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Review

Utility of protein electrophoretic analysis in the characterization of malignant tissues

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ABSTRACT

High-resolution electrophoresis of samples from malignant tissues and tumour cells has developed from a simple analytical tool to a high-tech system requiring a lot of satellite techniques. Though this developmental history now demands additional expensive instrumentation and a detailed knowledge of protein chemistry, the usefulness of this technique in tumour biology has been dramatically enhanced. Consequently, electrophoretic techniques combined with additional high-resolution and sensitive analytical tools can now be used to elucidate a particular phenotype of a cancer cell; moreover, the chemical nature of this phenotype can be revealed. The way from the protein backwards to the gene is now open!

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LIST OF ABBREVIATIONS

1-D, 2-D	One-, two-dimensional
1-DE, 2-DE	One-, two-dimensional electrophoresis
FACS	Fluorescent-activated cell sorter
HPIEC	High-performance ion-exchange chromatography
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
PAGE	Polyacrylamide gel electrophoresis
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
Tris	Tris(hydroxymethyl)aminomethane
SDS	Sodium dodecylsulphate

1. INTRODUCTION

Since the introduction of polyacrylamide as an electrophoretic matrix that allows an almost unlimited application in protein separation and analysis, much effort has been expended in attempting to characterize the particular properties of malignant cells and tissues via electrophoretic systems. One of the first researchers in this field was undoubtedly Busch [1–7]. In the early 1970s, he and his co-workers began a detailed electrophoretic analysis of proteins from normal liver, hepatomas and regenerating liver [1–8]. Using different methodologies they were able to characterize some tumour-associated nuclear proteins [4–8]. They focused their experiments on nuclear proteins, because even 25 years ago it had already been suggested that DNA-bound proteins might contribute to tumour-specific proteins, owing to the function of this particular protein class in gene activation and regulation [9,10]. During the following years, the availability of improved electrophoretic techniques that was achieved by the introduction of sodium dodecylsulphate (SDS) and tris(hydroxymethyl)aminomethane (Tris) [11], together with more widespread and sophisticated cell culture techniques, led to myriads of experimental approaches to characterize the malignant phenotype of a given cell or tissue using electrophoretic systems.

In 1975, O'Farrel [12] and Klose [13] described a high-resolution two-dimensional system that allows the simultaneous separation of several hundred to a

thousand polypeptides. The development of two-dimensional electrophoresis (2-DE), which combines isoelectric focusing (IEF) of protein samples in the first direction and conventional SDS electrophoresis in the second dimension, can be regarded as a landmark in protein electrophoresis. Within a few years this sophisticated technique has spread all over the world and led to euphoria about its prospects. Thus, during the early 1980s, several laboratories started research programmes to characterize malignant tissues, especially of human origin, using crude extracts from tumour bioptic material or simple cell extracts from permanent cell lines. Together with further development of the originally described technique, additional progress was made in protein staining techniques. The innumerable papers dealing with modifications of silver staining techniques reflect the confidence in high-resolution techniques during this period. However, protein patterns with up to 2000 spots appear too complex to the human eye, and much effort was undertaken to develop computer-supported densitometers to evaluate two-dimensional (2-D) patterns [14]. Besides the high cost of such systems, which is beyond most laboratories' means, the true reproducibility of 2-DE remained the limiting factor in computer-aided gel evaluation. To circumvent difficulties that occur, especially during the IEF step as a result of ampholyte diffusion, LKB introduced in 1982 immobilized pH gradients. Amphoteric buffers were covalently coupled to acrylamide, and different pH gradients were obtained by conventional gradient casting systems [15]. In contrast to carrier ampholytes, these pH gradients remain absolutely constant over extremely long periods thus allowing a true steady-state focusing [16]. The use of Immobilines successfully reduces variations that can occur in the protein profiles of identical samples separated in different laboratories. Consequently, an improved standardization of protein maps is now possible, which enables a direct comparison of data between different laboratories.

Apart from the development of a wide variety of methodologies for high-resolution electrophoretic systems, the availability of sophisticated sample-preparation techniques and the use of chromatographic fractionation methods contribute to the successful electrophoretic analysis of any biological material.

2. SUITABILITY OF HIGH-RESOLUTION ELECTROPHORESIS FOR CANCER RESEARCH

Despite many national health programmes designed to increase our knowledge of the events occurring during tumour development and progression, accurate characterization of the malignant phenotype of a tumour cell and of the parameters by which tumour cells differ from their normal counterparts remains an unsolved problem. A detailed analysis that compares all the biomacromolecules of tumour cells with the non-malignant form of the cell seems to be one avenue of great promise. Following the development of protein electrophoresis this methodological approach was considered to be one of the key techniques toward a complete tumour cell characterization. Owing to the limited separation

power of conventional one-dimensional (1-D) systems, however, this particular technique failed to reveal significant knowledge about the molecular mechanisms occurring during tumour development. Even high-resolution 2-D systems, which have been used to characterize the malignant phenotype of tumour cells, failed in this respect.

