Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cancer Letters 205 (2004) 121–131
CANCER Letters
www.elsevier.com/locate/canlet

Two-dimensional electrophoretic protein profile analysis following exposure of human uroepithelial cells to occupational bladder carcinogens

Mary Helen Kanitz[a,*], Santhanam Swaminathan[b], Russell E. Savage Jr.[a]

[a]*Division of Applied Research and Technology, Taft Laboratory, National Institute for Occupational Safety and Health, MS/C-23, 4676 Columbia Parkway, Cincinnati, OH 45226, USA*
[b]*Department of Pharmacology, University of Wisconsin Medical School, Madison, WI 53706, USA*

Received 15 January 2003; received in revised form 29 September 2003; accepted 30 September 2003

Abstract

Protein biomarkers to occupational carcinogens were investigated using a transformable human uroepithelial cell system, SV-HUC.PC. SV-HUC.PC was treated with *N*-hydroxy-4,4′-methylene bis (2-chloroaniline) (N-OH-MOCA) or *N*-hydroxy-4 aminobiphenyl (N-OH-ABP). Two-dimensional gel electrophoresis of cell lysates compared protein changes across treatments. Increasing N-OH-MOCA resulted in a dose-related increase in protein spots altered. Comparing cell profiles treated with either carcinogen revealed alterations in the expression of nine proteins, identified using the TagIdent database. These demonstrated isoelectric point shift (1) or quantity change (8). Our investigation may be useful in identifying biomarkers of effects of exposure to bladder carcinogens.

Keywords: Uroepithelial cells; Bladder carcinogen; Two-dimensional gel electrophoresis; Protein biomarkers

1. Introduction

Human urothelial tract tumors constitute a serious cancer problem in the United States with an incidence of over 50,000 new cases each year. Bladder cancer resulted in approximately 11,000 deaths in 1995 making it the fifth most common cancer among American men [1]. Bladder cancer development is generally multistage [2]. The prognosis is problematic since although as many as 10% of patients with indolent disease eventually develop malignant disease [3], there are no absolute clinical markers to predict disease progression [4]. For patients with metastatic disease cytotoxic chemotherapy is the only available option, and the chances of long-term survival are minimal. A better understanding of the mechanism of tumor initiation and progression remains an important research issue surrounding bladder cancer.

A higher incidence of bladder cancer in males compared to females (3:1) has been attributed in part to increased workplace exposure to unknown bladder carcinogens [5]. Specific epidemiological trends, e.g. higher incidence in urban versus rural areas, suggest

* Corresponding author. Tel.: +1-513-533-8488; fax: +1-513-533-8510.
E-mail address: mhk2@cdc.gov (M.H. Kanitz).

0304-3835/$ - see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2003.09.032

a role for industrial substances in the development of bladder cancer [6]. However, currently no method exists to determine if a worker has been exposed to an occupational carcinogen at a level which would compromise the integrity of the urothelial tract.

An occupationally relevant human model is necessary to determine putative carcinogenic risk for chemically exposed workers. We had previously employed an in vivo/in vitro transformation model to study chemical carcinogenicity in human uroepithelial cells (HUC) [7,8]. HUC cells were immortalized by SV40 virus to form a non-tumorigenic, near diploid clonal line of uroepithelial cells (SV-HUC) [9]. Bookland et al. [7] demonstrated that a variant cell line of SV-HUC, SV-HUC.PC, was reproducibly sensitive to transformation by bladder carcinogens. SV-HUC.PC cells were transformed in vitro following exposure, and transformation was confirmed in vivo by inoculating the cells into athymic nude mice and checking the mice for tumors. Although no palpable tumors appeared 24 weeks after inoculation of SV-HUC.PC treated with lower concentrations of *N*-hydroxy-4,4′-methylene bis (2-chloroaniline) (N-OH-MOCA; 2.5 or 5.0 μM), tumors were observed with either N-OH-ABP (5.0 μM) or the highest dose of N-OH-MOCA (10.0 μM) (one or two tumors, respectively, six sites tested) [7,10].

In this investigation we employed the SV-HUC.PC model to identify potential biomarkers for effects of exposure to known human bladder carcinogens. Perera [11] defined such a biomarker as a pre-clinical response or biochemical change in target cells associated with the occurrence of cancer. Quantitative alterations in cellular gene and protein expression are likely responsible for most differences between malignant and normal phenotypes [12]. We therefore employed two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the preferred method for separation and analysis of complex protein mixtures, to discover biomarkers for effect of exposure.

We chose two human bladder carcinogens, N-OH-ABP and N-OH-MOCA, to investigate intracellular alterations in SV-HUC.PC by 2D-PAGE following in vitro exposure. A previous epidemiological study showed that exposure to arylamine carcinogens resulted in increased risk of bladder cancer [13]. The arylamine 4-aminobiphenyl (4-ABP) is the most potent experimental bladder carcinogen known and is an ubiquitous chemical present in cigarette smoke [14] and synthetic fuels [15]. Exposed workers demonstrated an increased incidence of bladder cancer directly related to the intensity and duration of exposure [16]. Human bladder uroepithelium has shown 4-ABP–DNA adducts in vitro and in vivo [17–20]. Further, the uroepithelium has been identified as the prime target tissue type for arylamine carcinogenesis [21].

The International Agency for Research on Cancer (IARC) classified 4,4′-methylene bis(2-chloroaniline) (MOCA) as a probable human carcinogen in 1987 [22]. Due to its occupational relevance [23], the National Institute for Occupational Safety and Health (NIOSH) has accrued an abundance of data on this chemical. Here we employed N-OH-MOCA, the carcinogenic derivative of MOCA [24]. The procarcinogen is hepatically metabolized to the *N*-hydroxy derivative and presumably transported to the bladder to serve as the proximate carcinogen [25]. MOCA–DNA adducts in human uroepithelial cells and rat liver have been shown to have similar chromatographic profiles [26]. Hence this chemical was used in the in vivo/in vitro transformation study [27,28] and in the current investigation.

