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PROTEOMICS: A STUDY OFTHERAPY RESISTANCE IN CANCER CELLS

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The global analysis of expressed cellular proteins is commonly designated as "proteomics". Proteomics techniques provide powerful tools for the detailed comparison of proteins from normal and neoplastic tissue. In particular, cancer cell lines are suited for applying proteomics techniques, such as two-dimensional gel electrophoresis (2D-PAGE) and mass spectrometry (MS), to identify specific protein expression profiles and/or proteins that may be associated with a defined phenotype of the cancer cells. As an instance of such an application of proteomics techniques, the detailed proteome analyses of different drug-resistant and thermo-resistant cancer cell lines will be discussed. Following proteome analyses, the potential roles of newly identified factors have to be proven by functional studies. This experimental validation strategy will be discussed for the "transporter associated with antigen presentation" (TAP), a factor identified by 2D-PAGE analyzes of drug-resistant carcinoma-derived cell culture models.

15.1 Introduction

Following the completion of the human genome sequence, experimental endeavours have been more focused on a global analysis of the proteins. This approach has been commonly designated as "proteomics". The term "proteome" is defined as the total protein complement of a genome. The process of studying the proteome became known as proteomics. However, traditionally proteomics has been associated with displaying a large number of different proteins from a given origin by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In this sense, proteomics already dates back to the late 1960s when 2D-PAGE was introduced into biomedical research for determination of the protein composition in the ribosomal subunits of *Escherichia coli*. During the following years, the technique of 2D-PAGE was improved continuously.

There are several reasons for the intensified focus on the analysis of protein expression profiles: the mRNA expression level of a given gene frequently does not directly correspond to the cellular amount of biological active protein; although the amino acid sequence predicts potential modification sites within a given protein, the real post-translational modifications, that may be essential for biological function and activity, are not obvious; and reclusive genomic data do not reflect dynamic cellular processes. Moreover, proteomics includes the differential display of proteins for comparison of e.g. different physiological or disease states; it includes the characterization of protein localization; it includes the analysis of protein-protein and protein-nucleic acids interactions as well as the biochemical analysis of protein function. Although 2D-PAGE and mass spectrometry (MS) are currently the two most important proteomics technologies, several other techniques have been developed and are still under further development. These proteomics technologies include the yeast two-hybrid system (Y2H), protein microarrays, surface-enhanced laser desorption/ ionisation (SELDI), tissue microarray (TMA) technology, phage display method, and fluorescence resonance energy transfer (FRET) technique. Thus, the proteomic approach may have a major impact on the improved understanding of biological problems associated with clinical questions.

15. 2 Proteomics in cancer cell research

Various comparative 2D-PAGE experiments for analyzing differences in the protein expression pattern of human cancer cell lines have been performed. Cancer cell line-related investigations included pure protein expression studies, e.g. protein expression profiling in hepatocellular carcinoma (HCC) cells, gastric carcinoma cells, ovarian carcinoma cells, or mammary carcinoma cells. However, most of the cancer cell studies were performed on account of functional investigations, e.g. analyzes of invasion and metastasis, or proliferation and differentiation. Moreover, many of these functional studies concerned the cellular response of cancer cells against stress factors, including heat and drugs. Furthermore, proteomics appears as a promising strategy to compare the protein expression profiles in drug-resistant or other therapy-resistant cancer cell lines with those of non-resistant counterparts.

15.2.1 Therapy-resistant cancer cell lines

Therapy resistance, e.g. drug resistance, radiation resistance, or thermo-resistance, is the main cause of therapeutic failure and death in patients suffering from malignancies. Tumour cells can be naturally resistant to anti-cancer treatment, and they are able to develop acquired therapy-resistant phenotypes, which include the multi-drug resistance (MDR) phenomenon. The MDR phenotype is characterized by simultaneous resistance of tumour cells to various anti-neoplastic agents that are structurally and functionally unrelated. Besides the classical MDR phenotype, mediated by the enhanced expression of the adenosine triphosphate-binding cassette

(ABC) transporter MDR1/P-glycoprotein (P-gp), alternative forms of multidrug-resistant tumour cells have been described. Commonly used terms to designate this phenomenon are atypical MDR or non-P-gp-mediated MDR.

In recent years, some of the mechanisms leading to atypical MDR have been identified. These mechanisms include enhanced expression of alternative ABC-transporters, such as MRP1-MRP8 or BCRP, or alterations in apoptotic pathways. However, since all these mechanisms could not explain the MDR phenotype of all drug-resistant cells, other additional resistance mechanism must be operating in cancer cells. Furthermore, the current concept of MDR is based on the hypothesis that MDR is multifactorial and heterogenous.