Consequently, one has to ask whether a detailed electrophoretic analysis that is limited to proteins fulfils the requirements describing the malignant phenotype. Considering the molecular processes that determine the cellular programme during malignant transformation, one of the first events occurs at the DNA level. Mutations occurring within proto-oncogenes, as are well known for the ras-gene family, may be one of the most prominent examples of this mechanism [17]. It would be hardly possible to detect this mutated gene product (as a shift in p*I*) among thousands of structural proteins. Furthermore, point mutations occur as rare events related to only a limited number of cells (one stem cell!). From this point of view, it is clear that even high-resolution separation of tumour cell proteins represents only one of several approaches toward a successful characterization of the malignant cell. The detection of highly polymorphic DNA sequences, which can be used as molecular probes to detect mutations in the genome of tumour tissues, provides another promising elecrophoretic technique: variations that occur in additional DNA fragments in the restriction pattern between normal and tumour-derived tissues using such a polymorphic probe can now be cloned by polymerase chain reaction (PCR) techniques. But, in contrast to protein analysis of tumour cells, this approach fails to provide information about the gene product since DNA polymorphism can occur even within non-transcribed regions of the genome. However, the possibility of describing the specific malignant phenotype of tumour tissues or tumour-derived cell lines using a protein electrophoretic approach must be questioned. Even though thousands of papers that appeared during 1975–1985 try to describe the malignant phenotype of tumour cells, only some at least tumour-associated or proliferation-associated proteins were detected. This review comments on the limitations, advantages and problems associated with electrophoretic characterization of the protein patterns of malignant tissues.

3. BIOLOGICAL PREREQUISITES FOR THE MATERIAL

3.1. Tissues

In any electrophoretic separation system, the value of the results depends on the quality of the sample. Especially when bioptic tumour material has to be analysed, it must be remembered that most tumours consist of a heterogeneous cell population with respect to differentiation, proliferation and function [18,19]. Simple enzymic or mechanical disruption of tumour tissues produces a single-cell suspension representing the original material to an extent that is unknown to the

investigator. If highly specific markers exist for the cells that are to be analysed, one may perform a prefractionation step by using fluorescent-activated cell sorters (FACS) to enrich certain cell populations. To circumvent such laborious dissection steps of tumour biopsies, many researchers have performed 1-D or 2-D electrophoresis using small pieces of tumour biopsies (*e.g.* punch biopsies), which were frozen in liquid nitrogen immediately after surgery and subsequently solubilized in electrophoretic sample buffer. This simple strategy completely prevents any degradation that takes place during most of the time-consuming dissection steps. This approach has been used to obtain a lot of 2-D data from human tumour tissues during the past decade (see Section 6.2). But, even in the presence of 9 *M* urea (a concentration recommended in IEF buffer), without high concentrations of NaCl or KCl numerous proteins remain undissolved and/or bound to insoluble structures, such as membranes, cytoskeleton or nucleic acids. As an additional limitation, protein estimation remains difficult in the presence of a high concentration of urea, thus hindering an exact quantitative analysis. If only small amounts of tumour tissue are available without the possibility of culturing this material *in vitro,* such a direct preparation technique may be the method of choice. But tumour-specific antigens remain difficult to detect behind the bulk of "normal" housekeeping proteins.

3.2. Cell lines

To circumvent the problems that arise with bioptic material many laboratories favour electrophoretic analysis of cell cultures. One of the first cell lines to be subjected to an electrophoretic analysis was the HeLa line established from a cervical carcinoma in the 1940s. Using this particular cell line, Lenstra and Bloemendal [20] published a detailed 2-D study of cytoskeleton, mebranes, ribosomes, nuclei and soluble cytoplasm. Although such cell lines provide a bulk of obviously identical biological material, some precautions have to be taken when using permanent cell lines: owing to long-term cultivation, numerous sublines from the original cells exist, making any comparisons between different laboratories doubtful [21]. Furthermore, permanent cell lines fail to illustrate the biology of a normal cell.

In my opinion and to my knowledge, there is only one way out of this difficulty: if one is interested in characterizing "tumour-specific" or at least "tumour-associated" proteins, one must in the first place analyse several cell lines derived from this specific tumour type. It is recommended to use permanent cell lines since they are easy to handle and grow very fast, thus allowing numerous biological and biophysical prefractionation techniques to be used. On the other hand, it must be borne in mind that many cell lines are contaminated with mycoplasms, which can influence and change the cellular protein pattern [22,23]. If a particular elecrophoretic system has been selected to separate proteins from a given permanent (tumour) cell line, a protein map must be created from this cell type, either from whole cell extracts or from sub-cellular components.

In a second approach, primary cell cultures are established from these specific tumours and these cells have to be cultured over several passages. During this *in vitro* cultivation, the electrophoretic profiles or maps from these cell lines have to be monitored and compared with the results obtained from permanent cell lines. At the least, a single-cell suspension from bioptic material must be prepared using a sophisticated system. Even this material is subsequently subjected to an electrophoretic separation system. Additionally, it is recommended to observe the development of other tumour-specific parameters, such as the particular karyotype or clinical tumour markers to characterize tumour cell cultures. A comparison of profiles from these three different sources may yield information about those proteins that can be regarded as tumour-specific. However, to my knowledge no such comprehensive approach has yet been described in the literature.

4. PREFRACTIONATION TECHNIQUES

4.1. Dissection according to cellular functions

One of the difficulties that arise from electrophoretic analysis of whole cells or tissue extracts is to assign the results to particular biological structures. Consequently, prefractionation of cells according to natural compartments is used to reduce the number of protein spots (bands) and to obtain additional information about the function and/or localization of the separated proteins. One of the first cellular components subjected to high-resolution 2-D analysis were the ribosomes [24]. These particles are easy to separate and they bear only a comparatively small number of proteins. Later on, in the early development of 2-DE using IEF in the first dimension and SDS polyacrylamide gel electrophoresis (PAGE) in the second dimension, total soluble cellular extracts were often prepared (100 000 *g* supernatant). This fraction can be assigned to the cytoplasm, since under these conditions DNA-bound proteins and cytoskeleton structures remain insoluble [25,26]. Since the early 1970s, nuclear proteins have been the subject of intensive investigations in tumour biology [27], and numerous protocols were developed to prepare purified nuclei free of any (visible) cytoplasmic contamination [28–30]. Using salt extraction (0.4 *M* NaCl) of partially purified nuclei, nuclear proteins can be further fractionated from the remaining chromatin by increasing salt concentrations according to their function in the cell nucleus: tightly DNA-bound proteins, which participate in the chromatin structure, loosely DNA-associated proteins involved in gene regulation, and finally proteins attached to the nuclear matrix thus facilitating DNA replication [31]. In our own laboratories we have made use of this particular fractionation technique for several years. Figs. 1 and 2 show examples of how a 2-D protein pattern is made clearer if tumour cell nuclei are fractionated by increasing salt concentrations. As will be discussed in Section 4.2, chromatographic separation systems may be useful even in the prefractionation of nuclear proteins.

