Our studies were focused on the isolation and characterization of highly motile fraction of cells from hepatoma Morris 5123 population. Cells that underwent several migration cycles through Matrigel - coated filters were successfully cultured. The invasion index was determined by means of Matrigel invasion assay. Statistically significant increase in the value of invasion factor for selected cells variant in comparison to the parental population was observed. The considerable changes in the cell shape were followed by the reorganization of the actin cytoskeleton structure including a dense subcortical congestion in the distribution of β-actin isoform. The visualization of this protein in tumor cells was performed by immunostaining and scanning fluorescent confocal microscopy. The results were confirmed by densitometry analysis of Western blots. In addition, the increased state of actin polymerization in the cytoplasmic fraction of selected cells was determined as measured by filamentous to monomeric (F:G) actin ratio. Concluding, the selected fraction of hepatoma Morris 5123 cells with higher invasion capacity was characterized by rounded shape, remarkable increase of β-actin level, its submembrane concentration as well as with the increased state of actin polymerization with respect to parental cells population.

Key words: actin cytoskeleton, β-actin isoform, invasiveness, hepatoma

INTRODUCTION

The acquisition of motile phenotype by tumor cells, detachment from the primary tumor and invasion of the surrounding tissues is the first symptom of cancer malignancy. This phenomenon has been considered a pathology that is associated with deregulated cell migration and remarkable changes in the
functioning and architecture of actin cytoskeleton and regulatory actin binding proteins (1, 2).

Actin is a highly conservative protein, however it expresses molecular heterogeneity. It occurs in vertebrates in six isoforms. There are two isoforms in striated muscles - alpha-skeletal muscle actin (α-SKA) and alpha-cardiac muscle actin (α-CMA), two isoforms in smooth muscles - α-smooth muscle actin (α-SMA) and gamma-smooth muscle actin (γ-SMA). Two cytoplasmic isoforms occur in non-muscle cells - beta-cytoplasmic actin (β-CyA) and gamma-cytoplasmic actin (γ-CyA) (3, 4). Actin isoforms are products of separate genes, although the homology among them appears in the sequence of nucleotides as well as in the position of amino acid residues. The changes occur especially in the most variable acetylated N-terminal region of actin molecule. Actin isoforms express tissue but not species specificity. Actins from different tissues of the same species differ more than actin from the same tissue of evolutionary distinct organisms. Additionally, the level of different actin isoforms expression can be up- or down-regulated not only in neoplastic transformation and other cell pathologies, but also in the ordinary processes undergoing during embryogenesis and aging (for reviews see 5 - 7).

Every type of cell locomotion is driven by actin polymerization (2, 8, 9). The proportion of cytoplasmic actin isoforms is varying and depending on the cell type. Cytoplasmic β-actin was identified to accumulate in the region of moving cytoplasm and appear at the tips and edges of protrusive structures, whereas γ-cytoplasmic actin is rather likely to be found in the stress fibers, being responsible for the maintenance of cell shape and mechanical resistance (3, 5, 10). There are also some data showing that the basis of actin filaments rearrangement may result from the altered actin isoform expression (11, 12). The increased level of cytoplasmic actin was shown to accompany many tumor types such as chemically induced melanoma, hepatoma, lymphoma and human breast cancer, as summarized by Nowak and Malicka-Błaszkiewicz (6). Cytoplasmic β-actin seems to be overexpressed in many tumor cells, especially in actively moving, highly invasive cells (13, 14). In addition to this, β-actin is assumed to play a crucial role in ameba-like type of movement - characteristic for the process of intravassation and extravassation during metastasis formation (14, 15). Indeed, it was observed that there is a significant increase in the level of β-actin expression in invasive variants of colon adenocarcinoma (14) and sarcoma (13), to compare with parental cell lines. These changes were accompanied by submembrane distribution of β-actin, it's concentration in apical parts of pseudopodia and the increase in the state of actin polymerization (14). A special attention should be given to the research performed on the cells with different metastatic potential (13, 14, 16, 17). It would allow to verify factors responsible for the cell to become highly motile and affecting their ability to metastasis formation (18).

Our previous studies have demonstrated a remarkable increase in the level of total and polymerized actin during hepatoma Morris 5123 experimental tumor...
growth process. A significant increase in the state of actin polymerization coinciding with the first metastases formation to the lungs of tumor bearing rats was observed (19 - 21). At present the separation of highly motile fraction of hepatoma Morris 5123 cells and analysis of the correlation between the cells shape, the state of actin polymerization, β-actin expression, its subcellular distribution and invasive capacity of these cells were done.

