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Functional differences between two morphologically distinct cell subpopulations within a human colorectal carcinoma cell line

A.C.C. Solimene[1,2], C.R.W. Carneiro[2], I. Melati[2] and J.D. Lopes[2]

[1]Fundação Antônio Prudente, São Paulo, SP, Brasil
[2]Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil

Correspondence
C.R.W. Carneiro
Departamento de Microbiologia, Imunologia e Parasitologia
EPM, UNIFESP
Rua Botucatu, 862, 4º andar
04023-900 São Paulo, SP
Brasil
Fax: +55-11-572-3328
E-mail: crwcarneiro@ecb.epm.br

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Abstract

The LISP-I human colorectal adenocarcinoma cell line was isolated from a hepatic metastasis at the Ludwig Institute, São Paulo, SP, Brazil. The objective of the present study was to isolate morphologically different subpopulations within the LISP-I cell line, and characterize some of their behavioral aspects such as adhesion to and migration towards extracellular matrix components, expression of intercellular adhesion molecules and tumorigenicity *in vitro*. Once isolated, the subpopulations were submitted to adhesion and migration assays on laminin and fibronectin (crucial proteins to invasion and metastasis), as well as to anchorage-independent growth. Two morphologically different subpopulations were isolated: LISP-A10 and LISP-E11. LISP-A10 presents a differentiated epithelial pattern, and LISP-E11 is fibroblastoid, suggesting a poorly differentiated pattern. LISP-A10 expressed the two intercellular adhesion molecules tested, carcinoembryonic antigen (CEA) and desmoglein, while LISP-E11 expressed only low amounts of CEA. On the other hand, adhesion to laminin and fibronectin as well as migration towards these extracellular matrix proteins were higher in LISP-E11, as expected from its poorly differentiated phenotype. Both subpopulations showed anchorage-independent growth on a semi-solid substrate. These results raise the possibility that the heterogeneity found in the LISP-I cell line, which might have contributed to its ability to metastasize, was due to at least two different subpopulations herein identified.

Key words
- Carcinoembryonic antigen
- CEA
- Colorectal carcinoma cell line
- Laminin
- Fibronectin
- Desmoglein
- Metastasis

Introduction

Colorectal neoplasia is the second main cause of death by cancer in developed countries (1). In Brazil, it occupies fifth place among the most common forms of cancer, being responsible for an estimated death rate of 5.05% of all cancer patients for 1999 (2). Most of the knowledge about the genetic basis of the tumor and its drug sensitivity came from *in vitro* studies in which cancer cell lines were used. Although there are numerous colorectal cancer cell lines (3-5), they differ in many aspects due to the diversity of human tumors.

Heterogeneity is also found within the same tumor cell mass (6), this being the major barrier to the successful treatment of

metastases (7). Cells from individual tumors exhibit differences with respect to cell surface constituents, immunogenicity, growth rates, karyotype, sensitivity to cytotoxic drugs, as well as their ability to invade and metastasize. Heterogeneity is not restricted to cells in primary tumors, being also prominent among metastatic cells (8).

Metastasis is the result of a complex series of events, such as loss of intercellular adhesion, entry of tumor cells into lymphatic and blood vessels, survival in the circulation and arrest at the target organ. Several mechanisms are implicated in the process, including changes in adhesion properties that allow tumor cell detachment and migration (9,10). Adhesion of metastatic cells to the underlying extracellular matrix is very important (11), and the acquisition of metastatic competence by clones of cells with growth advantages is thought to be associated with an ability to coordinate the adhesive properties in every step of metastasis.