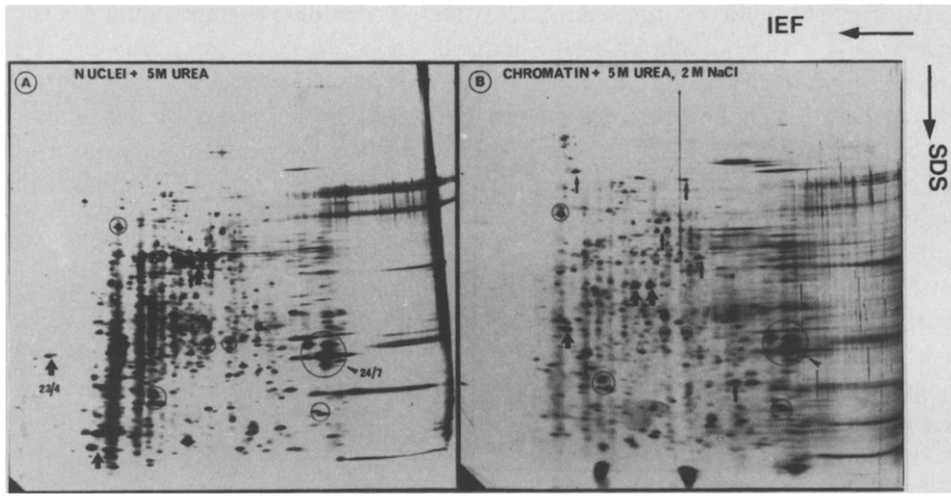

Fig. 1. 2-D PAGE of nuclear extracts from a human fibrosarcoma cell line (HT 1080) after (A) sequential extraction of nuclei with urea followed by (B) extraction of residual chromatin with urea/NaCl. Arrows indicate proteins enriched in one of the fractions. 23/4 and 24/7 indicate phosphoproteins that were characterized [55,111].

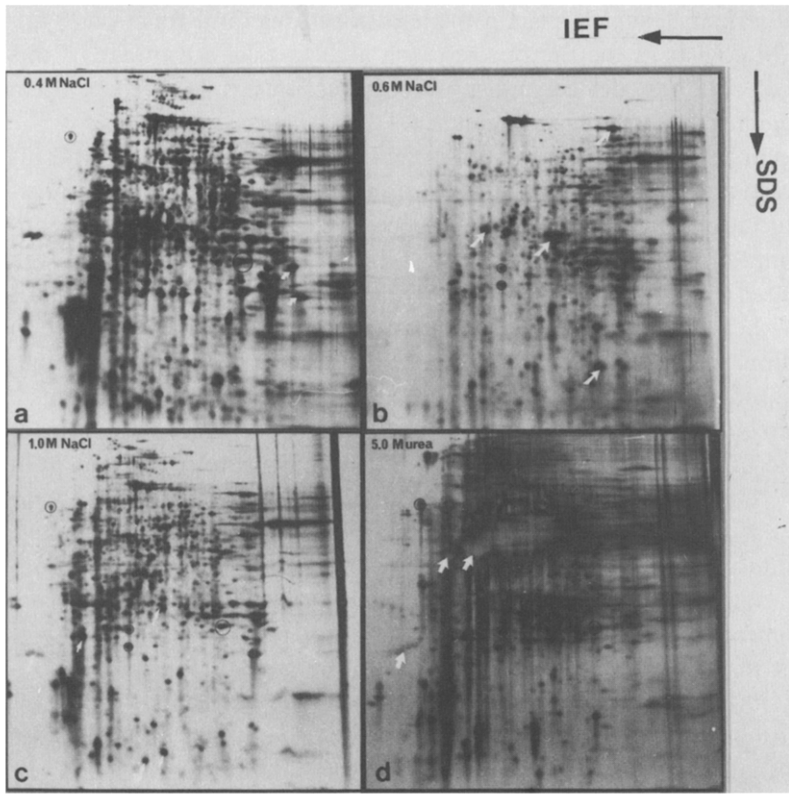

Fig. 2. 2-D PAGE of purified nuclei from a mammary carcinoma cell line (MCF-7). Purified nuclei were treated with increasing salt concentrations (a,b,c) and finally with 5.0 *M* urea (d) as indicated on the top. Using this sequential salt extraction, numerous proteins occur enriched in only one fraction (arrows).

Another subcellular component that attracted considerable interest during the past decade is the mitochondria. Like nuclei and ribosomes, these particles are easy to separate and they contain, apart from the nuclear coded proteins, a limited number of proteins coded by their own translation system. In particular, mitochondrial proteins isolated from tumour tissues attracted the attention of tumour researchers because this particular component seems to play a key role in tumour biology [32].

Thus fractionation according to subcellular components offers a simple and consequently reproducible method. However, contamination by other cellular proteins seems unavoidable, and prolonged purification protocols requiring large amounts of material are necessary. The necessity of enzymic markers to control the purity of the subcellular extracts must also be stressed (for a review of such methods see ref. 33).

