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STUDIES OF FIBRONECTIN-CANCER CELLS INTERACTIONS UNDER STATIC AND DYNAMIC CONDITIONS. DIFFERENT ADHESIVE BEHAVIOR OF HIGH METASTATIC AND LOW METASTATIC SUBCLONE OF NCI-H460

Maneva-Radicheva L[1]., Zlateva N.,[1] Kostadinova A.[1], Dimoudis N. [2], Altankov G. [1]

[1] Bulgarian Academy of Sciences, Institute of Biophysics, Department of Cell Adhesion, Acad.G.Bonchev Str. Bl.21, 1113 Sofia, Bulgaria

[2] Roche Diagnostics GmbH, Pharma Research Penzberg, Nonnenwald 2, D-82377 Penzberg, Germany

Author for correspondence:
Prof. Dr. G. Altankov
Institute of Biophysics
Bulgarian Academy of Sciences
Acad. G. Bonchev Str. Bl. 21
1113 Sofia, Bulgaria
Phone: +359-29792334
FAX: +359-297112493
E-mail: altankov@obzor.bio21.bas.bg

ABSTRACT. Metastatic tumor spread is pathologic process with series of adhesion/de-adhesion events. In this context, adhesion to, and detachment from the components of the ECM could be important measure for the invasive potential of cancer cells. Low (NCI-H460) and high (H-460-M2) metastatic lung carcinoma cell line were used to study the rates of adhesion to fibronectin under static and dynamic conditions. To measure the critical share force resulting in detachment of cancer cells from the protein-coated substrata, a special flow chamber was applied. Morphological immunofluorescence studies have been performed to better characterize the cellular adhesive interactions in static conditions. Thus, we found correlation between adhesive behavior and metastatic potential of cancer cells in respect of fibronectin in static and dynamic conditions: *(i)* In static condition both cell lines have the highest affinity to fibronectin in comparison to other matrix proteins

and preferably adhere to it. This affect was more pronounced for HM cells*; (ii)* in dynamic conditions, the low metastatic variant NCI-H460 showed weaker adhesiveness to fibronectin and subsequent instability to the flow rate; *(iii)* overall morphology of HM and LM cells showed differences between cell shape, cell spreading and the organization of focal adhesive contacts (vinculin); *(iiii)* the two subclones differ also in αvβ3 integrin expression, as HM cells demonstrated higher αvβ3 integrin receptor distribution on cell surface. Our results provide new evidences that in some distinct stage of invasion and metastasis tumor cells might perform different adhesive features.

KEY WORDS. cell adhesion, metastatic potential, fibronectin, integrin receptors

INTRODUCTION

An important feature of malignant cells is the invasive growth. This allows them to leave the compartment to which they normally are restricted, gain access to the connective tissue and the blood vessels and to complete the initial phase of the process of metastasis [1]. Metastasis is a multistep process that includes detachment of cells from the tumor mass, degradation of basement membranes, migration, adhesion and proliferation at the second site [2]. All these steps require series of cell adhesive interaction or loss of adhesion [3]. Tumor cell adhesion to the components of the extracellular matrix (ECM) and basement membranes is mediated by specific cell surface receptors, integrins [4] that bind to ECM adhesive proteins such as fibronectin (FN) [5], laminin and collagens [6]. Fibronectin is an adhesive heterodimeric glycoprotein present in the ECM of connective tissues in disulfide cross-linked insoluble fibrils and in the blood in dimeric soluble form [7]. *In vitro* transformed and tumor-derived cells failed to deposit a matrix, whereas the normal counterparts do have matrix [8, 9]. The addition of FN to tumor-derived cultured cells improves cell adhesion and induces ECM and cytoskeleton organization, supporting the normal cell morphology, FN has bee associated with the normal cell phenotype [10, 11]. The main FN-receptor, α5β1 is suppressed on the surface of cancer cells and predominantly they adhere via αVβ3-integrin receptor, known as integrin receptor for vitronectin [11, 12]. The integrin repertoire expressed in tumor cells differs also to that of their non-neoplastic counterparts [9, 10, 11] and the most consistent change accompanying tumor progression being the shift to expression of αvβ3 integrins [12-18]. It is well documented that cancer cells alter their adhesive properties to the extracellular matrix components [9-11, 18-21], and, particularly, the loss of adhesion to fibronectin is associated with neoplastic changes [7-18]. The present study is focused on the possible differences in the properties of cancer cells adhered to immobilized fibronectin, under static and dynamic conditions. For this purpose we chose NCI-H460 lung carcinoma cell line because of the existing of high metastatic subclone H-460-M2 [22]. Details of this investigation are reported herein.

