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Effect of Host-Organ Environment on the in vivo and in vitro Behavior of a Murine Mammary Adenocarcinoma

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Abstract

We investigated the role that organ environment may play in determining the homing of disseminated cells from a murine mammary adenocarcinoma moderately metastatic to lung (M3). Conditioned medium (CM) from normal lung was able to enhance both local and metastatic growth. It increased the number of lung colonies when inoculated together with tumor cells via intravenous or separately via intraperitoneal route. Several in vitro studies were performed in order to elucidate possible mechanisms. It was shown that lung CM stimulated the in vitro growth and the migration of M3 cells. Normal kidney and liver CM lacked all these capacities.

Key Words

Organ microenvironment
Migration
Cell growth
Metastasis

Introduction

Metastasis is a complex multistep process which includes the release of malignant cells from the primary tumor, intravasation, circulation of the released tumor cells and their lodging at the target organ where proliferation gives rise to a secondary tumor [1].

In 1889, Paget [2] proposed that the pattern of metastasis was not due to chance and he concluded that some cells ('seeds') had affinity for growing in the environment provided by certain organs ('soil'). This hypothesis has received considerable experimental and clinical support [3]. Specific host- and tumor-dependent factors involved in determining the site of secondary growth include the cell surface glycoproteins, which contribute to retaining the metastatic cells in the target organ [4], the adhesion of tumor cells to specific capillary endothelium [5] or parenchymal cells [6], as well as specific factors present in the target organs which modulate metastatic cell growth, migration or release of proteolytic enzymes [7–11].

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The purpose of the present work was to investigate the role that organ environment may play in determining the homing of disseminated cells from a murine mammary adenocarcinoma (M3) moderately metastatic to lung. Conditioned medium (CM) from lung was able to modify the tumorigenic and metastatic behavior of M3 cells. Several in vitro studies were performed in order to elucidate some possible mechanisms.

Materials and Methods

Tumors and Primary Cultures

We used a transplantable Balb/c mammary adenocarcinoma metastatic to lung (M3), which presented a 40% incidence of spontaneous lung metastases with an average of 6 nodules per mouse, after 25 days of subcutaneous transplantation [12].

To perform primary cultures, tumor cell suspensions from M3 solid tumors were enzymatically prepared according to Bal de Kier Joffé et al. [13]. The monolayers consisted mostly of epithelioid polyhedric tumor cells with less than 2% contaminating macrophages and fibroblast-like cells, as analyzed by morphological and cytogenetic criteria [13].

Murine embryo cell suspensions were prepared from 12-day embryos by trypsinization (0.25% in Hank's solution) for 30 min and the monolayers were grown in MEM plus 5% FCS.

Organ Conditioned Media

CM were prepared from lung, target organ for M3 dissemination, and from kidney or liver as control organs. Organs, obtained from 10-week-old Balb/c normal mice, were sliced into 1-mm^3 fragments and carefully washed. Homologous fragments from at least 5 animals were pooled and weighed. They were aliquoted in culture flasks at 0.125 g tissue per ml medium MEM 41500-018 (Gibco, Grand Island, N.Y.) containing 2 m*M* *L*-glutamine and 80 μg/ml gentamicin.

After 24 h of incubation at 37°C in 5% CO_2 in air, the CM were collected and spun at 720 *g* for 10 min. The supernatants were centrifuged again at 25,000 *g* for 30 min. CM were aliquoted and stored at −40°C. The protein concentration of CM, measured by Lowry's method, varied between 2.4 and 3.2 mg/ml.

Unconditioned medium alone was also processed in the same way to be used as control.

In vivo Studies

All in vivo studies were performed on 10- to 12-week-old male Balb/c mice.

Local Tumor Growth. To study the effect of CM on the local tumor growth, a suspension of 4×10^6 monodispersed cells prepared from M3 subcutaneous tumors were preincubated for 30 min in 1 ml of control, lung or liver CM. 0.05 ml of each cell suspension was injected into the left hind footpad of 10 randomized normal syngeneic mice per group.

Tumor growth was followed in terms of size by the measurement of footpad thickness. Latency period was defined as the time to reach a thickness higher than 1.85 mm. At 28 days postinoculation, mice were necropsied and tumors were weighed. The assay was repeated twice, with two different batches of CM.

Experimental Metastasis Assay. In order to study the effect of CM on lung colonizing ability of M3, two different experimental designs, each repeated twice, were performed.

In experiment A, 40 randomized mice were inoculated in the lateral tail vein with 2×10^5 cells/0.3 ml, treated according to the following scheme (see table 2). Group A1 received M3 cells preincubated 1 h with lung CM and coinjected with the same CM; group A2 were inoculated with M3 cells treated 1 h with lung CM, washed out and resuspended in control medium; group A3 were injected with cells treated 1 h with control medium, and group A4 received M3 cells preincubated for 1 h and coinoculated with liver CM.