4.2. Chromatographic separation of crude extracts

As a breakthrough in the history of chromatography, one may consider the introduction of high-pressure and high-performance liquid chromatography (HPLC). The development of new column supports, such as the FPLC system developed by Pharmacia, which sustain protein structures (and functions?) encouraged even biologists in the tumour research area to take advantage of this method. A lot of columns, devices and protocols became available for protein separation using chromatographic systems.

One of the pioneers in this area is undoubteldy M. T. W. Hearn: he and his co-workers investigatged carefully the separation behaviour of biomacromolecules on various chromatographic systems, especially on high-performance ion-exchange chromatography (HPIEC) columns [34,35]. With this method, even very closely related proteins can be separated and contaminating protein variants can successfully be removed. The authors showed much of their experience in protein separation and protein purification in a series of papers entitled "High-performance liquid chromatography of amino acids, peptides and proteins", published in this journal (*e.g.* refs. 36–39). In 1990, a comprehensive overview of the current state of the art in chromatography of biomacromolecules was published on the occasion of the *Ninth International Symposium on HPLC of Proteins, Peptides and Polynucleotides* in the same journal [40].

Recently, additional excellent papers and a review have been published concerning chromatographic methods in protein purification [41–48]. Aside from simple column chromatography using ion-exchange material, and molecular sieving with Sepharose, a combination of chromatography and biological fractionation has been developed. This is affinity chromatography, which takes advantage of the swiftness and reproducibility of chromatography but which provides additional information about the biological functions of the proteins as a part of the separation principle. A number of affinity materials have been developed to

separate phosphoproteins [49,50], DNA-binding proteins such as histones [51,52] and specific serum proteins [48]; CNBr-activated Sepharose, which can be coupled to a wide variety of molecules [53,54], has also been introduced. The advantage of affinity chromatography is that it utilizes a supposed function of protein(s) as the basis for the fractination process. Consequently this technique allows the separation of specific molecules present in low concentrations among the bulk of cellular proteins (see also refs. and 47 and 48).

A recent review referred to the necessity of such prefractionation steps to exhaust the resolving power of high-resolution 2-D gels [55]. Several published studies used DNA affinity chromatography of HeLa proteins [56] and reversed-phase chromatography of CHO cells [57]. In particular, Klose and co-workers [58,59] initiated a detailed study to generate a complete map of mouse proteins using different chromatographic fractionation systems and computer-aided evaluation of the 2-D gels. DNA affinity chromatography using short oligonucleotides coupled to CNBr-Sepharose and nuclear salt extracts has aroused considerable interest for the characterization of promotor-binding proteins [53]. Since we are interested in exploring the function of DNA-binding proteins in different human tumours, especially glioblastomas, we performed DNA affinity chromatography on placenta DNA coupled to CNBr-Sepharose to enrich those nuclear proteins that may recognize specific DNA sequences. Fig. 3 demonstrates the result of such a fractionation step: three protein groups present in the 5 *M* urea nuclear extract exhibit strong DNA-binding behaviour and consequently are easily separated from the bulk of non-binding proteins.

5. ONE-DIMENSIONAL SDS ELECTROPHORESIS

In 1971, Teng *et al.* [27] published the 1-D protein pattern of acidic, DNA-bound proteins to demonstrate the tissue specificity, phosphorylation and DNA-binding ability of this particular protein class. A year later, Stein and Borun [60] described an enhanced synthesis of chromosomal proteins preceding DNA synthesis in HeLa S-3 cells. Since high-resolution 2-D systems were not available at that time, these authors performed sequential salt extraction of purified nuclei to achieve an acceptable resolution in 1-D gels. In the past, numerous papers dealing with 1-D SDS electrophoresis of tumour cells or subcellular fractions were published. It is not surprising that this simple methodological approach failed to detect any tumour-specific or at least tumour-associated protein (*e.g.* refs. 61 and 62). Consequently, since the introduction of 2-D techniques the importance of the 1-DE method (with respect to the detection of tumour-associated proteins) has been confined to Western-blot analysis [63,64] (see Section 7.3), gel mobility shift assays (see Section 9) and to an analysis of chromatographic fractions. In some cases even phosphoproteins may be detectable with an acceptable resolution. Using *in vitro* phosphorylation and 1-D SDS-PAGE, Rayan *et al.* [65] described

