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Review

Cell-based proteome analysis: The first stage in the pipeline for biomarker discovery

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ABSTRACT

The early detection of a distinct disease is crucial for a successful treatment and depends on a sensitive as well as specific diagnosis. In last years tremendous attempts were undertaken to identify new biomarker applying proteomics, but no relevant candidate has been identified for clinical application. Although proteomics is enabling quantitative and qualitative analysis of proteins within complex mixtures it could not significantly contribute to this field. Therefore, different proteomics approaches have been established focusing on the direct analysis of cell populations involved in pathogenic processes to identify candidate biomarkers even for *in vitro* diagnosis.

Here, we will outline approaches applying cell- and tissue based proteome analysis as the first decisive step in the pipeline for the discovery of new diagnostic biomarkers. We will show examples for analysing precursor lesions of the pancreatic ductal adenocarcinoma (PDAC), nephron glomeruli and fibrotic and non-fibrotic liver tissue. This article provides also an overview about currently available techniques in the field of cell enrichment and quantitative proteome analysis of lowest amounts of sample.

1. Introduction

The discovery of new biomarkers is a multiple stage process and involves the application of different technologies. N. Leigh Anderson suggests a three-stage diagnostic pipeline comprising 1) discovery, 2) verification/validation, and 3) clinical implementation in which each stage includes different analytical technologies [1]. Preferentially, in the discovery stage quantitative proteomics techniques like e.g. LC–MS-based and 2-DE-based methods contribute to the identification of new candidate biomarkers.

The selection of candidate biomarkers for further verification/validation is often complicated due to the analysed sample type. Blood is the easiest available source for diagnostics, so that it is not surprising, that most proteomic approaches aim at identifying biomarkers directly in blood samples. Different studies analysing tens to thousands of plasma fractions have shown that currently such methods are limited in identifying new biomarkers or are not applicable for routine clinical tests [2,3]: plasma or serum exhibits an enormous dynamic range in protein abundance and is too complex to identify new biomarkers with non-targeted analytical strategies [4].

From our point of view the selection and analysis of the affected cell type is fundamental for a focused identification of new candidate biomarker with high specificity for a given disease (Fig. 1). If tissue specimens are used in differential proteome analyses the heterogeneity of these tissues is challenging for bioanalytical studies. In most pathological processes only a few cells or cell types are affected, while the other cells/cell types compromise the results by “diluting” the differences in protein amount. In regard to cancer it is desirable to separate tumor tissue from surrounding stroma or vascular areas. Looking at neurodegenerative diseases the case is even worse because the pathologic changes often affect only single cells scattered in certain brain regions. Normally, numerous, probably unspecific proteins are identified to be involved in the pathomechanism.

Therefore, we would like to introduce a further stage within the diagnostic pipeline in addition to the above mentioned three-stage process: to shorten the long lists of candidate biomarkers usually gained in these experiments it is mandatory to carefully select the tissue/cell type of interest already in the discovery phase and to focus on the proteins functionally involved in the pathological processes of the affected cells. Applying quantitative proteome techniques candidate biomarkers are identified at their place of activity (disease specific) and can subsequently be validated as a serum or histological biomarker in respect to their specificity and sensitivity in a targeted approach. We assume that disease specific serum biomarkers are released from diseased tissue into the blood stream. It is likely that these candidate biomarkers are more abundant at their place of origin and can be identified by available proteomics techniques [5,6]. Beside

Abbreviations: CA, carrier ampholytes; FACS, fluorescence activated cell sorting; FFPE, formalin fixed paraffin embedded; GFP, green fluorescent protein; LCM, laser-capture microdissection; MACS, magnetic-activated cell sorting; MRN, multiple reaction monitoring; PDAC, pancreatic ductal adenocarcinoma; SOP, standard operating procedure; TMA, tissue microarray