Experiment B was designed as follows: 50 randomized mice were inoculated intravenously with 2×10^5 M3 cells in 0.3 ml control medium. Group B1 simultaneously received a unique intraperitoneal inoculation of 0.3 ml control medium; groups B2, B3, B4 and B5 were inoculated intraperitoneally with 0.3 ml lung CM 3 h before, simultaneously, 3 or 24 h after cell injection, respectively. As a control (B6) 10 mice received intravenously 2×10^5 M3 cells preincubated with lung CM and injected in the presence of the same medium.

Mice from experiments A and B were sacrificed at 21 days postinoculation and autopsied. The number and size of lung colonies were recorded.

In vitro Studies

Organ CM preparations were tested for their ability to modify proliferation and migration of cultured M3 cells.

In vitro Cell Proliferation Studies. The effect of the CM on log-phase proliferating cells in the presence or absence of FCS was studied as follows: 24 multiwell trays (Falcon) were seeded with 5×10^4 tumor or murine embryo cells in growth medium (MEM plus

Table 1. In vivo growth of M3 cells coinjected with organ conditioned media in the footpad of syngeneic mice

	Control media	Lung CM	Liver CM
Latency	2/10	7/10[a]	1/10
Growth rate, mm/day	0.35 ± 0.07	0.53 ± 0.03[b]	0.39 ± 0.15
Tumor weight at 28 days, g	0.74 ± 0.57	2.32 ± 0.53[c]	0.91 ± 0.71

Data corresponding to one experiment. Similar results were obtained in a second assay. Latency was expressed as number of animals with tumor at 7 days/total of animals.

[a] $p < 0.01$ vs control and liver CM (Kruskal-Wallis nonparametric variance analysis and Dunn's procedure for comparison among groups).

[b] $p < 0.01$ vs. control and

[c] $p < 0.01$ vs. control and liver CM ($\bar{x} \pm SD$) (ANOVA, Scheffé for planned comparisons).

10% FCS, 2 m*M* *L*-glutamine and 80 μg/ml gentamicin). After 18 h, wells were washed with PBS. Then fresh medium, with or without 4% FCS, was added containing CM or control medium at varying dilutions in triplicate. As a positive control some wells were fed with medium plus 10% FCS. In some experiments, different concentrations of known purified growth factors or transferrin were assayed on tumor cell proliferation.

Cells were refed and CM samples added every 2 days. Cell growth was evaluated by the measurement of cell protein content, employing Oyama and Eagle's method [14]. This method was used because in previous assays a significant positive correlation between M3 protein content and both cell number or DNA concentration was shown. Each experiment was repeated at least 3 times with different batches of CMs.

Migration Test. We employed a method with gelatin-agarose plates modified from Alessandri et al. [15]. Plastic Petri dishes (60 × 10 mm, Falcon Lincoln Park, N.J.) were coated with a gelatin solution (Sigma) (2% w/v in PBS) and overlaid with 5.5 ml of agarose (BRL, Bethesda, Md.; gel electrophoresis grade) (1.2% w/v in DMEM plus 5% FCS, 80 μg/ml gentamicin and 2 m*M* *L*-glutamine).

Eight groups of three wells, 3 mm in diameter and spaced 2 mm from each other, were punched out per plate. 10 μl of M3 cell suspensions (4×10^4 cells) prepared from primary subcutaneous tumors were added to the central one of the three wells. At the same time, control medium, FCS, or CM were added to the lateral wells.

Dishes were incubated for 48 h at 37°C, in a humidified atmosphere of 5% CO_2 in air. Then the plates were fixed and stained with Giemsa solution prior to the removal of the agarose. Each sample was assayed in octuplicate.

Statistical Analysis

Data were analyzed by ANOVA. Scheffé's test was used for planned comparisons (F ratio). Latency and number of lung colonies after intravenously inoculation were analyzed by means of the Kruskal-Wallis nonparametric test and Dunn's procedure for comparison among groups. The number of mice with large colonies was analyzed by the χ^2 test.

Results

In vivo Studies

Conditioned medium from lung was able to enhance both local and metastatic growth. When M3 cells were coinjected with lung CM into the footpad of syngeneic mice, the latency period was significantly shortened, while the growth rate and the tumor size and weight were enhanced (table 1). On the other hand, liver CM did not modify the control growth pattern.

Lung CM was able to increase significantly the number of lung colonies after the inoculation of M3 cells in the lateral tail vein (ta-

Table 2. Effect of lung conditioned media on the lung colonizing ability of M3 cells inoculated intravenously

Group	Treatment		Lung colonies	
	pretreatment 1 h	inoculation with	median	range
A1	Lung CM	Lung CM	63	13–320[a]
A2	Lung CM	Control media	2	0–42
A3	Control media	Control media	0	0–15
A4	Liver CM	Liver CM	3	1–44

[a] $p < 0.01$ vs. groups A2, A3 and A4 (Kruskal-Wallis nonparametric test (n = 10 animals per group).