Lopes and colleagues (unpublished data) isolated a human colorectal cell line from a hepatic metastasis at the Ludwig Institute, São Paulo, SP, Brazil. This cell line (LISP-I) has been studied and compared to other established human colorectal cell lines (4,12-14) and has been shown to be resistant to growth stimulation by interleukins-1 and -6 (4) as well as by insulin-like growth factors-I and -II or transforming growth factor-α (12). In parallel, this lineage was shown to be susceptible to natural killer cell-mediated lysis (14), which is considered to be the most important immune mechanism for the destruction of metastatic cells. Taken together, these data indicated that a better understanding of LISP-I biology would be a new option for studying the metastatic phenotype. The purpose of the present study was to investigate some behavioral aspects of LISP-I based on its heterogeneous morphology. *In vitro* growth rate, expression of two cell adhesion molecules, interaction with two extracellular matrix proteins and *in vitro* tumorigenicity were the parameters chosen for comparison between two morphologically distinct isolated cell populations within the LISP-I cell line.

Material and Methods

Cell culture condition and cloning

The LISP-I cell line was kindly provided by Dr. R. Brentani, Ludwig Institute for Cancer Research, São Paulo, SP, Brazil. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% FCS (Cultilab, Campinas, SP, Brazil), 10 mM HEPES (Inlab, São Paulo, SP, Brazil) and 40 µg/ml gentamicin (Schering, São Paulo, SP, Brazil) at 37ºC in a 5% humidified CO_2 incubator until 80% confluence. Adherent cells were washed with PBS and detached by trypsinization (0.2% trypsin-0.02% EDTA, Instituto Adolfo Lutz, São Paulo, SP, Brazil). To obtain single cell-derived clones, cell suspensions were submitted to limiting dilution in 96-well culture plates (Costar, Cambridge, MA, USA).

Growth rate determination

Growth rate studies were carried out on single-cell suspensions derived from log phase cultures. LISP-A10 or LISP-E11 cells were plated in duplicate (10^4 and 3 x 10^4) in 24-well tissue culture plates. After 24 h, attached cells were washed, removed by trypsinization and counted by vital die exclusion with 0.4% Trypan blue (Sigma Chemical Co., St. Louis, MO, USA). Counts were performed every 24 h until day 6 and population doubling rates were calculated from exponential growth curves.

Flow cytometry

LISP-A10 and LISP-E11 were analyzed for cell surface expression of desmoglein

and carcinoembryonic antigen (CEA) as follows. Cells were harvested by trypsinization as described, fixed with 1% paraformaldehyde in PBS, washed and incubated for 1 h in the presence of 10 µg/ml anti-desmoglein mAb (Boehringer-Mannheim Corp., Mannheim, Germany), 60 µg/ml anti-CEA mAb (15) or 60 µg/ml unrelated antibody (pre-immune mouse IgG) at room temperature. After washing with 1% BSA (Inlab) in PBS, cells were resuspended in 200 µl of 1:50 (v/v) FITC-goat anti-mouse IgG (Sigma) in PBS and incubated for 45 min at room temperature. Cells were washed twice with 1% BSA (w/v) in PBS, resuspended in 500 µl PBS and analyzed with a FACScan apparatus (Becton Dickinson, San Jose, CA, USA). Dead cells were excluded based on forward and side scatter.

Adhesion assays

The laminin 1-nidogen complex from mouse Engelbreth-Holm-Swarm tumor was a gift from Dr. V.R. Martins, Ludwig Institute for Cancer Research. Fibronectin was affinity-purified from fresh human plasma as described elsewhere (16). Adhesion assays were performed in triplicate according to a classical protocol, with modifications (17). Briefly, 96-well culture plates (Costar) were coated with laminin or fibronectin at different concentrations, but in the same molar ratio, for 2 h at 37ºC. Wells were blocked with 1% BSA in PBS for 1 h at 37ºC and washed with PBS. Cells (5 x 10^4) were suspended in serum-free medium (DMEM) and added to each coated well. After the end of the incubation period (3 h at 37ºC), nonadherent cells were washed off by flushing with PBS three times. Remaining adhered cells were fixed with 100% methanol for 5 min, washed three times with PBS and stained with 0.2% crystal violet in 2% ethanol for 5 min. Wells were exhaustively washed with PBS and the absorbed stain was dissolved in 50% ethanol in a 0.1-M sodium citrate solution. Absorbance was measured at 550 nm.