MATERIALS & METHODS

Hepatoma Morris 5123 cells - isolation and culture

The experiments were performed on rat hepatoma Morris 5123 cells derived from experimental tumor rapidly growing on the left rear limb of Buffalo rats. Cells were isolated from biopsies of tumor by the cold cocktail method (22). The tissue was washed with culture medium supplemented with double strength penicillin (200 U/ml) and streptomycin (0.2 mg/ml), transferred into sterile dishes, cut into small pieces and placed in a cold enzymatic cocktail I (0.25% trypsin and 200 U/ml collagenase mixed in 2:1 ratio) followed by 4h incubation on ice. Then tube with cells was vigorously shaken for 2 minutes in 37°C bath. The liquid phase containing dispersed cells was neutralised with culture medium and centrifuged for 3 min. at 800xg. Meanwhile the tube with the rest of the tissue was replenished with the enzyme cocktail II (0.25% trypsin and 200 U/ml and 1 mg/ml DNase I mixed in 2:1:0.05 ratio), returned to the water bath and shaken. The fluid containing dispersed cells was again neutralised and centrifuged as above. Cells were counted and seeded in culture flasks at a density of 1-2x10^{5} cells/ml and were cultured in Dulbecco's Modified Eagle's Medium (supplemented with 10% Newborn Calf Serum, 1 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin) at 37°C and 5% CO_{2}, and used for the current experiments between 5th and 18th passage.

Separation of cells with higher migration ability and invasion assay

The separation of cells with higher migration ability was performed with invasion assay procedure according to Albini et al. (23) with the following modifications. The Falcon membrane systems with 5 cm² insert consisting of PET filters with 0.8 µm pore size were coated with 600 µl Matrigel® (0.3 mg/ml) that was allowed to polymerise for 5h. Afterwards, 4x10^{5} cells in serum free medium were seeded on the upper chamber and incubated for 24h to allow cells to migrate on the other side of the PET membrane whereby they were gently harvested with the rubber policeman. Cells which didn't overcome the barrier of Matrigel were previously removed with a cotton swab. The whole operation was repeated three times resulting in the separation and collection of a parental and highly motile fraction of hepatoma Morris 5123 cells population called later: HMP - the parental population and HM3M - the cells that underwent three migration cycles. We have also isolated additional third fraction of cells. At the end of the last migration cycle HM3M cells were not collected but incubated for next 24h and allowed to migrate to the bottom of the well which contained a glass coverslip. This subpopulation was called HM3M*.

The invasiveness of hepatoma Morris cells was evaluated with the same invasion assay procedure (23) with application of 0.63 cm² membrane coated with 80 ml of Matrigel® (0.3 mg/ml) and seeded with 2x10^{4} cells. Cells were fixed and stained with May-Grünwald dye and counted under 200x magnification. The invasion index was calculated as the percentage of cells
seeded onto upper surface of the filter system to the number of cells that passed to the other side of the membrane.

**Isolation of cytoplasmic fraction**

Cells grown on tissue culture flasks were collected and suspended in 3 volumes of freshly made monomeric (G) actin stabilizing buffer 10 mM Tris/HCl pH 7.4, containing 0.25mM sucrose, 1 mM DTT, 0.1 mM ATP and 0.1 mM CaCl₂. Cells were homogenised with a Dounce homogeniser and centrifuged at 105,000x g for 1h in 4°C (14). The high speed centrifugation supernatant was used as cytosolic fraction and kept frozen until actin quantitative assay and Western blotting analysis.

**Actin measurement**

Actin determination was based on inhibitory effect of actin on DNase I from bovine pancreas under standard assay conditions, as described by Malicka-Błaszkiewicz and Roth (24). The concentration of G actin was estimated by DNase I inhibition assay directly in cytosol samples. Total (T) actin content was measured after dilution of samples with the G actin stabilizing buffer until maximal inhibition of DNase I was reached. Filamentous (F) actin was calculated by subtracting the amount of G actin from T actin (F=T-G). The state of actin polymerisation was defined by filamentous to monomeric actin ratio (F:G). One unit of DNase I inhibitor (actin) is the amount which decreases the activity of 20 ng of DNase I by 10% under standard assay conditions (24). Actin concentration was expressed in units of DNase I inhibitor per 1 mg of sample protein.