MATERIAL AND METHODS

Cell Cultures

The low metastatic (LM) human lung carcinoma cell line H460 was purchased from NCI (Frederick Cancer Research Facility, Frederick, MD, USA; #503473), the high metastatic (HM) subclone H460-M2 was obtained from Corti et al, after intrasplenic injection in nude mice (from Novus Pharma, Monza, Italy). The cell lines were cultured in RPMI-1640 (RMB; #209945) with 10% FBS and 2 mM L-Glutamine and the cells were harvested with Trypsin-EDTA during the log-phase growth according to standart protocols [23] before seeding on FN-coated surfaces (see below).

Purifiaction of fibronectin

FN was prepared from human plasma by affinity chromatography on gelatin-Sepharose 4B columns according to the method of Engvall and Ruoslahti and stored in 4M urea. Before use, the FN was transferred to 120 mM NaCl (Boeringer), 50 mM Tris, pH 7, 3 by gel filtration with Sephadex G25, and stored at 4ºC.

Fibronectin Coating

FN was dissolved in PBS phosphate buffered saline of the following composition: 150 mM NaCL, 5, 8 mM Na_2HPO_4, pH=7, 4. The final concentration (20 μg/ml) was chosen to ensure surface saturation using protein adsorption data from the literature [23]. The adsorption procedure was performed as follow: glass coverslips (22x22 mm, Assistent, Germany) were placed in 6 well tissue culture plates (Costar) and coated with 20 μg/ml FN for 30 min at RT^o. Then the plates were washed with PBS three times and 1 ml suspension of $5x10^5$ HM or LM H460 cells was added left to spread for 4h in humidified CO_2 incubator. This protocol was used for study the static adhesion, as well as for immunofluorescent visualization of vinculin, and αvβ3-integrin receptor staining, respectively.

Overall cell morphology-vital staining with fluorescein diacetate (FDA)

For this purpose, 5 μl FDA (Sigma) diluted in acetone (concentration 5mg/ml) was added directly to the medium of preliminary adherent cells for 4 h. After 1-2 min incubation at 37ºC the cell media was removed and washed with RPMI1640. We observed and digitalized the pictures using digital camera PIXERA, adapted to inverted fluorescent microscope Axiovert, Zeiss. The images were processed via Pixera Softwear.

Cell Adhesion Assay

Glass coverslips were placed in 6 well tissue culture plates (Costar) coated with FN as above, as well as with collagen type I (concentration 100 μg/ml in 0,1% acetic acid), collagen type IV (concentration 50 μg/ml in 0,1% acetic acid), and laminin (concentration 10 μg/ml in PBS, pH=7,4). $5x10^4$ LM or HM H460 cells suspended in 1 ml RPMI medium containing 10% serum were added and leaved to attach for 4 h at 5%/CO_2/95% air humified atmosphere. Cell adhesion was quantified

via counting the mean cell number in 15 randomly chosen 10x10 mm squares on three low magnification (10x) pictures per sample. The squares were superimposed on the images using LSM 530 software. Statistical analysis was carried out using GraphPad InStat software and data are expressed as means and standard deviation (S.D.).