Migration assays

Migration assays were performed according to Jasiulionis et al. (18), with minor modifications. The outer membrane of Transwell® chambers (Costar) with 8-µm diameter pores was coated with 10 µg/ml of laminin or fibronectin for 2 h at 37ºC, washed with PBS, quenched with 1% BSA in PBS for 1 h at 37ºC and washed again. Cells (2 x 10^5) were suspended in serum-free medium and added to each of the inner wells of the chamber. Cells were allowed to migrate for 16 h at 37ºC in a 5% humidified CO_2 incubator. Chambers were then gently washed three times with PBS and cells were fixed with 100% methanol for 5 min. After washing with water, chambers were stained with 1% toluidine blue (Merck, São Paulo, SP, Brazil) in 1% sodium tetraborate (Sigma) and the remaining cells in the inner compartment were removed by scraping with a cotton swab. The absorbed stain present on membranes was suspended in 1% SDS by incubation for 30 min at 37ºC, and optical density was read at 570 nm. All experiments were performed in duplicate. Results were submitted to statistical analysis by comparing migration towards each protein with migration towards the negative control by the Student *t*-test.

Anchorage-independent growth

Anchorage-independent growth was assayed by suspending 5 x 10^5 cells of both cell clones in 0.6% agarose (Gibco BRL, Gaithersburg, MD, USA) over a 1% agarose base in 6-well culture plates (Costar). Agarose was made up with DMEM supplemented with 10% FBS. Following gellification, additional medium was overlaid on the agarose and the plates were refed every 7 days (19). Colonies were grown until multicell spheroids could be observed.

Results

LISP-I subcloning and growth rate curves

The highly heterogeneous cell line LISP-I was cloned and two morphologically distinct subpopulations were obtained, as shown in Figure 1. The cell clone named LISP-A10 displays an epithelial-like intrapopulational organization, whereas LISP-E11 is a fibroblastoid, with sparse intercellular contacts. After several passages in culture, subpopulations were compared for *in vitro* growth rates. Figure 2 shows that LISP-A10 duplicated within 38.3 h while LISP-E11 duplicated within 30.4 h.

Expression of cell adhesion molecules

Once the subpopulations displayed distinct cellular organization in monolayer cultures, the expression of two cell adhesion molecules, CEA and desmoglein, was investigated by indirect immunofluorescence with specific monoclonal antibodies, and analyzed by flow cytometry. Figure 3 demonstrates that both LISP-A10 and LISP-E11 expressed CEA on their cell surfaces, while desmoglein was present only on LISP-A10.

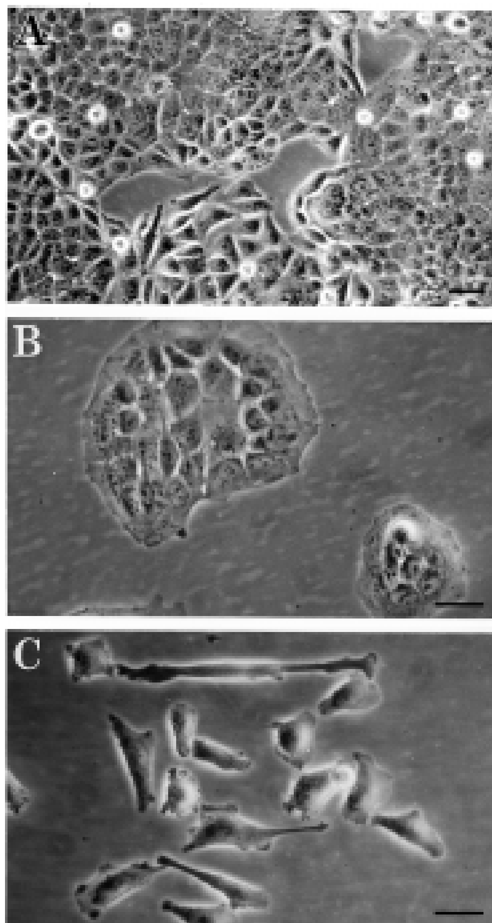

Figure 1. Phase contrast images of monolayer cultures of the LISP-I cell line and its cell clones: LISP-I (A); LISP-A10 (B), and LISP-E11 (C). Bars: 50 µm.