Protein changes that may occur in SV-HUC.PC cells following chemical treatment were detected by 2D-PAGE. The gels were analysed by PDQUEST, a software which automatically detected specific spots, matched the protein patterns of multiple gels and compared them for significant, quantitative differences. Using the SV-HUC.PC in vitro/in vivo transformation system and 2D-PAGE, we identified several putative biomarkers of effect of exposure to bladder carcinogens. The protein changes observed following treatment with the chemicals might be used as biomarkers to identify individuals at high risk of bladder cancer development due to occupational exposure.

2. Methods

2.1. Cell line and maintenance

The SV-HUC.PC cell line is isogeneic and derived from a clonal, near diploid line of SV-40 immortalized HUC [9,29]. Cytogenetic analysis (passage 23)

showed it retains a near diploid karyotype [30]. Cells were maintained in humidified incubators at 37 °C in 5% CO_2 and were grown in 1% FBS–F-12 +, i.e. 1% FBS (fetal bovine serum; Sigma Chemical Co., St Louis, MO) is added to a supplemented Ham's F-12 medium (Gibco, Grand Island, NY) developed specifically for the cells [7].

2.2. Chemical treatment

At approximately 90% confluency SV-HUC.PC were deprived of growth factors by changing to Hams F-12 medium devoid of serum. Cells were changed after 48 h into fresh serum-free medium containing either 0.5% dimethyl sulfoxide (DMSO; Sigma), 5.0 μM N-OH-ABP (Sigma), or varying concentrations of N-OH-MOCA (2.5, 5.0, and 10.0 μM). MOCA was obtained from Anderson Development Co., Adrian, MI, and N-OH-MOCA was synthesized at NIOSH by a modified method [26,31]. After 24 h incubation, the cells were harvested by trypsinization and stored at −80 °C.

2.3. 2D-PAGE

Analysis of soluble proteins in SV-HUC.PC was conducted as a modification of the method of Tollaksen et al. [32]. Treated SV-HUC cells were concentrated by centrifugation (30 min at 12,000 × *g*). The pellets were solubilized in buffer (pH 9.5) containing 2% NP-40 (Sigma), 9 M urea (BioRad, Hercules, CA), 0.5% dithiothreitol (DTT; Sigma) and 2% carrier ampholytes (pH 9 −11; Pharmacia, LKB, Piscataway, NJ). Solubilized proteins were resolved using the 20 × 25 cm^2 ISO-DALT™ 2D gel system (Hoefer Scientific Instruments, San Francisco, CA). Solubilized protein samples (20 μg) were applied to isoelectric focusing (IEF) gel tubes (25 cm × 1.5 mm; 4 gels/treatment group) containing 4% acrylamide (Crescent Chemical Co., Inc., Hauppauge, NY), 2% NP-40 (Sigma), and 2% ampholyte (2:1 ratio: pH 3–10, Pharmacia and pH 5–8, Sigma), pre-focused for 2000 Vh, and focused for 30,000 Vh at room temperature. Each first dimension IEF tube gel was loaded directly onto a second-dimension DALT slab gel (20 × 25 cm^2) held in place with polyester fabric wedges (Large Scale Biology Corp., Rockville, MD). Gradient slab gels were poured reproducibly using the Angelique™ computer-controlled gradient maker (Large Scale Biology Corp.) which enabled one to reduce run-to-run variability in the polyacrylamide gel concentration. The gels contained linear acrylamide gradients in which the top 5% of the gel was 9% acrylamide, and the lower 95% of the gel varied from 9 to 18%. Second-dimension slab gels were run in groups of 20 in a DALT slab electrophoresis tank (Hoeffer Scientific) at 10 °C for 18 h at 160 V. Molecular weight standards (Bio Rad) were co-migrated on the gel margin, while a carbamylated creatine kinase (Sigma) charge-train was used for internal charge standards. Following SDS (sodium duodecyl sulfate) electrophoresis slab gels were stained for protein using a silver staining procedure [fixed 1 h in methanol/acetic acid/water (40:10:50), 12 h in methanol/acetic acid/water (5:5:90), incubated 1 h in glutaraldehyde (5%), stained 1 h in alkaline ($NaOH/NH_4OH$) silver nitrate (8 g/l) solution, developed in 0.05 g citric acid/0.5 ml formaldehyde/1 l water].

2.4. Gel pattern analysis

Stained gels were digitised in green light at 169 μm resolution and scanned on a PDI 420 scanner (Protein DNA Imaging, Inc.). Images were processed using the PDQUEST software system (Bio Rad, Hercules, CA) to generate a parameter list giving *x*, *y* position, size and density of each detected spot. This procedure made use of digital filtering, mathematical morphology techniques and digital masking to remove background. It used full two-dimensional least-squares optimization to refine the parameters of a 2-D Gaussian shape for each spot.

