]Differential expression analysis of secreted protein spots between CAFs and NFs using the PDQuest software.

The final step consisted of the lysis and denaturation of the concentrated secreted proteins using a solution composed of 2 mol/l thiourea, 7 mol/l urea, 4% CHAPS, 40 mmol/l Tris and 65 mol/l DTT.

Although we achieved success in the sample preparation approaches, many problems remain to be solved. The major defect of the method used in this experiment involved the complex operation used to enrich the secreted proteins and the large amount of cell medium required; 50 ml of culture medium was needed for approximately 250 μl of the supernatant containing the secreted proteins. In order to enrich the secreted proteins, the supernatant containing the secreted proteins was collected repeatedly and the mixture was repeatedly put through ultrafiltration centrifugation. Obviously, it is extremely important to develop simpler and more efficient approaches such as the nanozeolite-driven approach for enrichment of secretory proteins (14). This technique not only simplifies the operation order, but also saves time. Non-gel electrophoresis technology may also have important application value in secretome analysis such as nanoproteomics (15), capillary ultrafiltration and multidimensional liquid chromatographic separation in combination with mass spectrometry(16). However, 2-D PAGE which is rapidly being modified plays an irreplaceable role in proteomic analysis and is still the classical and most widely used proteomics method.

Secreted proteins from CAFs may influence the NPC pathological process in different ways. Among these secreted proteins, Cu/Zn-SOD and fructose bisphosphate aldolase A are associated with cellular oxidative reaction and play an important role in protecting cells against reactive oxygen species injury. Cathepsin L, cystatin C, plasminogen activator inhibitor-1, heterogeneous ribonucleoprotein A1, Rho-GDP dissociation inhibitor-1, 14-3-3σ protein and annexin A5 are associated with signal transduction involved in NPC invasion and metastasis (17-19). As a result, CAFs are involved in the regulation of the NPC microenvironment through these secreted proteins by different processes including protein degradation, cell proliferation, invasion and metastasis. Galectin 1 is one member

of the family of β-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions. Galectin 1 may participate in the regulation of the nasopharyngeal carcinoma microenvironment and its regulating mechanism remains unclear and is yet to be further clarified (20).

In conclusion, CAFs may influence the biological behavior of adjacent normal epithelial cells and cancer cells through secreted proteins in an autocrine or paracrine manner to maintain tumor microenvironment balance. Therefore, the fibroblast cell is not a silent character in carcinogenesis, and it plays an important role in this complex process, particularly in the regulation of the tumor microenvironment. Although the 3D culture system is too complex for operation, its application and investigation warrant future efforts since it may have broad application prospects in the field of secretome analysis of CAFs.

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