Interactions with extracellular matrix proteins

Colorimetric adhesion assays were employed to evaluate the ability of LISP-A10 and LISP-E11 cells to adhere to laminin and fibronectin. Figure 4 shows that both subpopulations adhered to the two proteins in a dose-dependent manner with a tendency to saturation.

When LISP-A10 and LISP-E11 subpopulations were tested for their specific migration towards the two extracellular matrix components, both lineages were found to be able to migrate. Statistical analysis confirmed the specificity of migration of the two cell populations. Figure 5 shows that larger numbers of fibroblastoid cells LISP-E11 migrated more rapidly than LISP-A10 towards immobilized laminin or fibronectin.

Anchorage-independent growth

To investigate *in vitro* tumorigenicity, LISP-A10 and LISP-E11 were grown in soft agar cultures and Figure 6 shows that LISP-A10 cells formed spheroid-like structures, while LISP-E11 did not, despite their anchorage-free survival.

Discussion

The colorectal carcinoma cell line LISP-I is highly heterogeneous, presenting cell populations with different morphologies. Considering that this diversity may have contributed to the metastatic event, the aim of

the present study was to isolate different LISP-I subpopulations and to investigate some of their behavioral patterns. After cell cloning, the sublineages chosen for comparison displayed distinct morphology patterns, as shown in Figure 1. The epithelial-like population LISP-A10 indicates a more differentiated phenotype when compared to the fibroblastoid clone LISP-E11.

One of the first noticeable different characteristics of the cell clones was their growth in culture, with the fibroblastoid cell clone LISP-E11 duplicating its cell number within a shorter period of time than the LISP-A10 clone (Figure 2). The proliferation rates of cell populations are usually an indication of differentiation status, since poorly differentiated cell lines often proliferate faster than differentiated ones.

Biological heterogeneity is a common feature found among different tumors and, despite the monoclonal origin of most neoplasias, represents the major barrier to the treatment of cancer metastasis (7). The tumor mass contains several cell clones differing in morphology, growth rate and metastatic potential. Genetic and epigenetic mechanisms underlie this heterogeneity (6).

Fewer than one in ten thousand cells from the primary tumor are able to survive in the circulation, which makes metastasization a relatively rare event (20). It is thus considered a result of a selective process, in which heterogeneity could confer adaptive advantages to the tumor mass that escaped from its original organ (21,22).

Multiple sequential steps are required to enable a malignant cell to establish metastases at a distant site (23). As the first step, a tumor cell must be released from the primary tumor by loss of cell-cell adhesion. Intercellular contact is mediated mainly by adhesion molecules that include members of the immunoglobulin, cadherin, integrin, selectin and proteoglycan superfamilies (24). Cell-cell adhesion can also play a major role in the transduction of transmembrane signals, regulating cell differentiation, motility and fate. Thus, interactions among cells result in structural and regulatory functions (25).

CEA belongs to the immunoglobulin superfamily and is a widely known tumor marker that has been shown to play a role as a homotypic intercellular adhesion molecule (26,27). Also, CEA has been implicated in the development of hepatic metastases from colorectal cancers (23,28). It has been shown that poorly metastatic human colon cancer cell lines become highly metastatic when transfected with the cDNA coding for CEA by a mechanism not yet fully understood (29). To verify whether the epithelial morphotype of LISP-A10 was associated with higher CEA expression, immunodetection by flow cytometry was performed. Indeed,

