MOLECULAR CHARACTERIZATION OF NON-HISTONE CHROMATIN PROTEINS FROM EXPERIMENTAL TUMOURS *

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Using two-dimensional (2-D) electrophoresis, two non-histone chromatin protein fractions (NHCP1 and NHCP2) from three animal tumours (Kirkman-Robbins hepatoma, Morris hepatoma 7777 and Ehrlich ascites cells) and normal hamster liver were analyzed. Apart from many common components several tissue specific polypeptides of the NHCP1 and NHCP2 fractions were detected. It was found that some spots present in electropherograms of non-histone proteins of tumour cells \((M \times 10^{-3}/\text{p}1)\): 17-24/4.9-6.5 (NHCP1 and NHCP2); 34-41/4.9-6.0 (HCP1 and NHCP2); 44-46/5.3-7.5 (HCP2); 46-49/5.0-7.5 (NHCP1); 49/5.9-7.5 (NHCP2) and 102-134/5.6-7.0 (NHCP1) were absent from normal liver.

Non-histone proteins consists of heterogeneous proteins of the nuclear envelope, nuclear sap, chromatin, mRNP, and those proteins of nucleolus [1]. They are also main components of the interphase nuclear skeleton (matrix) as well as metaphase chromosome scaffold [2, 3].

It is well known that nuclear proteins by affecting organization of chromatin structure mediate the processes of gene expression and DNA replication. Therefore, an understanding of the changes of these proteins during transformation is of importance for elucidation of oncogenic process [4-8]. Many authors report on changes in non-histone proteins when cells are transformed from normal to neoplastic state both in vivo or in vitro [6, 7, 9-11].

The purpose of the present paper was to investigate the molecular characteristics of two fraction of non-histone chromatin proteins, i.e., NHCP1 and NHCP2 from three animal tumours as compared with those of normal hamster liver. The polypeptide pattern was analyzed using high resolution 2-D electrophoresis followed by Coomassie blue and silver staining.

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MATERIALS AND METHODS

Tissues. The following animal tumours used in this study were hamster Kirkman-Robbins hepatoma (cell line from Department of Oncology School of Medicine, Łódź, Poland), rat Morris hepatoma 7777 (Institute of Molecular Biology, Jagiellonian University, Kraków, Poland) and mouse Ehrlich ascites cells (Department of Tumour Biology, Institute of Oncology, Gliwice, Poland). Syrian hamster liver served as normal tissue.

Isolation of nuclei. Liver or tumour nuclei were isolated by the method of Chauveau et al. [12]. For each nuclear preparation, 8–10 tumour specimens or 8–10 livers totalling about 60 g of tissue were needed. The tissue was homogenized in 10 vol. of 0.25 M sucrose, 1 mM CaCl₂, 0.8 mM KH₂PO₄, 1 mM PMSF, pH 6.7, filtered through 8 layers of gauze and spun down at 800 × g for 7 min. The pellet was resuspended in the above solution and Triton X-100 was added to the concentration of 0.5% then the suspension was homogenized again and centrifuged at 800 × g for 7 min. The crude nuclear pellet was suspended in 10 vol. of 2.2 M sucrose, 5 mM MgCl₂, 1 mM PMSF and centrifuged at 40000 × g for 60 min. and then it was resuspended in 0.25 M sucrose, 1 mM CaCl₂, 0.8 mM KH₂PO₄, 1 mM PMSF, pH 6.7 and spun down at 800 × g for 10 min. The purified nuclei thus obtained were immediately used for isolation of non-histone proteins. Purity of nuclei was checked by phase contrast microscopy.

Isolation of non-histone chromatin proteins — NHCP1 and NHCP2. Non-histone proteins were isolated from nuclei and fractionated as described previously [13]. Briefly, the nuclei (50–70 mg based on DNA determination) were suspendend and homogenized in 2 M NaCl, 8% glycerol (v/v), 1 mM PMSF, 1% dimethylsulfoxide (v/v), 5 mM sodium phosphate, pH 7.9 (basic buffer), in a Teflon glass-pestle homogenizer. The homogenate was stirred overnight at 4°C. Then the solution (DNA content less than 0.8 mg/ml) was sonicated for 2 min (8 × 15 s) at 60 W with the ultrasonic homogenizer (model MSE MKZ) and stirred for 1 h in a cold-room. To the clear solution, 0.5 vol. of 20% dextran plus 0.5 vol. of 30% polyethylene glycol in the basic buffer was added. The highly viscous solution was shaken for several minutes and then spun down at 4000 × g for 15 min to remove nucleic acids. The upper, protein-rich phase was collected with a wide-tip syringe. The dextran phase was reextracted twice with fresh portions of polyethylene glycol in the basic buffer. The combined protein-rich phases were dialysed against two 4-litre portions of 2 M NaCl, 8% glycerol, 0.1 mM PMSF, 5 mM sodium phosphate, pH 6.8 in a cold-room. The dialysis residue was applied on hydroxyapatite column (2 × 20 cm) equilibrated with 2 M NaCl, 8% glycerol, 0.1 mM PMSF, 5 mM sodium phosphate, pH 6.8. Fractions of 2.5 ml were collected at a flow rate of 30 ml/h. Histones were eluted with the same buffer. Fractions of non-histone proteins: NHCP1 and NHCP2 were eluted with 50 mM (Na⁺
form) and 100 mM (K+ form) phosphate buffer, pH 6.8, in 2 M NaCl, 8% glycerol, 0.1 mM PMSF, respectively. All steps of elution from the hydroxyapatite column were performed in a cold-room.