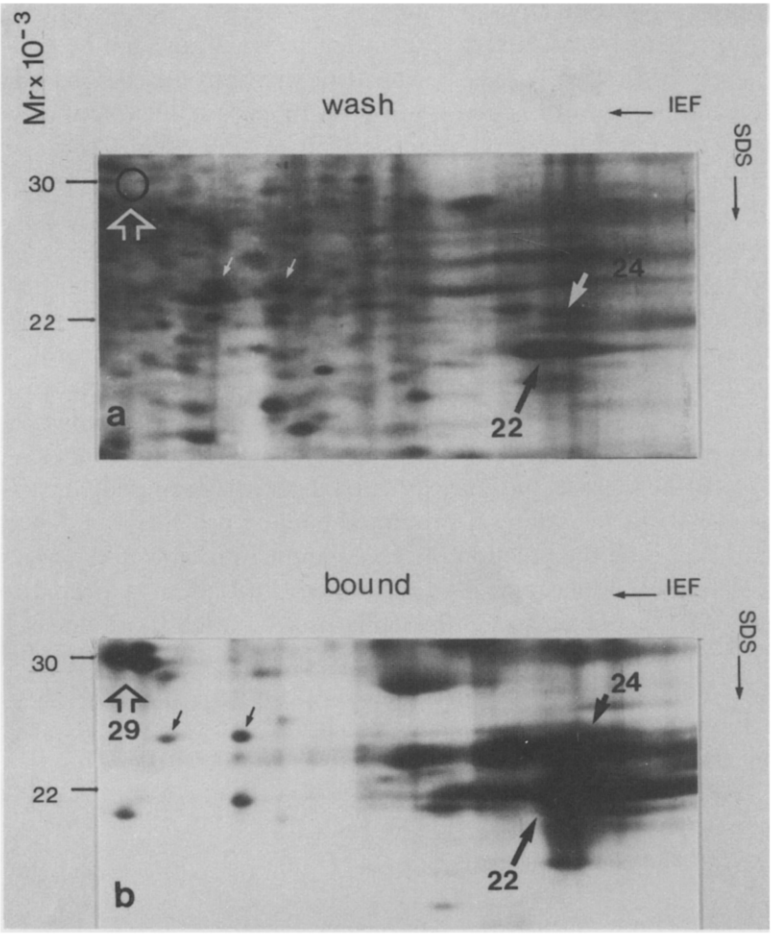

Fig. 3. (a) 2-D PAGE (close up) from HT 1080 nuclear extracts using urea. (b) The samples were applied to a DNA-Sepharose column containing human placenta DNA and eluted with increasing salt concentrations. Two protein groups (arrowed) in particular were retained on the column and eluted only at high NaCl concentrations.

an enhanced phosphorylation of chromatin-associated non-histone proteins in benign hyperplastic prostate. As will be discussed later 1-D electrophoresis is involved in most spot-processing techniques, but a characterization of malignant cells or tissues is not feasible using such a simple approach.

6. TWO-DIMENSIONAL ELECTROPHORESIS

6.1. Methodological improvements

Since the first description of 2-DE by O'Farrel [12] and Klose [13], numerous modifications of the original method have been described but only a few improve-

ments have been made. These include the development of Immobilines, which allow steady-state IEF [15,66]. Special arrangements and the use of giant gels have enabled the simultaneous run of multiple 2-D gels [67–69]. Some efforts have also been made to improve the separation of proteins in the first (usually IEF) dimension. Such an improvement was achieved by elimination of point streaking on silver-stained 2-D gels [70]. In order to enhance the capacity of 2-D gels, additives have been used that allow a high loading capacity (*ca.* 100 mg protein) [71,72]. However, the most striking progress in protein electrophoresis was achieved in separation technology rather than in detection systems and spot-processing methods, as will be described in detail in Section 7.

6.2. Two-dimensional electrophoresis of cancer cells

As mentioned in Section 2, high-resolution 2-DE can be regarded as a landmark in protein electrophoresis. Moreover, with the development of this technique scientists expected a complete and satisfactory description of the protein composition of a cell. They all had hopes of getting a description of tumour-specific proteins by a simple 2-D comparison of normal and malignant cells. We all participated in this euphoria during the early 1980s, at which time 2-DE came into use in laboratories all over the world as the primary tool in research applications, and a special issue of *Clinical Chemistry* dedicated to 2-DE was published in 1982. In this comprehensive issue some papers even dealt with the characterization of tumour tissues. Comings and co-workers [73–77] initiated a comprehensive study on human brain proteins, Thorsrud *et al.* [78] presented a comparison of mucosa, polyps and carcinomas of the large intestine, and Tracy *et al.* [79] investigated the patterns of normal colon mucosa and colon adenocarcinoma. The authors used bioptic material, frozen at –70°C and dissolved in IEF sample buffer containing 9 *M* urea. Comings and co-workers [73–77] presented a detailed protein map from human brain and polymorphisms, but the remaining papers described only similarities between protein patterns.

Some of the most excellent work in this area was carried out by Celis and Bravo [80] who used 2-DE to detect a proliferation-associated nuclear protein, which they termed cyclin. Together with their co-workers, they described in a series of brilliant experiments the cell cycle-dependent synthesis of this nuclear protein, as well as the dependence of cyclin concentration on the proliferation behaviour of cells [81–85]. The history of cyclin, now termed PCNA (proliferation cell nuclear antigen) remains a highlight among the myriad attempts to use 2-DE to characterize protein changes associated with cellular transformation. The discovery of PCNA (PCNA is known to be identical with the auxilliary protein for DNA-polymerase δ [86]) stresses the success of 2-DE techniques which led to the characterization of a cell cycle-related gene exhibiting a high structural conservation [87,88]. Recently, Waseem and Lane [88] detected some antigenetically distinct forms of PCNA, using monoclonal antibodies obtained

from expression-cloned PCNA, and promoter sequences involved in cell cycle-dependent PCNA expression have also been described [89].

During the past decade the success story of cyclin/PCNA has remained an isolated highlight. The history of 2-DE shows some conflict between continual references to its high resolving power and its apparent inability to detect tumour-specific proteins in malignant cells. Even such excellent 2-DE experiments on the brain and brain tumours published by the Jacobowitz's group remain superficial [90–92]. Though they carefully compared the electrophoretic patterns of punch biopsies from different astrocytomas, ependymomas and medulloblastomas, they failed to describe any tumour markers in detaill. One great disadvantage of their experimental approach is that they used a crude sample preparation: punch biopsies, frozen at −70°C were solubilized without additional disintegration in IEF buffer by sonification. This example reveals the dilemma that arises during high-resolution analysis of tumour tissues. If one intends to analyse a small, histologically well characterized tumour area, only such simple sample preparation techniques are available. On the other hand, additional prefractionation steps require certain amounts of identical (*i.e.* homogeneous) biological material, which can be obtained only from permanent cell lines because whole tumour pieces consist of a heterogeneous cell population. Additional problems occur with quantitative evaluation of silver-stained gels (see Section 7.1).