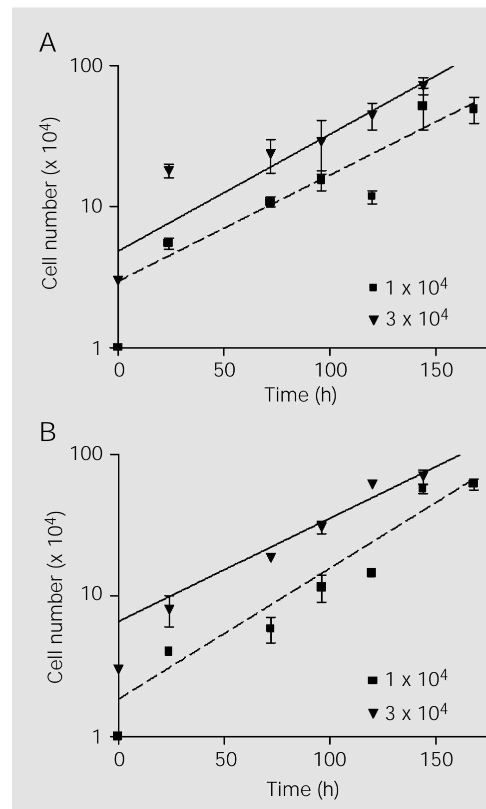

Figure 2. Growth curves of LISP-A10 (A) and LISP-E11 (B) clones. Cells (1×10^4 or 3×10^4) were plated on day 0 and growth curves were generated by counting viable cells every 24 h in 24-well culture plates. Population growth rates were calculated from these curves.

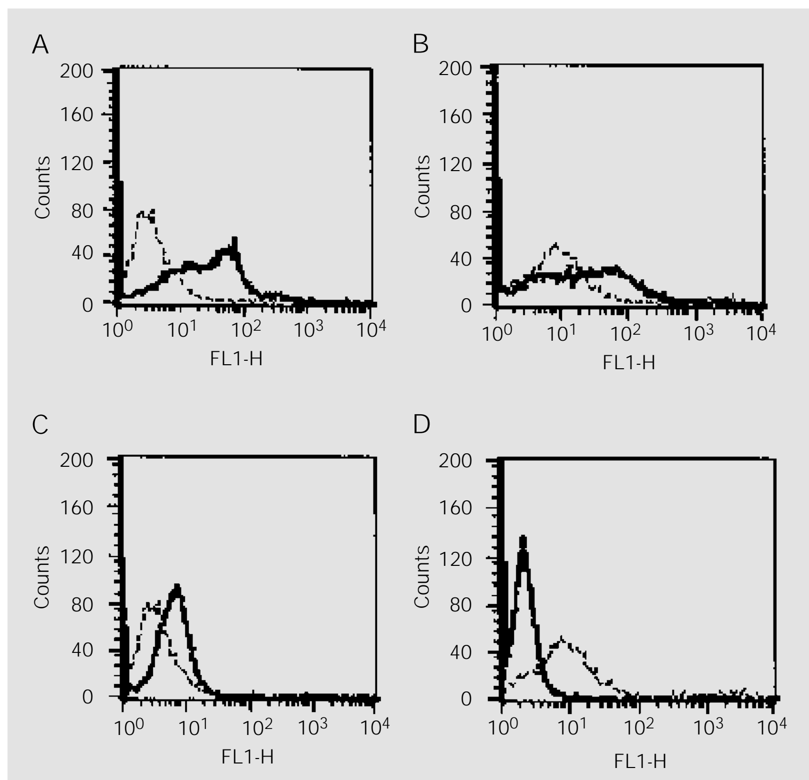

Figure 3. Carcinoembryonic antigen (A and B) and desmoglein (C and D) expression on the cell surface of the two clones, analyzed by flow cytometry using specific antibodies (solid lines). LISP-A10 (A and C), LISP-E11 (B and D). Dashed lines represent the negative control (normal mouse purified IgG). FL1-H: fluorescence intensity.