**Protein concentration**

Protein was determined by the standard Bradford assay procedure (25).

**Western blotting analysis**

Cytosol fraction (supernatant 105,000xg) was used as a source of actin in Western blotting experiments. It was isolated in identical conditions from the parental cell line and from the selected variant. The same amount of protein (30 µg) was subjected to analysis. Proteins were separated by SDS/PAGE according to Laemmli (26) and transferred to nitrocellulose sheets by the procedure of Towbin et al. (27). Monoclonal antibodies directed against β-actin (clone AC-15, Sigma) were used for actin identification. Immunoreactivity was shown by the extravidin-biotin peroxidase (Sigma) technique, using 3-amino-9-ethylcarbazole (AEC) (Sigma) as the substrate for peroxidase. The intensity of bands interacting with anti-β-actin antibodies was quantified by densitometry, using software Bio-1-D. The values of β-actin expression obtained for selected cells result from the comparison with the staining intensity measured for parental cell line. Significance of difference was calculated with Student's t-test, \( P < 0.05 \).

**Microscopic analysis**

**Light microscopy.** Differential interference contrast (DIC) was used to observe the behaviour of cells and their morphology under culture conditions.

**Fluorescent confocal microscopy.** Fluorescence analysis was applied for actin visualization and to study organization of microfilaments. Cells growing on coverslips were fixed with 4% paraformaldehyde for 20 min at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Unspecific binding sites were blocked with 3% BSA in PBS for 30 min. β-actin was visualized by a monoclonal anti-β-actin antibody (clone AC-15; Sigma) conjugated with FITC.
(fluorescein isothiocyanate), diluted 1:350 in PBS containing 1% BSA, at room temperature for 60 min. Actin microfilaments were visualized with rhodamine-conjugated phalloidin staining (Sigma) for 30 min. After washing with PBS cells were mounted in fluorescence mounting medium (Dako) and observed under fluorescent confocal laser scanning microscope Olympus FV500.

RESULTS

Hepatoma Morris 5123 cells were isolated from the biopsies of intensively growing experimental tumor and cultured in the conditions previously established in our laboratory (22). In order to separate highly motile population of cells we performed a three fold selection of hepatoma Morris cells and compared the properties of the parental population - HMP with its invasive variant - HM3M. The invasiveness of hepatoma Morris cells was evaluated by assessing the number of cells that reached the other side of the Matrigel-coated filter (23). The experiments were done in triplicate for three independent tumor biopsies.

Since the parental population of cultured cells appears to be very heterogeneous (Fig. 2a) in its morphology it was not surprising that after three migration cycles we were able to separate the population of the cells more actively invading through a Matrigel layer. The invasion index was expressed as a percentage of cells that were able to pass the barrier of Matrigel. It reached the value of 1,95 % (±0,49%) for HMP cells and it was similar for each of three analysed tumor biopsies. In contrast, during the same incubation time almost five times more HM3M cells migrated to the lower chamber of the filter reaching maximum value of 9.58% (±0,61%) (see Fig.1).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{The migration ability of hepatoma Morris 5123 cells. Cells were allowed to migrate through Matrigel coated filters for 24 hours in the direction of medium containing serum (1). Fixed cells were counted and the invasion index was calculated as a percentage of cells that migrated through the filter. * Indicates values significantly different from those for control cells as calculated by Student's t-test, P < 0.05.}
\end{figure}
Fig. 2. Hepatoma Morris cells - morphology and actin cytoskeleton organization. Morphology: (magnification 630x) - (a) parental HMP and (b) selected HM3M hepatoma Morris 5123 cells. Cytoskeleton organization: (d,g,j) filamentous actin, (c,f,i) β-actin distribution and (e,h,k) their merge; in (c,d,e) parental HMP cells, (f,g,h) HM3M cells and (i,j,k) HM3M*. Bars 10 µm.
The interesting data from Le et al. (13) supported also by our results obtained on human colon adenocarcinoma cell line variants with different metastatic potential (15) encouraged us to current research on cells isolated from biopsy. It was important to look for the correlation between invasiveness of selected cells, their behaviour and morphology, β-actin level and distribution and actin cytoskeleton remodelling. Differential interference contrast (DIC) and confocal fluorescence microscopy were applied (Fig. 2). Distinct differences in the phenotype and morphology of cell subpopulations HMP and HM3M were noticed including their adhesive properties, and proliferation rate (data not shown). Thus, parental HMP cells were mostly well spread and elongated with a polygonal shape producing distinct lamellipodial protrusions (Fig. 2 a,c,d,e). In contrast, HM3M cells were rounded (Fig. 2 b,f,g,h) and grew significantly slower than HMP cells although they had a higher affinity to the surface of culture dish. HM3M* cells were allowed to migrate to the bottom of the well (see Materials & Methods) and started to form pseudopods like structure (Fig. 2 i,j,k).