2D-PAGE patterns corresponding to each treatment were compared. The "master" pattern was used as a landmark dataset, a computer composite of protein patterns from all the treatment groups. It served as a baseline to which group patterns were compared to each other based on assigned spot numbers. During initial analysis a subgroup of specific protein spots was landmarked on each gel. Subsequently the software used an automatic program to match additional spots to the master pattern using the manual landmark data as a basis. After the automatic matching, the results were analysed for

quantitative and qualitative differences. Spots were judged to be quantitatively different if they met two criteria: (i) the mean intensities differed by at least a factor of 2; and (ii) the intensity distributions indicated that the difference was statistically significant (as determined using group-wise statistical parameters, e.g. two-tailed Student's *t*-test). These two criteria ensured a low number of false positives. Proteins satisfying these criteria were represented as highlighted spots on computer-plotted protein maps and stored as spot populations. To eliminate quantitative differences due to gel loading or staining, groups of gels were normalized or scaled together by a linear procedure based on specific spots. A coordinated decrease or increase in the magnitude of several hundred proteins compared to the master would likely be caused by a difference in total protein applied to the gel. The computer multiplied the spot abundances for each protein pattern by a scaling factor to account for loading differences. This allowed for valid comparisons of protein expression among groups.

Tentative identification of proteins was made using the TagIdent Protein Database, an electronic resource located at the following web site: http://www.us.expasy.org/tools/tagident.html. Specific biochemical data for each spot (relative molecular weight and isoelectric point) was entered, and candidate protein matches were identified based on these characteristics.

3. Results

Two-dimensional gels from treated SV-HUC.PC were studied using the PDQUEST system to automatically match multiple gel patterns. Fig. 1 depicts a representative gel illustrating the range of actual solubilized protein spots under investigation. A master profile (Fig. 2) illustrates the composite of all resolved protein spots and serves as a template to match single gel patterns between treatment groups. The average number of solubilized human uroepithelial proteins which could be reproducibly resolved and matched was 750. After individual gels were matched to the master, quantitative data for each matched spot was examined. Differences were studied after determining whether good gel–gel reproducibility occurred within a treatment group comprised of four gels. The number of matched spots with a coefficient of variation (CV)

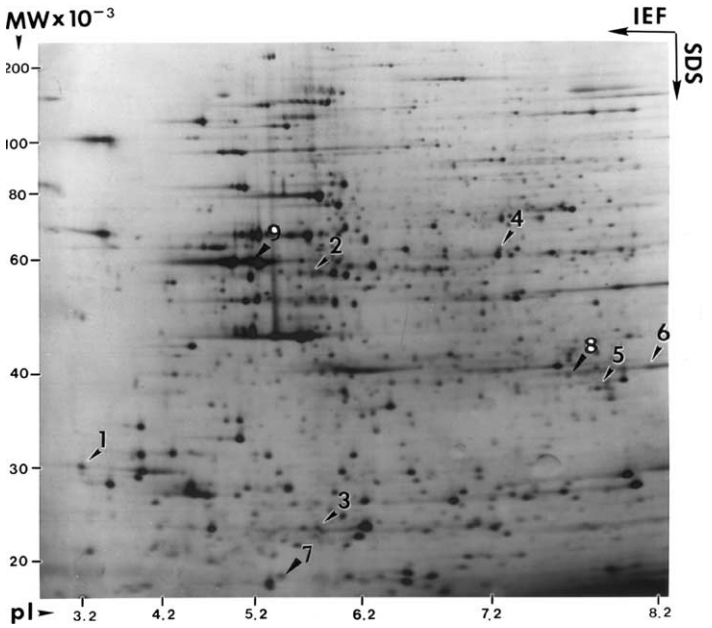

Fig. 1. Representative 2D-PAGE gel of DMSO-treated SV-HUC. The gel represents a vertical acrylamide gradient of 9–18% and a horizontal pH gradient of 3–8.2. Molecular weight and pI calibrations are based on co-migration with known standards. The spots exhibiting density alteration following exposure to N-OH-ABP (5.0 μM) or N-OH-MOCA (10.0 μM) are indicated by number.