Measurment of cell detachment under flow conditions

In previous study [23] we have useded special flow chamber for studying the strength of lymphocyte attachment to different protein-coated substrata. Here we apply the same method to characterize the tumor cells interaction with FN. The general construction of the flow chamber has been described elsewhere [24]. Briefly, PBS from an elevated reservoir was passed through a rectangular flow channel in which a glass coversleep formed the upper wall. The flow rate through the chamber was regulated by means of precision micrometer flow valve (Nunpro type M, purchased from North London Valve and Fittings, London, UK). Under the experimental conditions, laminarity of flow was confirmed both theoretically and practically for the conduit [24]. In this study the applied wall shear τ_w was calculated from the volumetric flow rate *Q* using the following equation:

$$\tau_w = -\mu \times \frac{\Delta V}{\Delta y} \tag{1}$$

with μ as absolute viscosity of the liquid and $\Delta v/\Delta y$ as shear rate. Since the velocity *V* of the liquid equals:

$$V = \frac{\Delta p}{2 \times \mu \times L} \times (b^2 - y^2) \tag{2}$$

with $y=2b$ as separation distance of the flow chamber and Δp as pressure drop along the conduit with length *L*, the shear rate $\Delta v/\Delta y$ is then:

$$-\frac{\Delta V}{\Delta y} = \frac{b \times \Delta p}{\mu \times L} \tag{3}$$

The volumetric flow rate *Q* was estimated as a function of flow valve position, keeping the hydrostatic pressure constant. The volumetric flow rate *Q* equals:

$$Q = \frac{2 \times \Delta p \times b^3 \times w}{3 \times \mu \times L} \tag{4}$$

with w as chamber width. Using Equations 1, 3, and 4, the applied wall shear force was calculated as follows:

$$\tau_w = \frac{3 \times Q \times \mu}{2 \times b^2 \times w} \qquad (5)$$

With this experimental set-up shear forces up to 10 N/m^2 were achieved.

The detachment measurements were carried out by injecting suspensions from HM or LM tumor cells (5x10^5 cells in serum–free RPMI1640 medium) into the preliminary warmed chamber so the cells could sedimentate on the pre-treated with FN coverslips. The chamber was incubated for 4 h at 37° C to accomplish the adhesion process. At the end of incubation the chamber was inverted so that weakly bound (dead) cells could detach from the cover slips due to the gravitational force. Counting the cells was performed by phase contrast microscopy. The first count was done after 5 min and used as control value. By opening the flow valve the attached cells were subsequently exposed to increasing shear force for 2 min. During this time a selected areas in the middle of the measuring chamber were observed to follow the detachment process. The flow was stopped and the actual number of remaining cells estimated. A special ocular micrometer scale has been used to count the cells in 4 different squares. Cell detachment was calculated in percent for each point investigated.

Immunofluorescent stainings

Immunofluoresecent stainings were performed according to standard protocols applying anti-vinculin antibodies to the 3% paraformaldehyde (5 min) fixed and permeabilized cells with 0.5% Triton X-100 (Pharmacia Biotech, Sweden) (see below Table 1). To study the organization focal adhesion contacts, glass coverslips were placed in 6 well tissue culture plates (Costar) coated with FN as above. The cells were incubated incubator in medium containing 10% serum for 4 h, as above, in a humidified CO_2. To study the organization of $\alpha v\beta 3$ integrin the fixed and permeabilized cells were stained as described in Table 1. For negative control experiments the primary or the secondary antibodies were omitted.

Table 1. Immunofluorescent staining protocols (section Materials&Methods)

Antigen	**Primary antibody**	**Dilution**	**Conditions**	**Pretreatment**	**Secondary antibody**	**Visualization**
Vinculin	Mouse monoclonal antibody from Sigma	1:100	20°C, 30 min	Permeabilization with 0.5% Triton X-100	Goat anti-mouse Cy3 labeled antibody from Jackson Immunoresearch	*Digital camera PIXERA on inverted fluorescent fluorescent microscope Axiovert 25, Zeiss*
Vitronectin receptor	Mouse monoclonal antibody against αvβ3 integrin from Chemicon	1:100	"	yes	"	

RESULTS

Adhesive phenotype of H-460-M2 (HM) and NCI-H460 (LM) cells under static conditions