Actin cytoskeleton was visualised in both parental and selected cells populations in confocal fluorescence microscopy after staining with rhodamine-conjugated phalloidin (Fig. 2 d,g,j) and by immunofluorescent technique with application of β-actin monoclonal antibodies (Fig. 2 c,f,i). Similarly to our former observations (22) hepatoma Morris 5123 cells - either parental or selected didn't form any stress fibres. In both populations (HMP and HM3M) actin was dispersed within the whole cell body and created a meshwork. In addition, in rounded HM3M cells actin was rather concentrated under the cell membrane (Fig. 2 f,g,h). In elongated HMP cells, showing some protrusion, actin microfilaments were diffused in the middle of cell body (Fig. 2 c,d,e). These clinch-shaped protrusions are clearly visible in HM3M* cells and appear to be very abundant in filamentous actin (Fig. 2 i,j,k).

The differences in the organization of actin cytoskeleton in parental and selected invasive cells have shown the involvement of β-actin isoform in the changes of locomotion abilities and morphology accompanying the process of invasion. We observed a very dense subcellular congestion of this isoform especially in invasive cells. The results on visualization of filamentous actin staining were compared with those for β-actin staining. Distinct β-actin submembrane localization is clearly noticeable in rounded HM3M cells forming a cortical ring (Fig. 2f) in contrast to the parental cells where β-actin concentrates in cell body and leading edges (Fig. 2c). Very dense β-actin architecture was especially clear in pseudopods of HM3M* cells, showing very dense network of actin (Fig. 2 i).

Cytosolic fraction, isolated from the parental and highly invasive subpopulations of hepatoma Morris 5123 cells was used to determine the level of β-actin expression. Proteins were separated by SDS/PAGE and β-actin was identified by Western blotting analysis with a monoclonal antibodies recognizing this isoactin. Quantitative blot analysis was done by densitometry after three
experimental cycles, each performed for each of three tumor biopsies (Fig. 3). A significantly higher level of β-actin was observed in the cytosol fraction of selected HM3M cells subpopulation.

To investigate whether there is any correlation between invasive capacity of tumor cells and the state of actin polymerisation, monomeric and total actin levels were measured in the cytosolic fractions of selected hepatoma Morris 5123 subpopulations by DNase inhibition assay (24). Filamentous actin and the state of actin polymerisation were then calculated as described in Materials & Methods. The data are presented in Fig. 4. The only statistically significant change was found in the ratio of filamentous to monomeric actin (F:G) in HM3M cells population of higher invasive ability.

**DISCUSSION**

Cell motility is a complicated process that involves dynamic rearrangements of actin cytoskeleton. The cell movement is driven by contractile forces generated by actin polymerization (8, 9, 28). This molecular machinery is controlled by a network of signals and action of actin binding proteins (2, 9, 28). Motile cells are key players in many biological functions of the organism such as immune response, wound healing and development of the organism (5, 6). Any alterations in the proper functioning of actin cytoskeleton may lead to the disease (29). Deregulated and enhanced tumor cells motility is lately most attentively studied disorder based on cell migration (1, 2). In contrast to tumor itself, metastases that invade and destroy normal tissue architecture have major contribution to cancer progression and a cause of patient's death (30).
The mechanisms that trigger an acquisition of invasive phenotype are not completely characterized yet nor are the basis of different types of migration. There are two major types of tumor cell migration identified so far (31, 32). Both types require an accession of protrusive structures. They can take a form of lamellipodia and filopodia that are characteristic for mesenchymal type of movement or pseudopods and invadopodia that were identified to take part in ameboidal movement (32 - 34). Because actin cytoskeleton drives protrusions it could be suspected that overexpression of actin could increase cell motility (13, 14). Consistently, it was proved that there is a correlation between the localization of β-actin mRNA and metastatic potential of cancer cell (35). Moreover, delocalization of β-actin mRNA from leading edge is followed by the loss of cell polarity and directional movement (36).