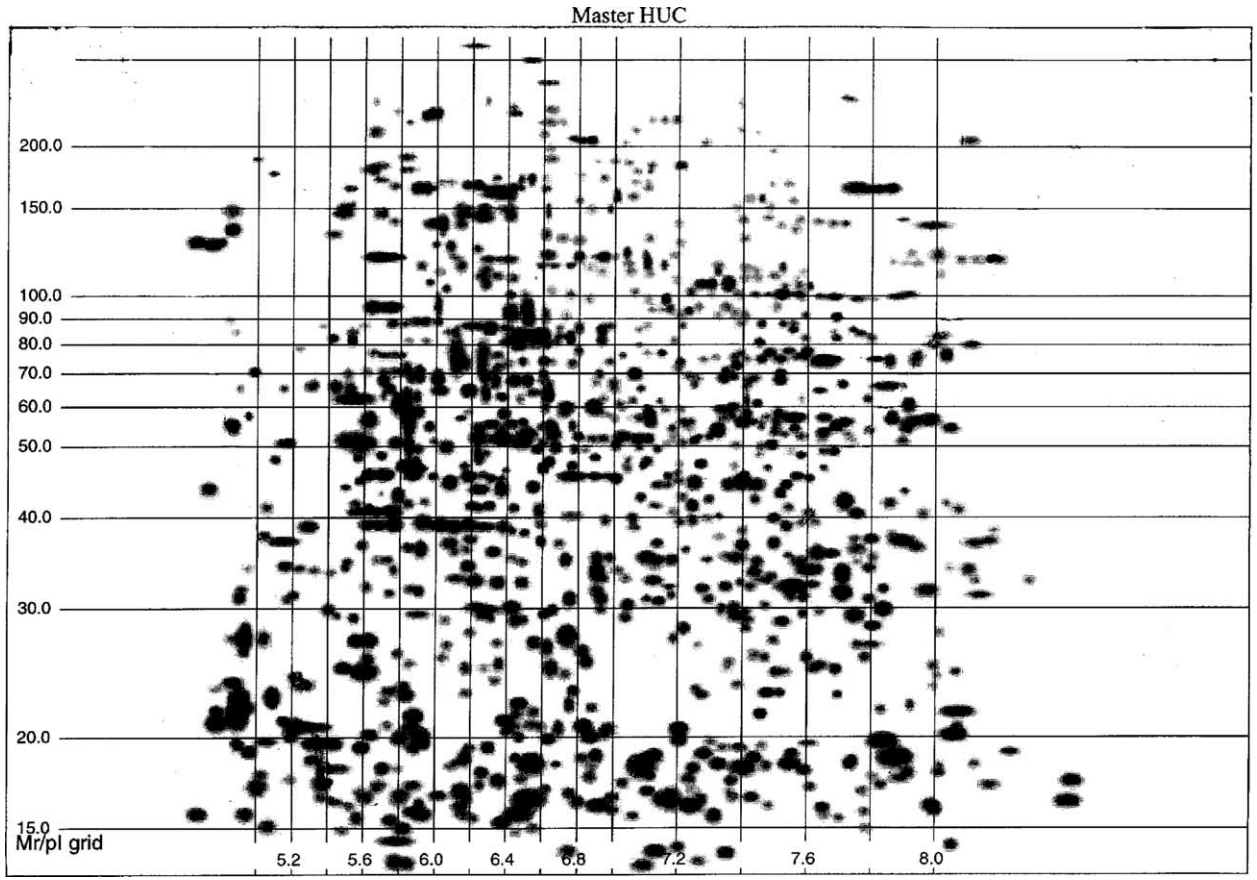

Fig. 2. Master SV-HUC.PC protein profile with Mr/pI (relative molecular weight/isoelectric point) grid. Grid demonstrates acidic and basic proteins oriented to the left and right, and low and high Mr proteins oriented to the bottom and top, respectively. A composite of all resolved spots and a template for matching individual gel patterns.

Table 1
SV-HUC.PC proteins altered following in vitro exposure to increasing N-OH-MOCA

N-OH-MOCA (μM)	Number of matched protein spots[a]	Percent matched protein spots (%)[b]
2.5	7	1
5.0	21	3
10.0	49	7

[a] Proteins altered in SV-HUC.PC following 24 h exposure to N-OH-MOCA ($P < 0.05$; Student's t-test).
[b] (Number of matched protein spots altered/total matched spots) × 100.

under 15% was 120, an indication of protein pattern consistency.

We investigated protein changes in SV-HUC.PC following 24 h exposure to varying levels of N-OH-MOCA. Suitable concentrations had been previously determined by cytoxicity analysis. Cytoxicity was dose-dependent, and 2.5, 5.0 and 10.0 μM N-OH-MOCA were selected for 10, 25 and 90% cell kill, respectively [10]. A statistical analysis of protein changes showed that significant alterations were noted at all concentrations of N-OH-MOCA. The number of proteins exhibiting a change increased with concentration at the $P < 0.05$ level of significance (Table 1).

We studied alterations in the matched protein spots by 2D-PAGE analysis of bladder carcinogen-treated SV-HUC.PC. Protein changes following in vitro exposure to the human bladder carcinogens, N-OH-MOCA and N-OH-ABP may be putative biomarkers for effects of exposure. The two-dimensional gel patterns of SV-HUC.PC treated with the highest concentration of N-OH- MOCA (10.0 μM) or N-OH-ABP (5.0 μM) was compared to that of DMSO-treated controls (0.5%). Following 24-h exposure to either carcinogen nine of the same spots were altered significantly relative to control ($P < 0.05$ Student's t-test). Fig. 1 shows the coordinate position, relative molecular weight (Mr) versus isoelectric point (pI), of these proteins. Following treatment with N-OH-MOCA or N-OH-ABP eight spot densities displayed a mean change of at least two-fold (Table 2). Most alterations resulted from increased abundance as shown by the densities of five spots from the carcinogen-treated cells. Protein nos. 1, 3, 4, 5, and 6 (Fig. 1) were expressed at significantly higher levels compared to control ($P < 0.05$; Student's t-test). Fig. 3 exemplifies the relative change observed across treatment groups for spot no. 1. In contrast, three protein spots, nos. 2, 7 and 8 (Fig. 1), were expressed at significantly lower level after N-OH-MOCA and N-OH-ABP treatment relative to vehicle control ($P < 0.05$; Student's t-test). Relative quantitative differences of each altered protein within individual gels across the treatment groups are shown by bar graphs in Fig. 4 ($n = 4$ chemical treatments/group).

A shift in pI of one protein spot, no. 9, was observed following N-OH-MOCA or N-OH-ABP treatment relative to control (Figs. 1 and 5). Fig. 5 illustrates the relative change observed for this protein following in vitro exposure to the bladder carcinogens.

4. Discussion

This investigation focused on the identification and characterization of putative protein biomarkers for effect of exposure to two known occupational bladder carcinogens. Previous epidemiological studies reported that the observed higher bladder cancer incidence in males compared to females (3:1) may be attributed to higher exposure to select workplace carcinogens. However, no known method exists to determine whether a worker has been exposed at a level sufficient to compromise the integrity of the urothelial tract. Since many bladder cancers are

Table 2
SV-HUC.PC proteins quantitatively altered following in vitro exposure to N-OH-MOCA and N-OH-ABP

Protein no.	Mr (relative molecular weight, kD)	pI (isoelectric point)	TagIdent match
1	31	3.2	–
2	55	5.6	Cytokeratin (CK20)
3	26	5.7	GST pi
4	70	7.3	–
5	38	7.8	–
6	41	8.1	–
7	16	5.4	FABP (fatty acid binding protein)
8	39	7.6	Annexin

Treated in vitro 24 h with N-OH-ABP (5.0 μM) or N-OH-MOCA (10.0 μM).