Two-dimensional investigations of malignant tissues have concentrated on only a few subjects: colon carcinomas, liver tumours and human leukaemic cells. The utility of 2-DE in cancer research with emphasis on leukaemia cell lines was recently reviewed by Hanash [93]. He and his co-workers initiated a program toward a standardization of 2-D gel studies, which is undoubtedly a prerequisite for any convenient use of 2-DE [94]. Most of the 2-D studies on malignant tissues were restricted to a preliminary description of differences between normal and neoplastic tissues rather than a use of this particular method as a starting point toward a precise analysis of individual spots [95–101]. Even in the literature spanning the past two years only such rather preliminary results were reported: 2-DE was used to characterize oesophageal carcinoma-associated proteins [102] and nuclear proteins in brain tumour cells [103], and in a comparison of duodenal and pancreatic cancer [104]. A more detailed study on specific differences between normal and malignant breast tissue was recently published by Wirth [105]. These authors used samples that had been fractionated in cytosolic and mitochondrial protein extract prior to electrophoretic separation. Their results describe six additional polypeptides present in the malignant material. Approximately twenty years ago, Kadohama and Turkington [106] reported on altered populations of acidic chromatin proteins in breast cancer cells by using 1-D SDS phosphate PAGE. However, one may suggest that high-resolution analysis of proteins derived from various tumour samples has come to a dead end. The description of additional or missing spots in a given tissue or cell line suffers from a lack of experiments that can reveal the function of these particular proteins. Unlike mo-

lecular genetics, which can make use of biological tools such as restriction enzymes and bacterial cloning of DNA fragments to characterize a gene, or at least a DNA sequence, protein chemistry lacks those elegant "natural" techniques. Further methodological advances in related areas are necessary to lead high-resolution analysis of tumour proteins from the glimmer of spot description to the glitter of gene analysis. In the following section, I shall discuss some of the most prominent detection systems, which limit and complete 2-DE. Of course, the use of these techniques is not restricted to an analysis of malignant tissues, but the inversion of the molecular dogma facilitates fascinating views even in this research field.

7. DETECTION SYSTEMS

A typical mammalian cell produces some 10 000 different gene products in ratios up to $1{:}10^6$, some of them probably at the rate of only a few copies per genome. This means that successful detection of true tumour-specific proteins is heavily dependent on the detection system. These devices can be divided into direct detection systems, such as staining procedures, and indirect invasive detection systems, which require spot (band) processing.

7.1. Staining methods

Even in a review article like this it is impossible to list all the staining protocols that have been published during the past decade. Readers with specific interest in this area are referred to some excellent reviews published in the journal *Electrophoresis*. However it is worthy of note that most silver staining systems are now easy to handle and produce reproducible results. In our own laboratory we modified the technique described by Blum *et al.* [55,107], and this procedure provides satisfactory gels with a sensitivity of a few nanograms of protein per spot. Since we are interested in a characterization of glioblastoma-specific DNA-bound proteins, rather than in computer-aided gel evaluation and spot quantitation, we shall the matter rest. The advantage of this particular staining protocol can be summarized as follows: (i) short staining time (20 min without fixation steps and preincubation); (ii) stability of the coloured spots over long periods (more than three years at 4°C); (iii) comparatively low background; and (iv) complete staining even in the acidic region of the gel.

7.2. Radioactive labelling

The search for tumour-associated proteins particularly concentrates on those factors that are synthesized during the time preceding the S-phase of the cell cycle. To facilitate the documentation of these newly synthesized proteins, metabolic labelling of cell cultures is recommended using [^{35}S]methionine and

[^{14}C]amino acids [108,109]. This approach is restricted to living cell cultures, because diffusion of radioactive amino acids in tissues is too slow to obtain a representative labelling. The use of post-transcriptional ^{125}I-labelling procedures remains restricted to a limited number of laboratories, and even the resolution of such labelled mixtures seems to be rather poor.

Since the discovery that cellular proliferation is associated with enhanced kinase activity, leading to an increase in protein phosphorylation, this particular assay has attracted considerable interest, especially in cancer research. In particular, *in vitro* phosphorylation assays are performed because these experiments require only small amounts of radioactivity compared with the labelling of whole cell cultures. Fig. 4 demonstrates the 2-D phosphoprotein pattern from mitochondria: normal human mucosa was compared with preparations from colon carcinomas, and isolated mitochondria were subjected to *in vitro* phosphorylation without exogeneous kinase. Whereas the proteins of normal and tumour tissues look quite similar, marked differences occur in the phosphoproteins. Consequently, the number of papers dealing with *in vivo* and *in vitro* labelling with ^{32}P, and high-resolution separation of phosphorylated proteins, increased dramatically during the 1980s, and 1-D and 2-D studies of numerous cancer-related phosphoproteins were published [110,111]. The discovery that even non-tumour-related proteins become modified at the tyrosine residues has reduced the value of this approach, but high-resolution analysis of phosphoproteins, together with an analysis of phosphoamino acids, may be still an important methodology in tumour biology [111].