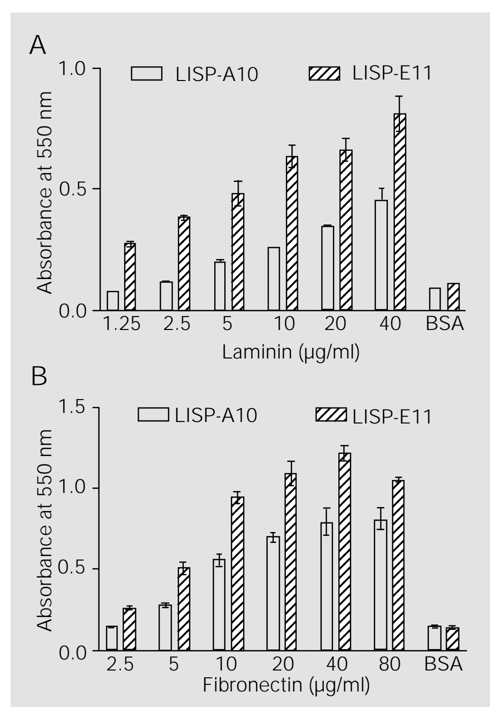

Figure 4. Adhesion of 5 x 10^4 cells from LISP-A10 (A) and LISP-E11 (B) cell clones in response to increasing concentrations of laminin, fibronectin or 1% BSA (control) adsorbed to the solid phase. The graphs show a representative result from four experiments. Error bars indicate the standard deviation.

LISP-A10 expresses higher amounts of CEA on its cell surface than LISP-E11 (Figure 3A and B).

The other intercellular adhesion molecule studied was desmoglein, an important cadherin superfamily member, which is essential for cell-cell adhesion since it is one of the main components of desmosomes (30, 31). Again, it was observed that the epithelial morphotype LISP-A10 produces desmoglein, while LISP-E11 does not (Figure 3C and D). It has been proposed that the morphological characteristics of tumors (32) as well as CEA expression (14,29) are directly related to their degree of differentiation. Based on the present observations concerning *in vitro* morphology and expression of intercellular adhesion molecules, it may be suggested that LISP-A10 displays a more differentiated phenotype than LISP-E11.

Another important step in the metastatic process is the interaction of tumor cells with the surrounding extracellular matrix. In this study, the adhesive properties of LISP-A10 and LISP-E11 to laminin and fibronectin were analyzed by colorimetric adhesion assays (Figure 4). Both cell populations adhered to these extracellular matrix components, although LISP-A10 adhered to a lesser extent. This finding could be explained by the formation of aggregates by cells from this population, thus reducing cell surface availability to interact with proteins in the solid phase. Correlating the adhesion behavior to the degree of differentiation, Daneker Jr. and colleagues (32) found similar differences, with poorly differentiated colorectal carcinoma cell clones adhering better to laminin and fibronectin than a well-differentiated one. Interestingly, LISP-E11, which is poorly differentiated, readily spread on fibronectin, but not on laminin, after contact with these proteins (data not shown). This finding is in accordance with another report that showed spreading of poorly differentiated tumor cells on extracellular matrix components (32). A possible explanation for this

observation is that the LISP-E11 cell clone has been adaptively selected for its ability to rapidly recognize, adhere to and spread on fibronectin, which is present in the space of Disse in the liver. The space of Disse contains a discontinuous extracellular matrix composed of collagen types I and IV and fibronectin, but not laminin (23). However, laminin is present in the basement membranes of the endothelium of central and portal veins. One may speculate that the tumor cell mass that reached the liver adhered to laminin on the basement membrane, degraded it and migrated on the digested matrix to the space of Disse. Then, there would be a switch of the enzyme specificity resulting in the activation of fibronectin-specific enzymes. This hypothetical switch would have been the result of a selective pressure on cells that conquered the new environment of the secondary metastatic site. If so, all these phenomena would have facilitated the metastatic event that originated the LISP-I cell line.

Even if they can attach to and degrade extracellular matrix, tumor cells will still fail to complete the invasion process if they are unable to migrate. Laminin and fibronectin can serve as substrata for cell attachment and spreading as well as a signal for cell migration. Active migration towards extracellular matrix components is one of the most important requirements for the success of metastasization (33) and can be analyzed by simple *in vitro* systems. However, depending on their concentration, extracellular matrix molecules may have opposite effects on cell migration (34). In the present report, colorimetric assays of migration were performed with an optimal protein concentration, based on previous results of adhesion assays. Thus, LISP-A10 and LISP-E11 subpopulations were compared for their locomotion ability. The fibroblastoid cell morphotype LISP-E11 was shown to migrate towards immobilized laminin or fibronectin, which is in accordance with its poorly differentiated phenotype. On the other hand, LISP-A10 presented a discrete migration towards these proteins, as shown in Figure 5. This behavior could be explained by the strong aggregates formed by LISP-A10 cells due to the expression of intercellular adhesion molecules, which prevent cell migration.