To improve response rates of cancer patients to chemotherapeutic treatment, in recent years chemotherapy has been combined with experimental treatment regimens, e.g. hyperthermia. Good responses have been reported with combined thermo-chemotherapy in several experimental tumour models as well as in advanced cancer patients including tumour cells exhibiting a MDR phenotype. Thus, it turned out that chemotherapy combined with hyperthermia might be considered as a promising approach. The clinical success of this combined anti-cancer treatment may be limited by the induction of MDR phenotypes and additionally by the development of thermoresistance. Consequently, the elucidation of the biological mechanisms involved in drug resistance and thermo-resistance is of urgent importance to develop new treatment modalities and improve response rates in advanced tumours.

In order to gain further understanding of therapy resistance in human neoplasms, various *in vitro* model systems derived from many tumour entities were established in recent years. For this approach, commonly cancer cell lines were exposed to stepwise-increased concentrations of different antineoplastic agents for several months resulting in the selection of drug-resistant sublines, respectively. In analogy, thermo-resistant cell lines were established by exposure to increasing temperatures. In various biochemical studies using these *in vitro* systems, distinct differences between the therapy-sensitive parental cells and the corresponding therapy-resistant sublines have been described. However, since these studies could not explain all therapy-resistant phenotypes of cancer cells in detail, other additional mechanisms must contribute to drug resistance as well to thermoresistance. A powerful strategy to identify new factors that could play a role in therapy resistance of neoplastic cells is the proteomic approach. Applying 2D-PAGE or alternative proteomics techniques provide ideal tools to compare the protein expression patterns in parental sensitive cancer cells with those in different drug-resistant, thermoresistant, or radiation-resistant cancer cell lines.

15.2.2 Proteomic analyzes of therapy-resistant cancer cell lines

The first 2D-PAGE studies using cancer cell lines and corresponding drug-resistant sublines were already performed in the mid 1980s. In these experiments, expression patterns of [35S]-methionine-labeled proteins prepared from parental KB cells and multidrug-resistant variants selected for resistance against colchicine, doxorubicin, or vinblastine, were analyzed. Protein alterations in the multidrug-resistant lines included the decreased prevalence of members of a family of proteins of molecular mass in the rage of 70-80 kDa, pI 4.8-5.0, and the increased expression of a 170 kDa protein in membrane preparations of these cell lines. Moreover, in the colchicine-selected multidrug-resistant KB cell variant KB-Ch, the increased synthesis of a protein of molecular mass 21 kDa, pI 5.0, could be observed. Although, Western blot experiments indicated that the increase in the expressed 170 kDa protein is probably identical to P-gp, the identity of the differential expressed proteins was not determined.

In the last years, systematic proteomics studies were performed for identifying potential proteins involved in drug resistance and/or thermoresistance by using cell culture models derived from breast cancer, cervix carcinoma, colon carcinoma, fibrosarcoma, gastric carcinoma, hepatoma, lung cancer, melanoma, and pancreatic carcinoma. The sensitive parental cell lines and their therapy-resistant sublines were analyzed for differences in the protein expression patterns by 2D-PAGE. For this approach, several independent 2D-PAGEs were regularly performed. Using PDQUEST software the different gels were scanned. Commonly, the scanned gels were used for calculation of cell line-specific master gel images. Decreased or increased protein levels were determined by comparing differences in the optical density of corresponding protein spots in cell line-specific gel images. Proteins showing differences in expression level were identified by MALDI-TOF MS, or microsequencing after enzymatic hydrolysis in the gel. Subsequent to this procedure, for some of the proteins the differential protein expression level was confirmed by alternative, more specific techniques.

Figure 1 illustrates an example of this strategy: the protein expression patterns of the parental human pancreatic carcinoma cell line EPP85-181P and its thermoresistant derivative EPP85-181P-RT were analyzed by 2D-PAGE. The over expressed protein spot indicated in Figure 1A, was hydrolyzed with trypsin and the MALDT-TOF MS (Figure 1B) identified the spot as the endoplasmic reticulum (ER) protein reticulocalbin.