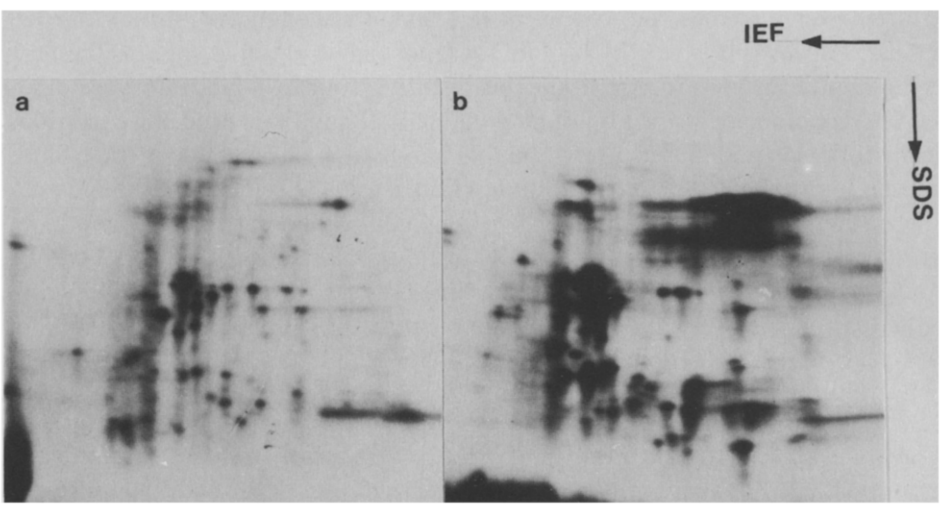

Fig. 4. 2-D PAGE and autoradiography after *in vitro* phosphorylation of mitochondrial proteins from (a) normal human mucosa and (b) colon carcinoma from the same patient. Whereas the protein compositions of the two samples are indistinguishable by silver staining, the increased incorporation of ^{32}P into the colon carcinoma proteins is obvious from these patterns. (C. Welter and G. Unteregger, in preparation.)

7.3. Electroblotting

Even high-resolution electrophoresis of malignant tissue seems to be an unsatisfactory tool if the nature of the interesting polypeptides remains unknown. Immunostaining, using mono/polyclonal antibodies prepared by the injection of crude cellular extracts (chromatin, nucleoli) into animals, or the use of serum from patients carrying autoantibodies, can help to elucidate the specificity and function of proteins separated by 2-DE. Using such an approach, Chiu *et al.* [112] detected colon adenocarcinoma antigens in rat chromosomal proteins after 1-D SDS-PAGE. Studies by Freeman *et al.* [113] revealed a proliferation-associated nucleolar antigen present in a wide variety of tumours. These authors used a HeLa nucleolar preparation, which was blocked by normal anti-nucleolar serum prior to immunization. As well as its role in the immunological detection of proteins transferred to nitrocellulose, electroblotting has proved to be an essential tool in the study of DNA–protein interactions. Chromosomal proteins extracted from several tumours or tumour cell lines are separated using conventional 2-D PAGE and transferred to nitrocellulose. Sequence-specific DNA-binding proteins (*e.g.* with affinity for oncogene–promoter sequences) can be detected by incubating the nitrocellulose sheet with radioactively or digoxygenine-labelled DNA in the presence of competitor DNA [114–116]. As is well known from studies on the transcriptional regulation of the oncogene c-fos, eukaryotic transcription factors often contain amino acid sequences favouring complex formation with zinc [117]. Assuming that zinc-binding proteins represent such transcription factors, their occurrence, especially in nuclear extracts from tumour cells, may be followed by incubating the proteins transferred onto nitrocellulose with ^{65}Zn [118].

7.4. Spot processing

7.4.1. Protease digestion

High-resolution electrophoresis is not only an excellent analytical tool in protein chemistry; owing to progress in related field, electrophoretic separation may also be successfully used as a quantitative system. Especially in tumour research, one question is often asked: How similar are spots/bands that exhibit almost identical locations within the gel when tumour and normal tissues are compared? There are several possible approaches to this problem. One of the most elegant techniques was recently published by Zivy and Granier [119], who employed protease digestion of proteins separated by 2-D PAGE. Proteins were cleaved by proteases (ficin) by shaking the gel in the appropriate solutions. After this digestion step, the gels were returned to the apparatus and electrophoresis was continued in the opposite direction. Using this procedure, digested proteins form several bands and the profiles help to check proteins for their identity on different gels. A similar approach is that spots/bands extracted from a gel are subjected to

protease digestion and the polypeptides obtained are separated by second 1-D SDS-PAGE. The latter approach was used for the development of a database of amino sequences for human colon carcinoma proteins [120].

7.4.2. Protein microsequencing

One of the most exciting developments has undoubtedly been the possibility of microsequencing of proteins separated by 1-D or 2-D electrophoresis and transferred to appropriate supports, such as activated glass fibres and polyvinylidene difluoride (PVDF) membranes by electroblotting [120–123]. Like protease digestion of single spots, this technique makes use of the resolving power of 2-DE as a preparative technique to obtain pure proteins, even at low concentrations. The ability to obtain a partial amino acid sequence from as little as a few picomoles of protein after 2-DE opened up a new area in molecular biology. Moreover, this method efficiently combines several advantages. First, it is technically straightforward and inexpensive; second, it is sensitive (when combined with gas-phase sequencing) and generates amino acid sequence information from starting amounts of protein of as little as 1–10 μg (corresponding to a spot clearly visible with Coomassie Blue staining). Coupled with *in situ* protease digestion, this technique provides additional biochemical data from the separated protein. Moreover, since these data (amino sequences, peptide pattern) do not depend on individual techniques that may differ between laboratories (like 2-DE), any data exchange is now easy to carry out. Coupling of high-resolution electrophoresis with databases and microsequencing was successfully initiated some years ago by Eckerskorn *et al.* [124]. Since then this highly sophisticated technique has become a standard tool in many laboratories, and even some protein data have been published with the emphasis on tumour-specific proteins. This was recently done by Ward *et al.* [120] for a human colon carcinoma cell line. Although this paper fails to present biochemical data from a colon carcinoma-specific protein, the strategy used emphasizes how to proceed. As a consequence of those developments, 2-DE analysis of tissues or cell lines should not be used as the sole method of investigation. Electrophoretic techniques intitially developed as fast and easy protein separation systems should now be coupled with highly sophisticated and expensive techniques such as microsequencing. Furthermore, sequencing of proteins in the low picomole range demands not only specific equipment but also experienced researchers capable of interpreting the results. Moreover, as discussed in the next section, databases have to be created to describe the protein composition of a given cell or tissue type.