2-D electrophoresis. For 2-D electrophoresis the method of O’Farrell [14] was slightly modified. The non-histone protein fraction after precipitation with trichloric acid were dissolved in 9.5 M deionized urea - 2% Nonidet P-40 - 2% 2-D Pharmalyte (pH range 3.0-10.0). The composition of the isoelectric gel in glass tubes 10 × 0.2 cm was as follows: 3.78% acrylamide, 0.22% bisacrylamide, 9 M urea, 2% Nonidet P-40, 2% 2-D Pharmalyte (pH range 3.0 to 10.0). The protein samples (about 250 µg) were electrophoresed in the first dimension for 14 h at 300 V and for 2 h at 400 V; the electrolyte solutions were: 0.02 M NaOH (cathode) and 0.01 M H2PO4 (anode). The pH of the “blank” focused gels was measured at 0.5 cm intervals in 0.01 M KCl using Beckman pH-meter. After isoelectric focusing the gels were equilibrated for 1.5 h in the buffer containing 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 0.0625 M Tris/HCl (pH 6.8). Electrophoresis in the second dimension was carried out in 11.2% polyacrylamide gel according to Laemmli [15]. The slab gels were stained either with Coomassie brilliant blue R-250 by the Fairbanks et al. [16] technique or with silver nitrate according to Wray et al. [17]. The proteins used as standards were as follows: phosphorylase B (Mr 94,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 30,000), soybean trypsin inhibitor (Mr 20,100) and α-lactalbumin (Mr 14,400).

Other methods. Protein was estimated by the method of Lowry et al., [18], and DNA was determined spectrophotometrically.

Reagents. Acrylamide was a product of Koch-Light Lab. (Colnbrook Bucks, England). N,N-Bisacrylamide, ammonium persulphate and hydroxyapatite were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Ampholine was from LKB-Producer AB (Bromma, Sweden), and Coomassie brilliant blue R-250 and dimethyl sulfoxide from Sigma Chem. Co. (St. Louis, MO, U.S.A.). Dextran T-500 (Mr 500,000) and molecular weight standard proteins kit were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). PMSF was from Calbiochem (San Diego, CA, U.S.A.). Polyethylene glycol, type 6000 (Mr 6000-7500) was a product of Serva Feinbiochemica (Heidelberg, F.R.G.). SDS specially pure was supplied by B.D.H. Chemicals, Poole (Dorset, England). Other reagents were analytical grade products supplied by POCh (Gliwice, Poland).

RESULTS AND DISCUSSION

Recently electrophoretical and immunological methods are widely used in research on tumour-specific or at least tumour-associated nuclear non-histone proteins, especially in human neoplastic cells and animal tumours [6, 8, 11, 19-23].
For comparison purpose with normal cells we have used animal tumours: hamster Kirkman-Robbins hepatoma, rat Morris hepatoma 7777 and mouse Ehrlich ascites cells.

Molecular distribution of the peptides of the NHCP1 and NHCP2 fractions was tested by 2-D electrophoresis followed by Coomassie blue and silver staining. The use of 2-D electrophoresis and highly sensitive silver staining of polyacrylamide gels [25] still improved by Wray et al. [17] enables both high resolution and reproducibility of separation of nuclear proteins.

Most of the components of NHCP1 and NHCP2 fractions were located in the pH range from 5.5 to 7.5 and the molecular zone above $M_r$ 40 000 (Figs.