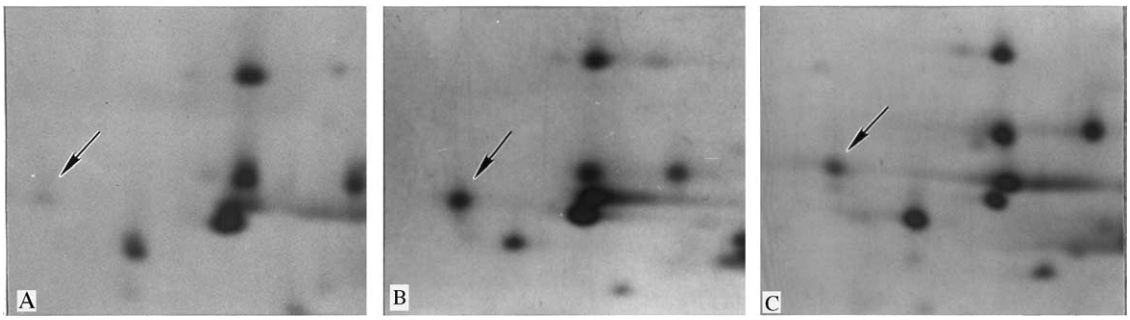

Fig. 3. Enlarged (2 ×) region of 2D-PAGE gel to display protein spot no. 1 (Mr, relative molecular weight, 31; pI, isoelectric point 3.2). Taken from SV-HUC.PC gels following 24 h treatment in vitro with (A) DMSO (0.5%), (B) N-OH-MOCA (10.0 μM), (C) N-OH-ABP (5.0 μM).

indolent [2], there is a need to develop appropriate biomarkers which reflect early effects initiated by occupational carcinogens. Identifying specific markers that may occur in carcinogenesis may help to identify groups at risk for bladder cancer.

We attempt to identify such biomolecular correlates using an SV-transformable human uroepithelial cell model previously shown to be ideal for assessing carcinogenic effects of experimental exposures that mimic occupational exposures. In vivo/in vitro studies were done with sublines of SV-HUC to assess the effects of exposure to a carcinogen [7]. Following the inoculation of SV-HUC.PC cells treated in vitro with N-OH-ABP (5.0 μM) or N-OH-MOCA (10.0 μM), tumors were observed in athymic mice [27,28]. In this investigation, we use the same human bladder carcinogens, relevant to occupational exposures, to directly investigate biochemical changes occurring within the target cell.

We illustrate the use of automated 2D-PAGE to detect protein changes, potential biomarkers that may occur in SV-HUC.PC cells following treatment with known bladder carcinogens. The technique resolves hundreds of cellular proteins based on their acidic and basic amino acid content (IEF) and molecular weight (SDS electrophoresis). In combination, a two-dimensional protein pattern uniquely characteristic for a given cell line is produced. Individual proteins within the pattern can be analysed for alterations in abundance, charge, and molecular weight. The ability to simultaneously determine changes in large numbers makes possible the selection of specific proteins that deserve further study and identification. Assigning specific spots to known proteins allows one to make mechanistic interpretations of the observed changes and may provide insight into the mechanisms associated with occupational carcinogen exposure.

We studied two-dimensional gels using the PDQUEST computerized system to automatically match multiple gel patterns. This approach shows significant differences obscured by the minor changes in spot intensity that arise between separate cell cultures. Further, computer analysis of the gel patterns allows one to search for changes that are consistent and statistically significant. However, analysis is complicated by intraclonal variability in the synthesis of different proteins [33]. That is, the intensity of a large fraction of the spots in parallel cultures may be measurably different due to the accumulation of minor, but uncontrollable variations between different cultures. To control for this we pooled the results of four gels per each treatment group and searched for spots that are different to the 95% significance level and where the magnitude of the change (two-fold or greater) makes them unlikely to be the result of intraclonal variability.

The master SV-HUC.PC cell profile illustrates a universally representative pattern for proteins under all treatment conditions and serves as a template to match single gel patterns. After individual gels are matched to the master, quantitative data for each matched spot are examined. The levels of N-OH-MOCA used clearly have altered the pattern of human uroepithelial protein expression. Interestingly, a concentration-dependent increase in the number of quantitative protein alterations was observed following increasing N-OH-MOCA (Table 1). Accordingly, in the in vivo/in vitro