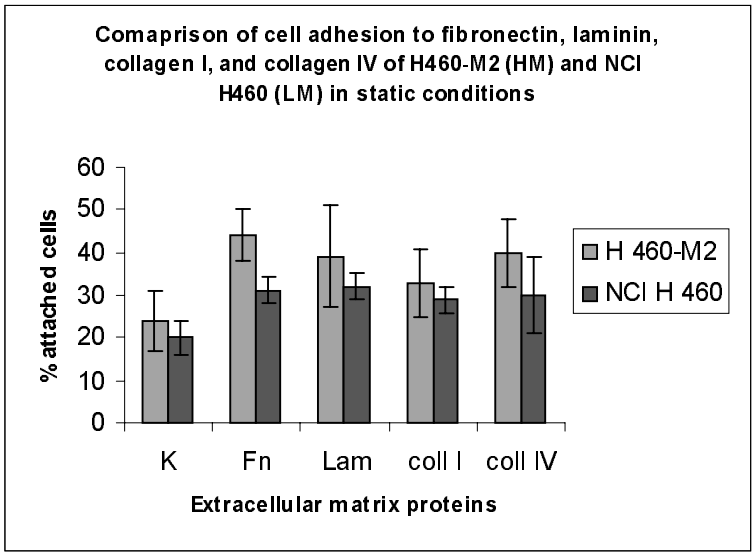

Fig.1. *Comparison of cell adhesion behavior in static conditions. LM and HM subclones of lung carcinoma cell line were seeded on different adhesive proteins such as fibronectin, laminin, collagen I and collagen IV.*

This chart represents the adhesive behavior of the two subclones of lung cancer cells on different extracellular matrix proteins, including (FN), laminin (LN), collagen I (coll I) and collagen IV (coll IV). In comparison to the control (plain glass without pre-coated protein), both cell lines show clear tendency to adhere well on these proteins, but fibronectin appears to be preferable adhesive subtsrate, particularly for HM subclone. This is was an additional reason to focus our further studies on adhesive interactions of cancer cell with FN.

Overall cell morphology of H-460-M2 (HM) and NCI-H460 (LM) cells on fibronectin (vital staining with FDA)

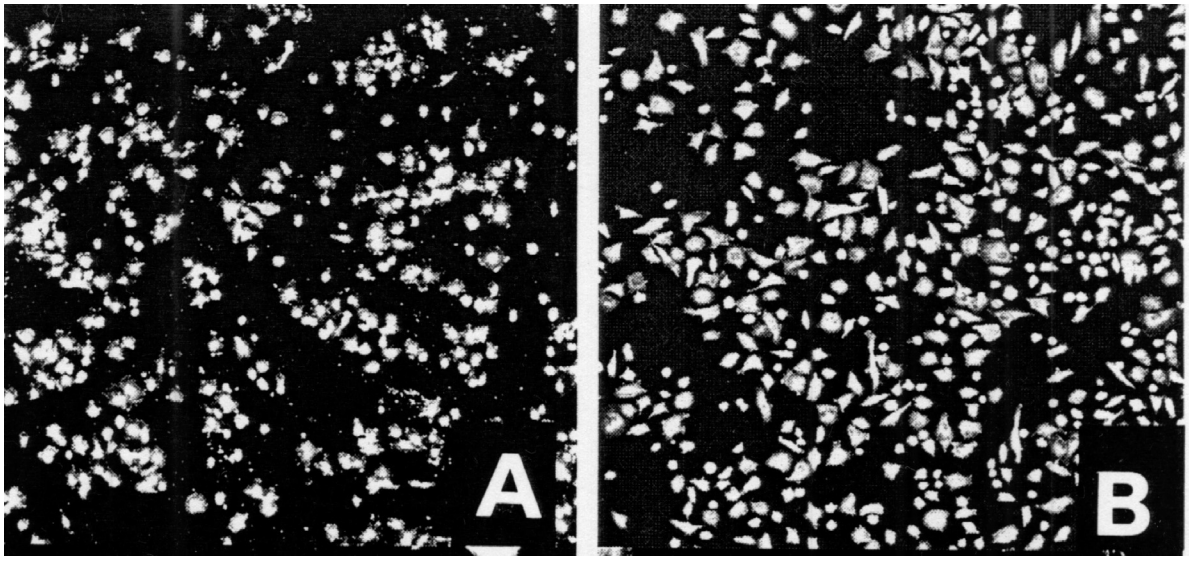

Fig.2 *Overall cell morphology of HM and LM cells. The cells were subjected to vital staining with fluorescein diacetate (FDA) (see Materials&Methods).* **A/** *HM cells tend to form homotypic aggregates;* ***B/*** *Most of the LM cells have polarized cell shape-a typical feature of high motility cells. There are less aggregates, the cells are predominately single and uniformly dispersed on the substrata.*

Vital staining with FDA allows us to compare the morphological characteristics of HM and LM cells. HM (H-460-M2) cells are rounded and tend to form homotypic aggregates, whereas the LM (NCI-H-460) cells are elongated and show clear tendency for polarization. Since polarized cell shape is not thypical for this cell type we refer it to increased motility of the LM cells that could be a sign for acquisition of invasive phenotype.