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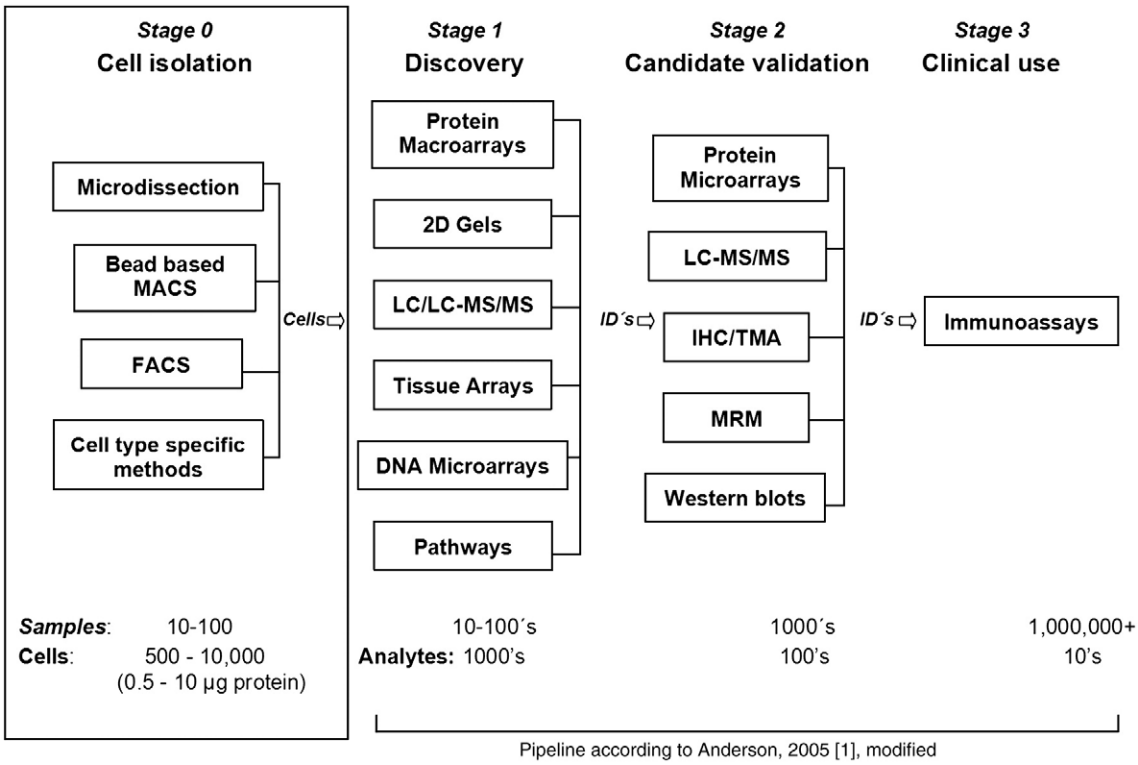

Fig. 1. Schematic overview of the diagnostic pipeline according to Anderson (modified) [1] advanced by the cell isolation stage (stage 0, box) representing different cell-specific isolation techniques. In the discovery phase (stage 1) the applied quantitative techniques must allow the analysis of lowest amount of sample (0.5–10 µg protein) from ten to hundreds of samples.

the classical secretory pathway there are hints from the literature that several processes contribute to the release of proteins into the blood stream upon a certain pathological condition. A common example is the release of troponin T – a biomarker of myocardial infarction – as consequence of tissue damage [7]. One alternative processes contributing to the release of proteins is ectodomain shedding. An example is the elevated release of soluble cadherines in several cancers [8]. The transport of proteins by exosomes are currently discussed as a further relevant mechanism releasing possible biomarkers into the blood stream [8].

2. Isolation of specific cell types

Selection and analysis of disease affected cells using quantitative proteomics is the first stage in the diagnostic pipeline according to this cell-based approach. Depending on the scientific questions and the available sample material different techniques have to be considered to successfully identify new candidate biomarkers. Because tissues are heterogeneous conglomerates of different cell types, selection of specific cell populations can be realised using microdissection. If cells can be separated easily out of tissues or if blood cells are analysed, fluorescence activated cell sorting (FACS) or cell separation using beads are suitable strategies. Here, we will outline recent techniques for the isolation of specific cell types.

2.1. Microdissection

Until now the isolation of a specific cell population is challenging and very labour intensive. The most common way to get highly homogeneous cell populations is using microdissection. This technique is widely used for genomic experiments but can also be used for subsequent proteomic studies. Several different techniques for tissue microdissection have been developed including manual microdissection [9], laser-capture microdissection [10–12] and laser microdissection [13]. For all methods slices of tissue biopsies or tissue blocks are used that have been mounted on glass slides. In the case of manual microdissection fine needles are used to scrape the areas containing the cells of interest under visual control using a microscope. Subsequently, the cells are directly transferred into lysis buffer [9,14]. Laser-capture microdissection uses a thermoplastic film on a so-called cap, which is applied to the surface of the tissue section. An infrared laser pulse is used to heat the film, which becomes focally adhesive and fuses the cells of interest to the film. So the cells fused to the film can be selectively removed. The diameter of the laser beam (>7.5 µm) defines the resolution, which can be applied to successfully select single cells. In the case of laser microdissection a pulsed UV-laser (diameter down to 0.5 µm) follows a pre-drawn line and cuts out the area of interest very precisely. The advantage is that the specimen is not heated and both single cells and larger areas can be selected. If a non-manual system is used the system can be equipped with software offering image analysis, cell recognition and automatic sampling.

2.1.1. Tissue treatment

Using microdissection procedures to collect samples for proteome analysis the pre-treatment of the tissue is an important issue to keep in mind. This includes both the process of tissue fixation and tissue staining. The best choice for proteomics experiments is to use freshly frozen unfixed tissue. Unfortunately, most tissue banks contain mainly formalin fixed tissue, embedded in paraffin (FFPE). These tissues exhibit a high degree of covalently crosslinked proteins. The cross-linking hampers many of the standard proteomics methods based on the analysis of whole proteins like e.g. 2D-GE. Nevertheless, some groups developed methods making an analysis of these tissue specimens possible. E.g. Hood et al. used stable isotope labelling and subsequent RPLC-MS/MS analysis on tryptic peptides released from the fixed tissue [15]. A comparison of proteomes from microdissected prostate cancer cells and cells from microdissected benign prostate hyperplastic tissue revealed that quantitative proteome analysis from