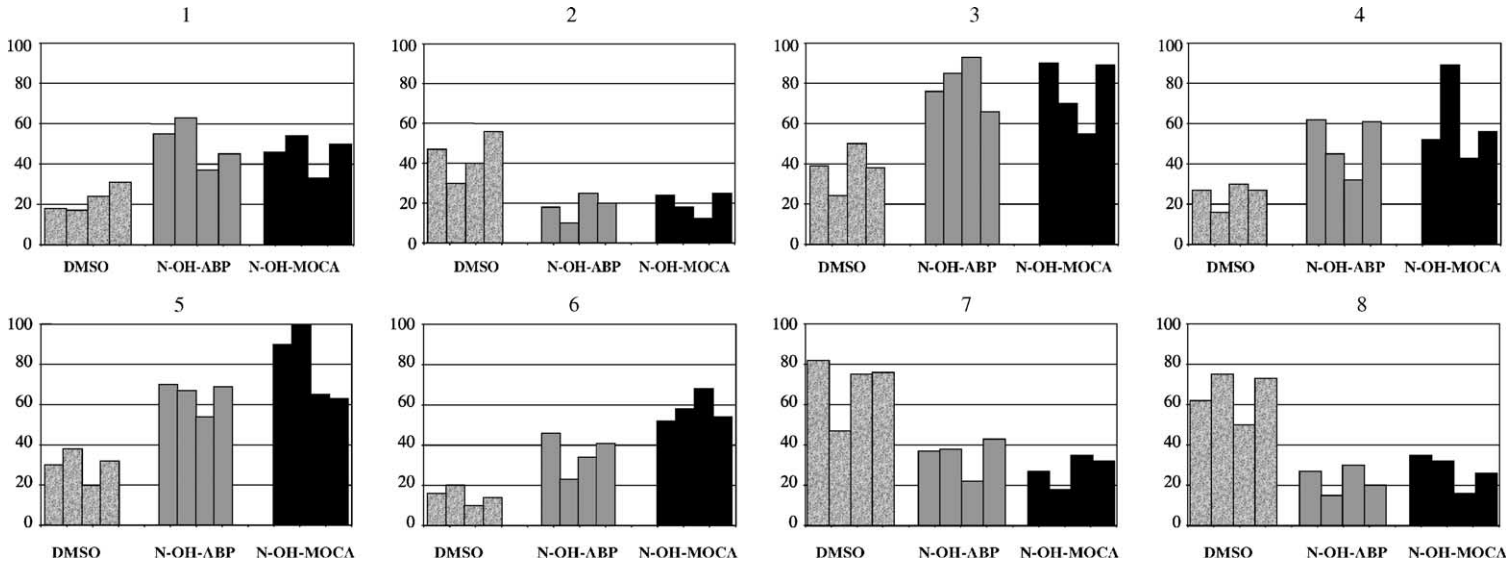

Fig. 4. Bar graphs of SV-HUC.PC proteins significantly altered two-fold or greater following in vitro exposure to N-OH-ABP (5.0 μM) and N-OH-MOCA (10.0 μM) ($P < 0.05$; Student's *t*-test). Each panel shows data for one protein spot (spot number in the upper corner). Bars within each panel are grouped by chemical treatment. Single bars represent the relative abundance for one gel ($n = 4$ treatments per group).

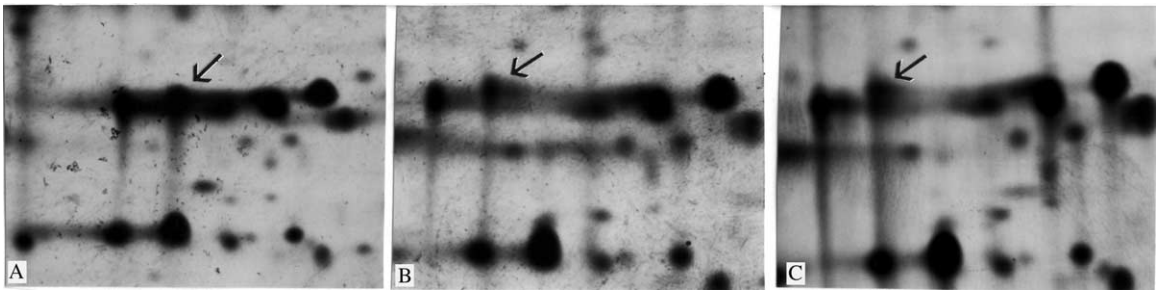

Fig. 5. Enlarged (2 ×) region of 2D-PAGE gel to display protein spot no. 9. Taken from SV-HUC.PC gels following 24 h treatment in vitro with (A) DMSO (0.5%), (B) N-OH-MOCA (10.0 μM), (C) N-OH-ABP (5.0 μM).

HUC transformation model athymic mice inoculated with cells exposed to lower doses (2.5 and 5.0 μM) N-OH-MOCA did not develop tumors, while tumors were observed following cells treated at the highest dose (10.0 μM) [10].

We also compared the two-dimensional gel patterns of SV-HUC.PC cells treated with either N-OH-MOCA (10.0 μM) or N-OH-ABP (5.0 μM) to vehicle-treated cells. A subset of proteins were altered by both carcinogens, N-OH-ABP and N-OH-MOCA, suggesting that changes in the quantity of these specific proteins may have implications as predictive biomarkers for bladder carcinogen exposures in general. Following in vitro carcinogen treatment eight spot densities displayed a mean change at least two-fold. Fig. 1 demonstrates the relative position of the proteins within the gels. Five spot densities increase, and three decrease after carcinogen treatment ($P < 0.05$; Student's *t*-test). A difference in mean density presumably reflects alterations in protein abundance and suggests altered regulation of the genome, change in protein turnover rate, change in protein stability or post-translational modifications following treatment. An apparent shift in pI in one protein was also observed following carcinogen treatment of SV-HUC.PC. This shift reflects protein charge modifications or changes in normally expressed micro-heterogeneities and suggests post-translational protein alterations may have occurred. We used the TagIdent database to match the carcinogen altered proteins in this study with proteins previously found to be significantly altered in human bladder carcinogenesis or progression (Table 2). Protein no. 2 (Mr, 55 kD; pI, 5.6), apparently down regulated after carcinogen exposure, had similar biochemical characteristics to cytokeratin (CK20 or keratin 20). Cytokeratin, expressed in both normal uroepithelia [34] and in transitional cell carcinoma (TCC) [35], is associated with cell differentiation. Protein nos. 7 (Mr 16 kD, pI 5.4) and 8 (Mr 39 kD, pI 7.6) which decreased in abundance with chemical treatment correspond biochemically to TCC-expressed proteins, fatty acid-binding protein (FABP), which plays a role in growth control, and annexin, a calcium-binding protein, respectively. Interestingly, FABP and annexin have been found to be down regulated with progression of bladder carcinoma [36].