Organization of focal adhesion contacts of H-460-M2 (HM) and NCI-H460 (LM) cells cultured on FN (vinculin staining)

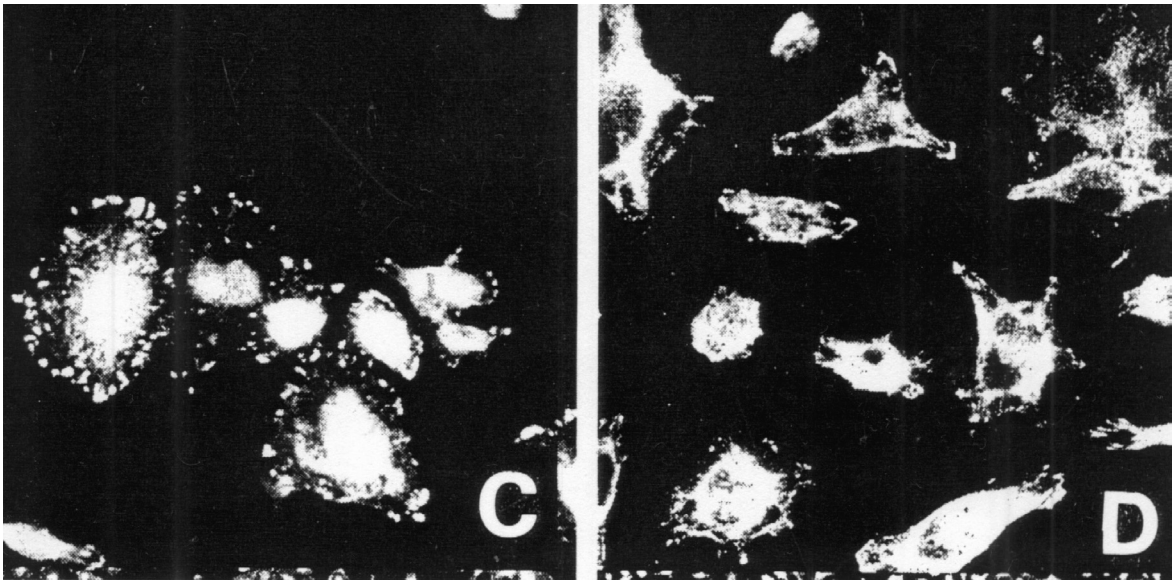

***Fig 3**. Visualisation of focal adhesion contact via vinculin). **3C**/H460-M2 (HM) cells demonstared better developed focal adhesive contacts **4D/** NCI -H460 (LM) cells seems to be less adhesive and elongated. High magnification 100x.*

The cells adhere to the substrate by focal adhesive contacts, formed via interactions among the integrin receptor protein, ECM-ligands and cytoskeleton. Therefore, their organization is an important criterion for cell-substrate interactions. In order to explain the observed differences in the overall cell morphology we performed immunofluorescent staining for vinculin, an obligatory persisting protein involved in focal adhesive contacts. Our results suggest that HM cells are more adhesive to FN, because of the higher number of focal adhesive contacts (more intense staining, see Fig. 3D), focused at the cell edges, in comparison to the LM subclone (Fig3C). We speculate that this difference in focal adhesion contacts reflecting the stability of attachment to FN could refer to the mechanisms of metastasis. These result confirmed the data shown above for static adhesion.