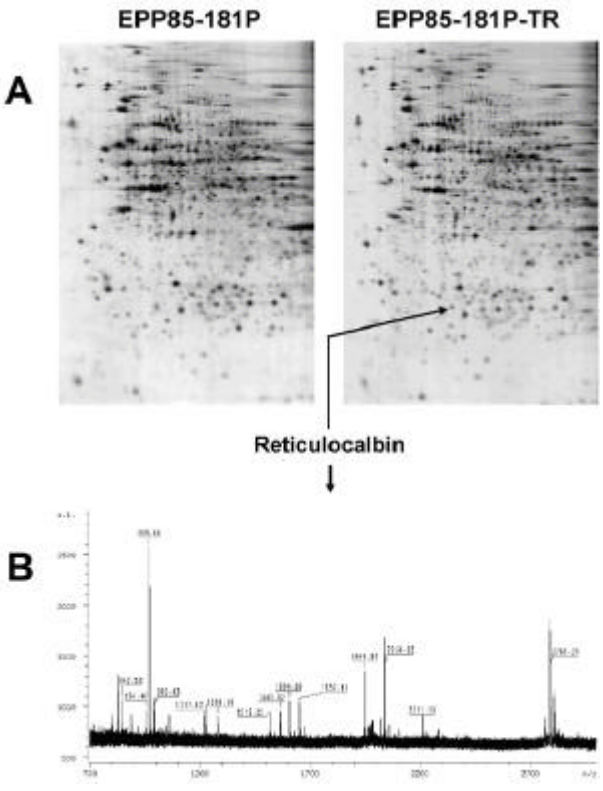

Figure 1. Enhanced expression level of reticulocalbin in thermoresistant pancreatic carcinoma EPP85-181P-TR cells. (A) 2D-PAGE analysis of silver stained protein expression patterns in parental EPP85-181P cells and the thermoresistant counterpart EPP85-181P-TR. (B) Mass spectrum (MS) of reticulocalbin following in-gel

digestion with trypsin. (Data are from Lage (2004) Pathol. Res. Pract. 200: 105-117; the 2D-PAGE images were kindly provided by Pranav Sinha, Klagenfurt, Austria; the reticulocalbin-specific MS image was kindly provided by Martina Schnölzer, DKFZ, Heidelberg, Germany).

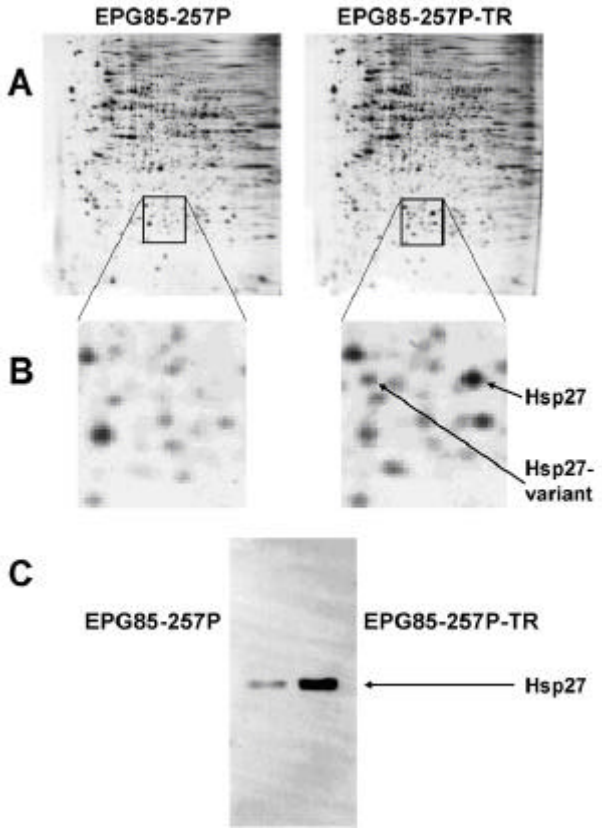

Figure 2 Analysis of protein expression by the proteomic approach in the thermosensitive, parental gastric carcinoma cell line EPG85-257P and in its thermoresistant variant EPG85-257P-TR. (A) 2D-PAGE analysis of silver stained protein expression patterns in both cell lines. (B) Detail magnification of 2D-PAGE images. In the thermoresistant cell line EPG85-257P-TR additional protein spots could be observed. MALDI-TOF MS identified one of them as Hsp27 and another spot as variant of Hsp27. (C) Confirmation of differential Hsp27 expression by Western blot. (Data are from Lage (2004) Pathol. Res. Pract. 200: 105-117; the 2D-PAGE images were kindly provided by Pranav Sinha, Klagenfurt, Austria).

A further example is shown in Figure 2: the protein expression profiles of parental human gastric carcinoma EPG85-257P cells and the thermoresistant counterpart EPG85-257P-RT were analyzed by 2D-PAGE. Evaluation of the silver-stained gels using the PDQUEST software revealed at least 19 MALDI-TOF MS-identified proteins exhibiting alterations in the expression level. Figure 2B shows increased expression of the small heat shock factor Hsp27 and of a variant of Hsp27 in the thermoresistant variant EPG85-257P-TR. As shown in Figure 2C, the increased expression of Hsp27 was confirmed by Western blot analysis. Since expression of Hsp27 may be the result of increased temperature, the data are conclusive.