7.4.3. Antibody production

Although the above-mentioned approach provides biochemical data on proteins separated onto 2-D gels, researchers are often interested in following protein in malignant tissues and in cell cultures using immunological techniques. Amino sequence data can be used to produce synthetic peptides that can be used to

immunize animals for antibody production, but this approach is laborious and time-consuming, as well as requiring specific technical equipment. Additionally, proteins may be blocked at the amino terminus, thus hindering microsequencing. Monospecific antibodies against proteins of interest detected within a 2-D gel may be obtained by direct injection of nitrocellulose pieces and/or homogenized spots containing a few micrograms of protein after conventional Western blotting. This technique was successfully used by various authors [125,126]. In order to avoid any contamination, the appropriate protein has to be clearly separated from any others. If additional chromatographic steps are not available (loss of material) some improvements can be achieved by simply changing the pH gradient of the IEF gel. Fig. 5 shows how the separation of a particular spot (55/5.9, M_r/pI) is improved by a shift in the pH gradient.

8. DATABASE ISSUES

Since the advent of high-resolution 2-DE, one problem has been how to manage all the data that are obtained from a 2-D pattern. Whereas the evaluation of 1-D gels is relatively simple, the recording of 2-D gels demands more sophisticated equipment. In the early 1980s, several research groups started to develop

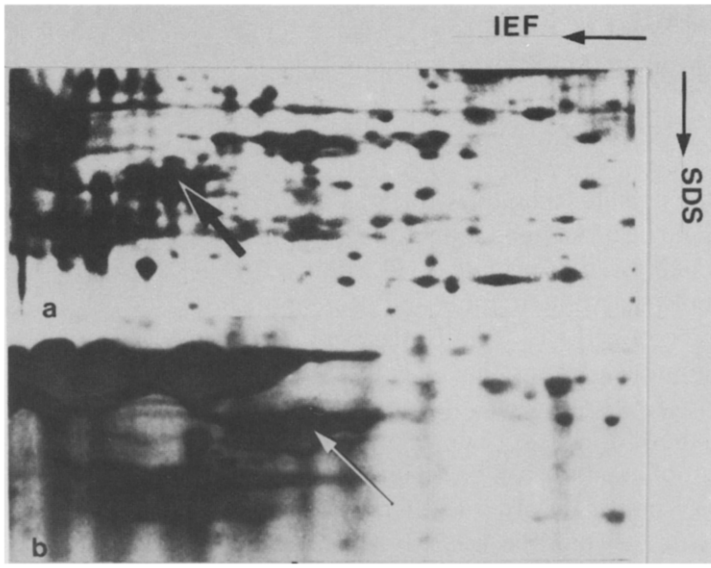

Fig. 5. 2-D PAGE (close up) with silver staining. DNA-bound proteins from a human glioblastoma cell line (U 373) were separated on either (a) standard or (b) modified carrier ampholyte gradients in the first direction. IEF-gels in (a) contain a standard mixture [55] that allows only a poor separation in the pH range 5–6. To enhance the separation from a specific protein (arrowed) ampholytes 5–7 (Serva) were used instead of ampholytes 3–10. Note the marked shift in the protein pattern toward the cathodic side of the gel.

computer programs that were able to recognize and to store 2-D patterns. Owing to differences in the protein patterns even from two identical samples in the same laboratory, these programs have become very complex. Differences result from variations in temperature, ampholyte composition (*i.e.* type or charge), handling of the IEF gel during the mounting step onto the second-dimension gels, staining procedures and, of course, the protein composition itself. Whereas most of these parameters responsible for pattern variations can be standardized, some remain unpredictable. Initially developed as a tool to identify peaks, to calculate their optical density (*i.e.* spot quantitation) and to list these proteins according to their molecular masses and isoelectric points, databases now contain information concerning the various properties of all the proteins of a particular cell type (physical, chemical, biochemical, physiolgoical, genetic, immunological, etc.). Together with microsequencing, those comprehensive databases provide a link between proteins and genes. Most laboratories have established databases from transformed rat-cell lines [127–129], from mouse cells including different organs [130], from human amnion cells [131], from human embryonic lung fibroblasts [132], and from secreted proteins from lung fibroblasts [133]. Such databases have even been created from benign hyperproliferative diseases, such as psoriasis [134]. The strategy developed by Klose [130] also encompasses the location of proteins, *i.e.* they recommend 2-DE on subcellular components. At this time, however, only the REF 52 database established by Garrels and Franza [127–129] and the colon carcinoma database of Ward *et al.* [120] are designed for the determination of tumour —or at least proliferation/transformation related— proteins.

9. FUTURE TRENDS

The development of satellite techniques, such as databases, protein microsequencing and immunological methods, offers the possibility of identifying the molecular phenotype of a given protein detected within a 2-D gel. Furthermore, using conventional molecular genetic tools and some more sophisticated ones, such as PCR, it may be possible to identify the corresponding gene in cDNA libraries [135]. This approach means that scientists have now started to work backwards from the protein to the gene, in contrast to the practice of genetics. However, only a few laboratories as yet can make use of these satellite techniques. Even the establishment of database can be achieved only for permanent cell lines rather than for bioptic samples. In order to characterize tumour-associated proteins in malignant tissue, it would be necessary to fractionate the cells, *i.e.* to reduce the number of spots on a gel, to establish short-term primary cell cultures, and to identify the tumour cell type with appropriate markers like cytogenetics. The protein content of these primary cell cultures has to be compared with the pattern obtained from direct tissues preparations. Proteins of interest have to be compared for their homology by protease digestion, by comigration, and of course finally by microsequencing. Chromatographic separation systems may be

introduced during all these steps to enrich the particular proteins on the gel. Protein spots that cannot be further analysed by analytical techniques may be scraped off or eluted from the gel, and used directly for immunization procedures.

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