Protein no. 3 (Mr 26 kD, pI 5.7) increased in abundance with treatment and biochemically corresponds with glutathione-*S*-transferase (GST pi) which is typically expressed in low grade TCC [35]. The alterations in GST might be of importance in bladder carcinogenesis since the function of GST enzyme has traditionally been considered to be the detoxification of electrophiles. Since 4-ABP and MOCA are known to generate reactive electrophiles and reactive oxygen species, GST is likely to be involved in neoplastic transformation and the progression of bladder cancers [28]. Although there are several isoforms of GST, the major isoform in urinary bladder has been identified as GST pi (Swaminathan, unpublished data). GST pi exhibits polymorphism in humans and its expression is altered in cancer patients [37]. Recent data suggests that GST pi might be involved in the inhibition of jun N-terminal kinase and thus cell proliferation [38]. In view of the polymorphism

and functional importance of GST pi in carcinogenesis, it is conceivable that GST pi could play a determinate role in bladder cancer susceptibility.

The assigning of spots to known proteins will enhance the ability to make mechanistic interpretations of the observed spot changes and provide greater insight into the mechanisms associated with occupational carcinogen exposure. This capability could have clinical or public health benefits. Measurements of phenotypic profiles may be useful in identifying groups at increased risk of bladder cancer due to chemical exposure. Bladder cells may slough from the epithelium and appear in the urine as exfoliated cells or the proposed biomarker proteins may be liberated from anchored cells into the bladder lumen and subsequently appear in the urine. In either case, a biomarker of effect may be detectable in a biological specimen (i.e. the urine) which is obtained by non-invasive techniques. Once validated for disease the biomarkers could be used to identify groups at high risk for bladder cancer development and possibly cancer patients who may benefit from aggressive intervention or chemoprevention.

References

[1] P.A. Wingo, T. Tong, S. Bolden, Cancer statistics, CA J. Clin. 45 (1995) 8–30.

[2] H. Rubben, W. Lutzeyer, D.M.A. Wallace, The epidemiology and etiology of bladder cancer, in: E.J. Zingg, D.M.A. Wallace (Eds.), Bladder Cancer, Springer, New York, 1985, pp. 3–49.

[3] F.M. Torti, B.L. Lum, The biology and treatment of superficial bladder cancer, J. Clin. Oncol. 2 (1984) 505–531.

[4] P.D. Abel, Prognostic indices in transitional cell carcinoma of the bladder, Br. J. Urol. 62 (1988) 103–109.

[5] American Cancer Society, Cancer Facts and Figures, Publ. 95-375M-No5008.95, ACS, Atlanta, GA, 1995.

[6] D.T. Silverman, P. Hartget, A.S. Morrison, Epidemiology of bladder cancer, Hematol. Oncol. Clin. North Am. 6 (1992) 1–30.

[7] E.A. Bookland, S. Swaminathan, K.W. Gilchrist, R. Oyasu, C.A. Reznikoff, Tumorigenic transformation and neoplastic progression of human uroepithelial cells after exposure in vitro to 4-aminobiphenyl or its metabolites, Cancer Res. 32 (1992) 1606–1614.

[8] C.A. Reznikoff, C. Kao, E.M. Messing, M. Newton, S. Swaminathan, A molecular model of human bladder carcinogenesis, Sem. Cancer Biol. 4 (1993) 143–152.

[9] B.J. Christian, L.J. Loretz, T.D. Oberley, C.A. Reznikoff, Characterization of human uroepithelial cells immortalized in vitro by simian virus 40, Cancer Res. 47 (1987) 6066–6073.

[10] R.E. Savage Jr., D.G. DeBord, S. Swaminathan, M.A. Butler, J. Snawder, M.H. Kanitz, et al., Occupational applications of a human cancer research model, J. Occup. Environ. Med. 40 (1998) 125–135.

[11] F.P. Perera, Molecular cancer epidemiology: a new tool in cancer preventions, J. Natl. Cancer Inst. 78 (1987) 887–898.

[12] G.L. Nicolson, Gene expression, cellular diversification and tumor progression to the metastatic phenotype, BioEssays 13 (1991) 337–342.

[13] L. Rehn, Blasengeschwulste bei Anilinarbeitern, Arch. Klin. Chir. 50 (1895) 588–600.

[14] L.H. Kuller, L. Garfinkel, P. Correa, N. Haley, D. Hoffmann, S. Preston-Martin, D. Sandler, Contribution of passive smoking to respiratory cancer, Environ. Health Perspect. 70 (1986) 56–69.

[15] D.A. Haugen, M.J. Peak, K.M. Shuhrbler, V.C. Stamoudis, Isolation of mutagenic aromatic amines from a coal conversion oil by cationic exchange chromatography, Anal. Chem. 54 (1982) 32–37.

[16] W.F. Melick, J.J. Naryka, R.E. Kelly, Bladder cancer due to exposure to para-aminobiphenyl: a 17-year follow-up, J. Urol. 106 (1971) 220–226.

[17] S. Swaminathan, J.F. Hatcher, Identification of new DNA adducts in human bladder epithelia exposed to the proximate metabolite of 4-aminobiphenyl using 32P-postlabeling method, Chem. Biol. Interact. 139 (2002) 199–213.

[18] J.F. Hatcher, S. Swaminathan, Detection of deoxyadenosine-4-aminobiphenyl adduct in DNA of human uroepithelial cells treated with *N*-hydroxy-4-aminobiphenyl following nuclease P1 enrichment and P32-postlabeling analysis, Carcinogenesis 16 (1995) 295–301.