Organization and surface expression of αvβ3 integrin receptor of H-460-M2 (HM) and NCI-H460 (LM) cells cultured on FN

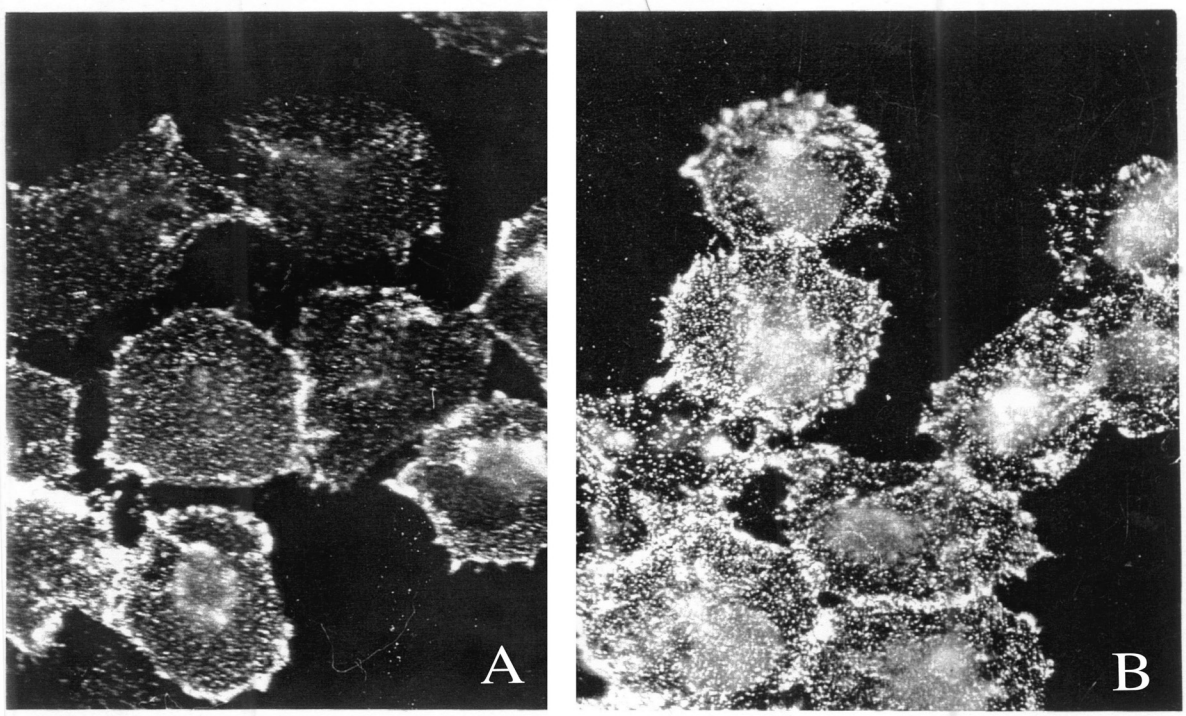

Fig. 4. *Cell surface expression and organization of αVβ3 integrin receptor proteins of HM and LM cells on FN. High magification 100x.* ***A****/LM cells shows lower expression and diffuse distribution of αVβ3 integrins (weaker signal)* ***B****/HM have better expression and tend to cluster in the points of focal adhesion contacts (intense staining)*

To study which integrin receptors are involved in cell adhesion of HM and LM cells to FN we performed immunofluoresent visualization to study the surface expression and organization. First, we stained cells for the main FN-receptor α5β1, but it was very low signal (data not shown). As it was reported before (Corti et al., 1996), however, there is a not significant difference in expression of α5β1-integrin receptor, proved via FACS analysis. Therefore, we assumed the participation of alternative integrin receptor for FN that could be responsible for the adhesion of cancer cells, and stained for αVβ3 integrin receptor. Moreover, it have been reported that increasing of malignancy is associated with increasing in expression of αVβ3 integrin receptor [16-19]. Most likely reason of that is the participation of αVβ3 integrin in MMP's-activation, enzymes that are responsible for ECM degradation in both normal and pathological processes, such as tissue regeneration, or neoplastic transformation. In our study, differences in expression and organization of HM and LM cells have been obtained. LM cells demonstrated lower expression, lack of organization to the cell borders, and diffuse distribution of αVβ3 integrins at the cell surface (weaker fluorescent signal, see Fig.4A), and almost do not cluster in cell periphery, suggesting particular or missing involvement in focal adhesive contacts. HM, conversely, have higher expression and clear tendency to cluster in the points of focal adhesion contacts (intense staining, see Fig. **4B**)