To investigate the anchorage-independent growth ability of both lineages, cells were grown in soft agar cultures. This assay is considered to be an *in vitro* method that correlates with *in vivo* tumorigenicity (35). Figure 6 demonstrates that LISP-A10 was able to form spheroid-like structures, while LISP-E11 was not, despite its survival. These observations indicate that both cell subpopu-

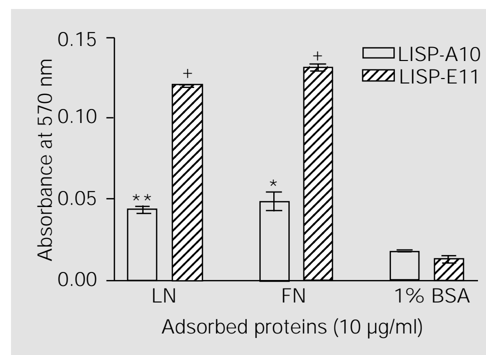

Figure 5. Migration of LISP-A10 and LISP-E11 cell clones towards laminin (LN) and fibronectin (FN) in comparison to 1% BSA (control) immobilized on the outer surface of a modified Boyden chamber. Migration was observed after 16-h incubation at 37ºC. Experiments were performed in duplicate and analyzed by the Student t-test (*P = 0.03; **P = 0.007 and +P<0.001). The graph shows one representative experiment. Error bars indicate the standard deviation.

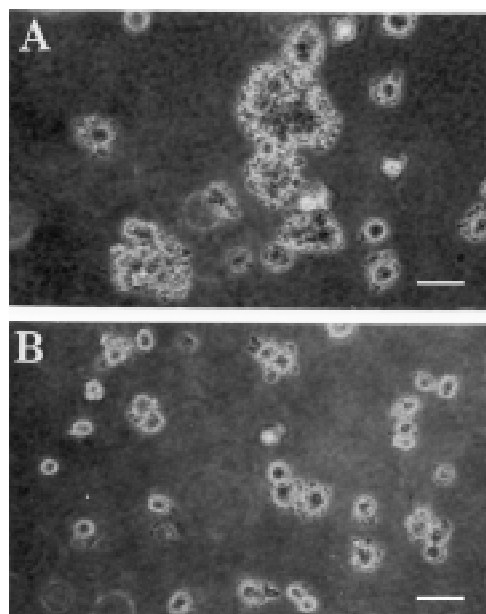

Figure 6. Anchorage-independent growth of LISP-A10 (A) and LISP-E11 (B). Known numbers of log-phase cells in single-cell suspensions were plated onto 0.6% agarose. Colonies were grown for 14 days and spheroid formation was noticed after 7 days, only for LISP-A10. Bars: 50 µm.

lations present a malignant phenotype. Interestingly, recent data have shown that the two clones maintain their proliferation after nearly one month in this condition with no medium replacement (Carneiro CRW and Guariniello LD, unpublished results), suggesting the secretion of an autocrine growth factor. This finding is under investigation in our laboratory.

In the present study we identified two morphologically distinct subpopulations within the LISP-I cell line. Morphological differences were shown to correlate with diverse functional properties that probably contributed to the metastasis from which the LISP-I cell line was isolated. Thus, the heterogeneity displayed by the LISP-I cell line may have been a positive selected feature, with LISP-A10 contributing to the maintenance of cell aggregates in the circulation, and LISP-E11, with its migration ability, contributing to the invasive steps of metastasization. The cell sublineages herein reported represent new models that may be used to address biological questions concerning tumorigenesis and metastasization.

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