Hsp27 may act in signal transduction pathways and is an ATP-independent powerful molecular chaperone, its main chaperone function being protection against protein aggregation. Its activity contributes to mechanisms that enable tumour cells as well as normal cells to survive and recover from stressful conditions by as yet uncompletely understood mechanisms. Hsp27 is of special clinical interest because of data suggesting its role in thermoresistance by acting as an antiapototic protein. Thus, it is not astonishing that the expression of Hsp27 is differentially regulated in the thermoresistant cell variant. However, the exact molecular mechanism of Hsp27, e.g. modulation of apoptotic signals or correct refolding of drug-damaged proteins, by that Hsp27 contributes to thermoresistance, is not yet clear.

A large number of differentially expressed proteins could be identified by comparing the 2D-PAGE protein expression patterns of sensitive and therapy-resistant cancer cell variants. Only a few of the factors identified in these 2D-PAGE studies have been previously linked to drug resistance or thermoresistance. So far it is not known how these proteins might be involved in therapy resistance, or whether they are merely co-regulated, or the alterations in expression may be the result of unspecific events. Thus, it is absolutely essential to evaluate the data to find out whether the potential new factor is functionally involved in therapy resistance, or, e.g. in the case of a specific co-regulation, is useful as diagnostic or prognostic marker.

15.2.3 Validation of the biological relevance of the potential new factor "TAP"

2D-PAGE analyzes of a gastric carcinoma-derived drug resistance model demonstrated various alterations in protein expression profiles in the drug-resistant cell lines. Microsequencing of a protein spot found to be overexpressed in the mitoxantrone-selected atypical multidrug-resistant gastric carcinoma cell line EPG85-257RNOV revealed amino acids sequences exhibiting similarity to the "transporter associated with antigen processing" (TAP) 1. Northern and Western blot analyzes confirmed that the expression levels of TAP1 as well as of TAP2 are indeed increased in the atypical multidrug-resistant gastric carcinoma cell line. TAP represents an additional member of the ABC-transporter superfamily. TAP, a heterodimer formed by TAP1 and TAP2 subunits, physiologically plays a major role in major histocompatibility complex (MHC) class I–restricted antigen presentation by mediating peptide translocation over the endoplasmic reticulum (ER) membrane. TAP1 and TAP2 are homologous polypeptides, each possessing a hydrophobic N-terminal domain and a C-terminal nucleotide-binding domain. Both monomers are required for peptide binding and translocation, preferentially peptides of 8-15 amino acid residues. It has been reported previously that over-expression of TAP could be detected in MDR cell lines by using a TAP1-specific antiserum. This study demonstrated that expression of rat cDNAs encoding TAP1 and TAP2 subunits in the TAP-deficient lymphoblastoid cell line T2 could lead to a slightly elevated tolerance to etoposide. Consistent with these data, a cDNA microarray study analyzing the mRNA expression profiles in different drug-resistant human hepatoma cell lines, likewise identified TAP1 as associated with resistance against mitoxantrone.

For functional validation of the potential role of TAP in the mitoxantrone-selected atypical MDR phenotype of the gastric carcinoma cell line EPG85-257RNOV, both TAP subunits encoding cDNA molecules, TAP1 and TAP2, were transfected into the drug-sensitive parental counterpart EPG85-257P. This experimental design conferred a 3.3-fold resistance to mitoxantrone but no cross-resistance to other antineoplastic agents. Furthermore, cell clones transfected with both, but not singularly expressing TAP1 or TAP2, reduced cellular mitoxantrone accumulation. The data indicate that the heterodimeric TAP complex possesses characteristics of a xenobiotic transporter and that the TAP dimer is functionally involved in atypical MDR of human cancer cells.

However, whether TAP is possibly useful as a diagnostic or prognostic marker for drug resistance, has to be evaluated in further studies using clinical specimens.

15.3 Conclusions

Proteomics provides powerful tools to study pathological processes or clinically important problems at the molecular level and will have a major impact in the future. Since cell culture models are widely used and characterized to a large extent, cell lines, especially cancer cell lines, represent the ideal object to evaluate and improve proteomics techniques. A specific and highly reproducible manipulation of these models, e.g. an acquired drug-resistant phenotype, can be analyzed in detail by methods such as 2D-PAGE. Although functional studies could confirm that potential factors that were identified by proteomics techniques are indeed involved in the phenotype of interest, other investigations, analyzing the role of a potential new factor, failed. Thus, expression data obtained by proteomics studies should be considered as preliminary. It is absolute necessary to perform hypothesis-driven biochemical experiments to evaluate the potential role of a protein of interest. Moreover, considerable technological innovations are necessary to improve the repertoire of proteomics technologies for applying them for better diagnostics and introduction into clinical practice.

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