Detachment of LM cell line from fibronectin coated-substrata under dynamic flow conditions

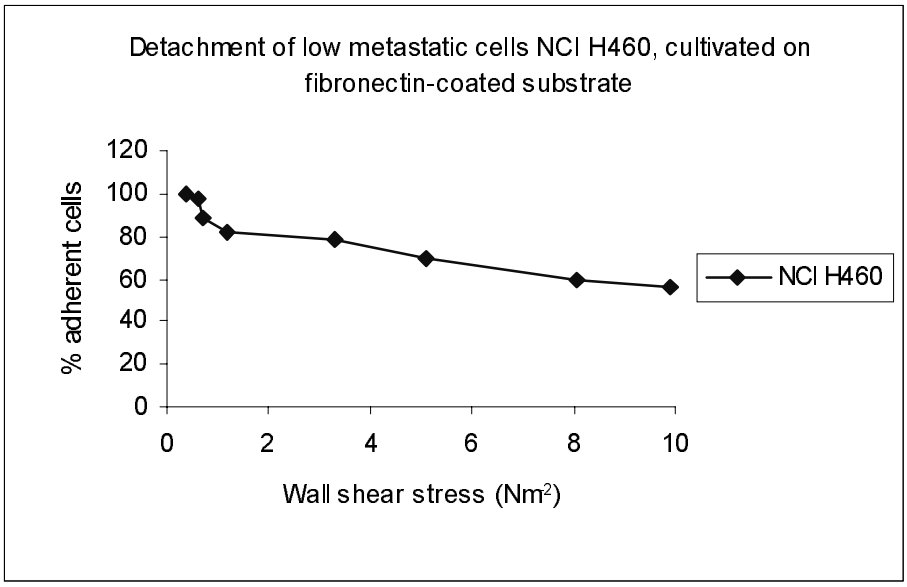

Fig. 5. *Deatchment of NCI -H460 (LM) from FN under dynamic flow conditions*

The LM (NCI-H460) again demonstrated weaker adhesive interaction as in static adhesion assay. Approximately only half of the cells remains attached to the FN-substrate (56%) after applying the maximum shear force (10 N/sm$^{2)}$).

Cell detachment of LM cell line from fibronectin coated-substrata under dynamic flow conditions

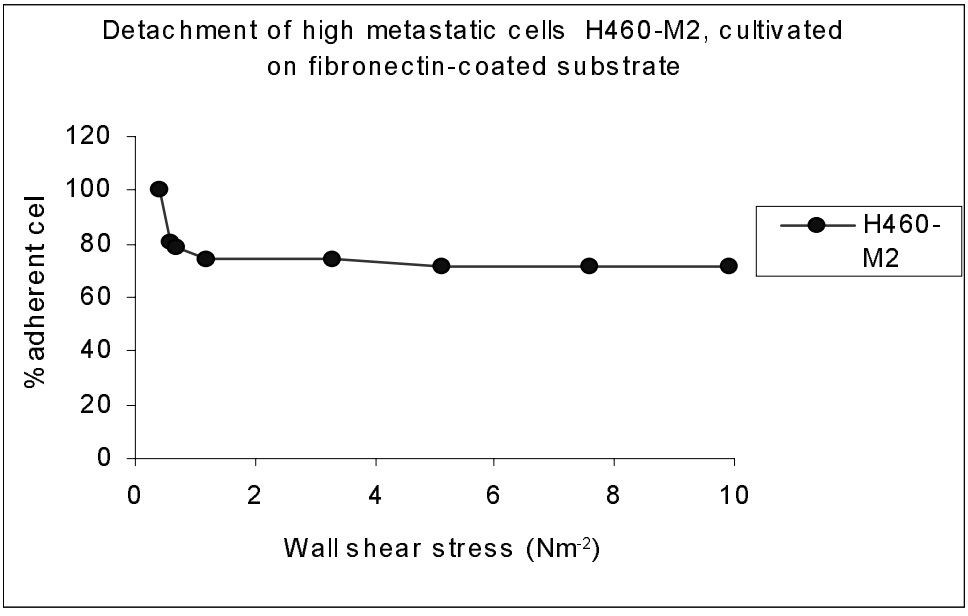

Fig.6. *Deatchment of H460-M1 (HM) cultured on FN under dynamic flow conditions (see Materials&Methods).*

This chart demonstrates the detachment kinetics of H460-M1 (HM) cell with increasing of the dynamic flow rate. The percent of adherent cells remaining on the substratum at the maximal shear force (10 N/sm²) is significantly higher, more than 73%, suggest better interactions with FN in the comparison to the LM cells.