Fig. 4. Changes in actin level and the state of polymerisation in parental and selected hepatoma Morris 5123 cells. Actin was measured as an inhibitor of DNase I from bovine pancreas. Actin concentration was expressed as arbitrary units per mg of sample protein. The different actin forms and the state of polymerisation (F:G) were determined as described in Materials and Methods. Bars represent the mean for data obtained from three independent experiments. *Indicates values significantly different from those for control cells as calculated by Student's t-test, P < 0.05.
Our studies on hepatoma Morris 5123 cells have shown that increased cell locomotion is accompanied by increased level of β-actin and produces a qualitative change in migratory mechanism especially in the type of cell extensions. Many questions have been arised regarding the issue of intracellular distribution of actin isoforms and the existence of isoform specific actin binding proteins (ABP). Different regulatory proteins are able to distinguish between one actin isoform from another, thereby causing assorted regulation of actin polymerization dynamics and their recruitment to specific cellular localization (2, 5). Thus, we observed different organization of β-actin microfilaments and their subcellular distribution in highly motile cells what may probably occur due to altered actin binding proteins expression (33). It appears that higher level of β-actin in invasive tumor cells could be one of the parameters allowing cell to invade Matrigel in ameboidal manner by the mode of bleb-like structures not a classical lammellipodium.

The role of actin binding proteins (ABP), their up and down regulation in many types of cancer cells in relation to actin cytoskeleton organization is under current investigation (2, 28). They may also participate in the observed increased state of actin polymerization measured as filamentous to monomeric (F:G actin ratio) in highly HM3M motile cells. Actin didn't form any stress fibers in parental population as well as in selected one. Moreover, in rounded highly motile HM3M cells filamentous actin was concentrated under the membrane, forming a subcortical ring, whereas in outstretched polygonal cells actin was dispersed within the cell body. Special attention should be focused on the results of studies on actin expression in invasive and non invasive cancer cells. Some data on the contribution of rounded bleb-like type of movement in the process of cancer progression are still accumulating (33). They are consistent with our former observations on human colon adenocarcinoma cell sublines characterized by different metastatic potential, where the correlations between metastatic potency, the state of actin polymerization, actin cytoskeleton organization and β-actin expression were also seen (14, 15). The behavior and morphology of selected HM3M cells resembles EB3 subline of human colon adenocarcinoma cells (derived from LS180 epithelial line) as well as KINE lung cancer cells (derived from non-small cell lung cancer H460 line) lately isolated by Fok et al. (16).

Moreover, selected HM3M* cells (see Materials & Methods) tent to push distinct pseudopodial protrusions rich in actin. Perhaps they could be regions of a stronger attachment where focal adhesions are already acquired or a newly lammellipodium is formed. Several models of types of motility were suggested to unravel the phenomenon of tumor progression (32, 35, 37). Tumor cells turned out to be a very flexible enemy. They are probably able to switch between modes of motility migrating on different substrates (18, 31, 32). Because there is no one model to describe cell motility and this process was shown to depend on cell type and the environment it is so difficult to combine efforts in effective therapy.

It is well documented that a complex network of signals associated with extracellular matrix digestion and cytoskeleton architecture remodeling have to
be mobilized by cancer cells (9) to get through the barrier of vessel wall in the process of intravasation and extravasation. In addition to this, we have demonstrated a correlation between invasive capacity of the cancer cells, higher level of β-actin and the state of actin polymerization. The considerable rounding of the cell shape was accompanied by the reorganization of the actin cytoskeleton structure - including noticeable, subcortical condensation of β-actin. In summary, it was the first attempt to isolate the variant population of hepatoma Morris 5123 cells. The selected fraction of tumor cells differs from the parental population in distinct manner. The selected hepatoma Morris 5123 cells should be implemented back to experimental rats to examine the possible changes in their invasiveness and metastatic capacity. Our research should widen the knowledge on molecular basis of tumor growth and metastases formation as well as answer the question if tumor malignancy is connected with the changes in β-actin expression and actin cytoskeleton reorganization. This could facilitate in future in the construction of anti-tumor drugs affecting cell motility.

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