![Fig. 1A. 2-D electrophoresis of the NHCP1 peptides of hamster liver (L), and hamster Kirkman-Robbins hepatoma (K-RH) followed by Coomassie blue (I) and silver staining (II). The protein samples (about 250 µg) were applied on gels. The isoelectric focusing in polyacrylamide gel (IFPA) was in the horizontal dimension and electrophoresis in polyacrylamide gel containing SDS was in the vertical dimension. Molecular weight standards (from top to bottom): phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactoalbumin. The symbols indicate: tissue specific, *, common for neoplastic tissues, <; and main common for all tissues spots, !]
1 and 2). Although a great majority of NHCP1 and NHCP2 peptides were common for the tissues investigated some qualitative and quantitative differences were noticed.

In the electropherograms of all NHCP1 fractions analyzed a prominent band of the $M_r \times 10^{-3}$/pI 54-57/5.0-5.3 was always observed (Fig. 1A and 1B). The components of NHCP1 fraction migrating in the molecular zone of 34/6.2, 35/6.0-6.2, 60/5.4 appeared exclusively in the normal liver hamster whereas the spots 31/5.1, 33/4.6, 34/4.7 (visible only after silver staining), 55/5.6, 70/5.2 and 85/5.0 only in Kirkman-Robbins hepatoma (Fig. 1A). The spots of 23/6.8, 37/5.9 and 44/5.9-7.3 were characteristic only for the NHCP1 of Morris hepatoma 7777 (plate 1B). The NHCP1 fraction isolated from Ehrlich ascites cells contained tissue specific components of 21/4.7 (visible only after Coomassie blue staining), 52/5.9-6.2 and 95/5.5-5.6 (Fig. 1B). The polypeptides in the molecular zone 17-24/4.9-5.5, 34-41/4.9-6.0, 48-49/5.0-7.5 and

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Fig. 1B. 2-D electrophoresis of rat Morris hepatoma 7777 (MH) and mouse Ehrlich ascites cells (EA) NHCP1 followed by Coomassie blue (I) and silver staining (II). For further explanations see Fig. 1A.
132-134/5.6-7.0 present in NHCP1 from the tumour cells studied were absent from hamster liver.

In the NHCP2 fraction (Fig. 2A and 2B) two main components located in the molecular zone of 85-90/5.3-5.7 and 43-58/5.8-7.5 (especially elevated in liver) are common for the tissues analyzed. The polypeptides of 23-24/6.8, 7.0, 7.3, 30/7.0, 40/5.9, 6.1, 6.2, 63/7.1 and 67/6.4, 6.8 seemed to be specific for liver while 60/5.4-5.7 and 70/5.6-6.3 — for hamster hepatoma (Fig. 2A). The spots of 30/5.6 (stained only with Coomassie blue) and 52/5.4-5.5 are characteristic for the Morris hepatoma 7777 (Fig. 2B). In the electrophoretic pattern of Ehrlich ascites cells the polypeptides of NHCP2 at 55-60/5.9, 6.0, 6.1 and 47/6.2-6.5 appear to be specific for this tissue (Fig. 2B). The NHCP2 components migrating in the molecular zone of 17-20/5.2-6.5, 38-41/4.9-5.2 in Morris hepatoma 7777 and Ehrlich ascites cells 44-46/5.3-7.5, and 49/5.9-7.5 were found in the investigated neoplastic tissues.

Fig. 2A. 2-D electrophoresis of hamster liver (L), and hamster Kirkman-Robbins hepatoma (K-RH) NHCP2 followed by Coomassie blue (I) and silver staining (II). For further explanations see Fig. 1A.
Fig 2B. 2-D electrophoresis of rat Morris hepatoma 7777 (MH) and mouse Ehrlich ascites cells (EA) NHCP2 followed by Coomassie blue (I) and silver staining (II). For further explanations see Fig. 1A.

Besides the above mentioned qualitative differences, significant quantitative differences were found between the NHCP1 and NHCP2 fractions studied of tumour and normal cells, concerning mainly the peptides of $M_r$ of about 24 000-36 000, 40 000-46 000, 55 000-60 000, 65 000-70 000 and 85 000-90 000. It is worth noting that non-histone proteins of the tissues examined contain also some slightly staining spots (with shades of yellow or light brown colour visible on the original gels but indistinguishable on the black/white photography).

The results of our studies indicate that presence of some non-histone protein components characteristic for experimental tumours, absent from normal tissue. The research aiming at their further characterization is in progress.

Clinical and experimental studies on neoplasia are lacking reproducible and well-characterized markers of the transformation events. The increasing awareness of the role played by non-histone chromatin proteins, has recently led to a search for these chromatin proteins as markers of transformation [6, 7, 9, 11, 19, 20, 22, 26, 27].
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REFERENCES