[19] J.F. Hatcher, S. Swaminathan, ^{32}P-Postlabeling analyses of DNA damage in human uroepithelial cells exposed to proximate metabolites of the environmental carcinogen 4-aminobiphenyl, Environ. Mol. Mutagen. 39 (2002) 314–322.

[20] G. Talaska, M. Schamer, P. Skipper, S. Tannenbaum, N. Caporaso, L. Unruh, et al., Detection of carcinogen–DNA adducts in exfoliated urothelial cells of cigarette smokers: association with smoking, hemoglobin adducts, and urinary mutagenicity, Cancer Epidemiol. Biomarkers Prev. 1 (1991) 61–66.

[21] F.F. Kadlubar, F.A. Beland, Chemical properties of ultimate carcinogenic metabolites of arylamines and arylamides, in: R.G. Harvey (Ed.), Polycyclic Hydrocarbons and Carcinogenesis, ACS Press, Washington, DC, 1985, pp. 341–370.

[22] The International Agency for Research on Cancer, Suppl. 7, 91 (1987) 1–42.

[23] C.A. McQueen, G.A. Williams, Review of the genotoxicity and carcinogenicity of 4,4′-methylene-dianiline and 4,4′-methylene-bis-2-chloroaniline, Mutat. Res. 239 (1990) 133–142.

[24] K.C. Morton, M-S. Lee, P. Siedlik, R. Chapman, Metabolism of 4,4′methylene-bis2chloroaniline (MOCA) by rats in vivo

and formation of N-hydroxy MOCA by rat and human liver microsomes, Carcinogenesis 9 (1988) 731–739.
[25] F.F. Kadlubar, J.A. Miller, E.C. Miller, Hepatic microsomal N-glucuronidation and nucleic acid binding of N-hyroxyarlamines in relation to urinary bladder carcinogenesis, Cancer Res. 37 (1977) 805–814.
[26] D.G. DeBord, K.L. Cheever, D.M. Werren, T.M. Reid, T.F. Swearingen, R.E. Savage Jr., Determination of 4,4′-methylene-bis (2-chloroaniline)-DNA adduct formation in rat liver and human uroepithelial cells by the ^{32}P-postlabeling assay, Fundam. Appl. Toxicol. 30 (1996) 138–144.
[27] S. Swaminatham, S.M. Frederickson, J.F. Hatcher, C.A. Reznikoff, M.A. Butler, K.L. Cheever, R.E. Savage Jr., Neoplastic transformation and DNA binding of 4,4′-methylenebis(2-chloroaniline) in SV-40-immortalized human uroepithelial cell lines, Carcinogenesis 17 (1996) 857–864.
[28] S. Swaminathan, C. Reznikoff, Biochemical and molecular carcinogenesis, in: N.J. Vogelzang, P.T. Scardino, M.U. Shipley, D.S. Coffey (Eds.), Comprehensive Textbook of Genitourinary Oncology, Williams & Wilkins, Baltimore, MD, 2000, pp. 289–297.
[29] L.F. Meisner, S.-Q. Wu, B.J. Christian, C.A. Reznikoff, Cytogenetic instability with balanced chromosome changes in an SV40 transformed human uroepithelial cell line, Cancer Res. 48 (1988) 3215–3220.
[30] C.I. Pratt, R. Oyasu, C.A. Reznikoff, Neoplastic progression by EJ/*ras* at different steps of transformation in vitro of human uroepithelial cells, Cancer Res. 52 (1992) 688–695.
[31] T.H. Chen, B.I. Kuslikis, W. Braselton Jr., Hydroxylation of 4,4′methylenebis (2-chloroaniline) by canine, guinea pig and rat liver microsomes, Drug Metab. Dispos. 17 (1989) 406–413.
[32] S.L. Tollaksen, N.L. Anderson, N.G. Anderson, Operation of the ISO-DALT System, 7th ed., US Dept. Energy Publ. ANL-BIM-84-1, US Department of Energy, Argonne, IL, 1984.
[33] M.J. Miller, D.M. Schwartz, S.S. Thorgeirsson, Inter- and intraclonal variability of polypeptides synthesized in a rat hepatoma cell line: quantitative two-dimensional gel analysis, J. Biol. Chem. 263 (1988) 11227–11236.
[34] J.E. Celis, P. Celis, M. Ostergaard, B. Basse, J.B. Lauridsen, H.H. Rasmussen, et al., Proteomics and immunochemistry define some of the steps in the squamous differentiation of the bladder transitional epithelium: a novel strategy for identifying metaplastic lesions, Cancer Res. 59 (1999) 3003–3009.
[35] A. Celis, H.H. Rasmussen, P. Celis, B. Basse, J.B. Lauridsen, G. Ratz, et al., Short-term culturing of low grade superficial bladder transitional cell carcinoma leads to changes in the expression levels of several proteins involved in key cellular activities, Electrophoresis 20 (1999) 355–361.
[36] J.E. Celis, M. Ostergaard, B. Basse, A. Celis, J.B. Lauridsen, G.P. Ratz, et al., Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas, Cancer Res. 56 (1996) 4782–4790.
[37] R.C. Strange, M.A. Spiteri, S. Ramachandran, A. Fryer, Glutathione-*S*-transferase family of enzymes, Mutat. Res./ Fundam. Mol. Mech. Mutagenesis 482 (2001) 21–26.
[38] V. Adler, Z. Yin, S.Y. Fuchs, M. Benezra, L. Rosario, K.D. Tew, et al., Regulation of JNK signalling by GSTp, Eur. Mol. Biol. Org. J. 18 (1999) 1321–1334.