DISCUSSION

The location of secondary tumors is determined, in part, by anatomical blood flow and by interactions between tumor cells and host organs [1, 2]. The adhesive molecules, such as integrin receptors and matrix proteins are involved in the tumor progression. These adhesive interactions are between the tumor cells themselves, as well as between tumor cells and the target tissue [13]. The interaction with fibronectin seems to play important role, because it reported to be strongly influenced by the process of carcinogenesis [3-5]. It is difficult to follow these adhesive interactions *in vivo*, and in this work we developed a model cell system that is closer to in vivo conditions. The cell line used in this investigation provides another advantage, an opportunity to compare adhesive behavior of cells with different metastatic potential in vivo.

Our data demonstrated that high metastatic cells have higher affinity to FN. This results is contradictory to the expectations that HM cells should be less associated to the matrix, and hence, easier to leave the place of primary tumor. Thus, it became obvious that cancer dissemination (metastasis) reflect not only the invasion it self. The penetration of basal lamina, degradation of ECM and persistence in the blood circulation are necessary, but are not enough to allow the cancer cell to gain the distant target organ.

On the other hand, metastasizing cancer cells need to be attached, hence to increase its adhesiveness to the ECM. This is why integrin-mediated interactions between tumor cells and ECM in particular host organ thought to be important for organ-specific metastasis formation. Indeed, our results demonstrate that in lung carcinomas αvβ3-integrin expression differs significantly depending on the metastatic potential. Thus, we hypothesize that HM subclone H-460-M2 stucked more successfully to some tissues because of its higher expression of intergrins that can bind to FN. However, to gain distinct organs, cancer cells must invade the basal lamina of the primary tumor and that is its first obstacle. In this stage of invasion matrix metalloprotease (MMP) seems to play crucial role since they degrade ECM and "create a path" for cancer cells to move forward. This fact offers a possible explanation of some puzzling results in clinical trails of using MMP-inhibitors, showing unexpected increased in the number of micrometastasis in vivo.

We also obtained morphological differences between high and low metastatic variants, adhering on fibronectin that is another sign for differences in their in vivo behavior. The high metastatic cells are round shaped and tend to form homotypic aggregates, whereas the low metastatic cells are polarized presumably in attempt to develop a motile morphology that is another sign for higher invasive abilities.

Hydrodynamic shear forces also appear to influence adhesive properties of lung carcinoma cells. Under dynamic flow conditions the low metastatic NCI-H460

cells show weaker adhesiveness to fibronectin that might result in the subsequent instability to the flow rate.

In conclusion, our results point to the possibility that in some distinct stage of progression tumor cells gradually change their adhesive phenotype. It likely appear in the moment of "switch" from invasion to metastasis, reflecting the series of adhesion-de-adhesion events of cancer cells with surrounding ECM matrix.

CONCLUSIONS

1. In static conditions both high metastatic and low metastatic cells adhere to FN, but the HM subclone demonstrated higher affinity in comparison to the low metastatic variant.

2. In dynamic conditions the low metastatic variant shows weaker adhesiveness to fibronectin presumably reflecting their subsequent instability to the flow rate in vivo

3. We obtained morphological differences between high and low metastatic variants adhering on fibronectin. High metastatic cells are round shaped and tend to form homotypic aggregates, whereas the low metastatic cells are polarized presumably as an attempt to developed motile (invasive) morphology.

4. The high metastatic subclon showed higher expression and better organization of αVβ3-integrin that could refer to their higher metastatic potencial *in vivo*.

5. A new model has been developed for studying the adhesive behavior of cancer cells that could be useful for *in vitro* testing of anticancer drugs.

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