Table 3. Effect of the intraperitoneal inoculation of lung conditioned media on the colonizing ability of M3 cells

Group	2×10^5 M3 cells inculated i.v. with	Lung CM i.p. (0.3 ml)	Colonies per mouse	
			median	range
B_1	Control medium[a]	–	3	0–11
B_2	Control medium	– 3 h	5	3–17
B_3	Control medium	0 h	41	3–91[b]
B_4	Control medium	+ 3 h	31	14–93[b]
B_5	Control medium	+ 24 h	32	20–87[b]
B_6	Lung CM	–	89	18–178[b]

[a] This group of animals also received 0.3 ml of control medium i.p. simultaneously with cell injection.
[b] $p < 0.01$ vs. B_1 and B_2 (Kruskal-Wallis test; n = 10 animals per group).

ble 2). This effect was observed when M3 cells were incubated and inoculated together with lung CM (group A1). But when M3 cells were treated for 1 h with lung CM, washed out, resuspended in control medium and then inoculated into syngeneic mice (group A2), the number of lung colonies was similar to that obtained with control medium (group A3). Liver CM was unable to modify the number of lung colonies.

Besides, we investigated whether lung CM could modulate the lung colonizing ability of M3 cells through a systemic pathway. Mice were administered intraperitoneally with lung CM before, simultaneously and after the intravenous inoculation of M3 cells. Table 3 shows that the inoculation of lung CM simultaneously, and 3 or 24 h after the intravenous injection of M3 cells significantly increased the number of lung colonies. However, when lung CM was inoculated 3 h before the tumor cells, the number of lung colonies was similar to the control.

Multiple treatments with lung CM given intraperitoneally between 10 and 21 days after intravenous cell injection had no effect either on the number or on the size of lung colonies [data not shown].

When the size of lung colonies was analyzed, it was observed that 81% mice receiv-

ing M3 cells preincubated and intravenously inoculated with lung CM presented lung colonies larger than 1 mm diameter versus only 44% of the mice that were inoculated intraperitoneally with lung CM ($p < 0.05$).

In vitro Studies

In order to elucidate some possible mechanisms to explain the in vivo enhancing effects of lung CM on tumor and metastatic growth, we performed several in vitro studies.

Effect of Conditioned Media on Tumor and Normal Cell Proliferation

We studied the ability of 20% v/v CM to modulate the in vitro proliferation of tumor and normal cells in log-phase in the presence of 4% FCS. It was found (fig. 1a) that lung CM stimulated M3 growth in a similar way as 10% FCS. Liver CM showed a markedly inhibitory, though reversible effect, while kidney CM did not modify the growth of primary M3 cell monolayers.

Figure 1b shows that the growth of normal primary mouse embryo cells in the presence of 4% FCS was also stimulated by lung CM and inhibited by liver CM.

Lung CM was also able to stimulate M3 cell growth in the complete absence of serum. The dose-dependent effect was evident with doses as low as 2.5% v/v, reaching a plateau between 10 and 20%, as shown in figure 2.

Preliminary Characterization of the Enhancing Proliferative Activity of Lung CM

The proliferative activity of lung CM was characterized by physicochemical methods. Lung CM was treated under different conditions and then its growth promoting activity in the absence of serum was tested.

To test for sensitivity to enzymes, aliquots were incubated at 37°C for 2 h with 50 μg/ml trypsin (type XI, Sigma Chemical Co., St. Louis, Mo.) and the reaction was stopped by the addition of 100 μg/ml of soybean trypsin inhibitor (Sigma). Other aliquots were treated with protease (insoluble enzyme attached to 4% cross-linked beaded agarose, type XI-A, Sigma) at a concentration of 4 μg enzyme/μg CM, for 30 min at 37°C. Neither the trypsin nor the protease modified the proliferative action of lung CM. The mitogenic component of lung CM was relatively heat- and acid-stable with exposure to 80°C for 10 min or to pH 2 for 1 h, reducing the activity to 58.3 and 56.3% of the control levels, respectively. The proliferative activity was nondialyzable and highly stable to room temperature and lyophilization.

Lung CM was separated by ultrafiltration (Centricon 30, Amicon) employing membranes with a cutoff of 30 and 10 kDa. It was found that the mitogenic activity was mainly present in the fraction of MW higher than 30 kDa.

Lung CM prepared from mice perfused with saline solution to eliminate blood cells did not alter the CM proliferative activity. Lung CM prepared from allogeneic mice also enhanced M3 cell growth.

Effect of Growth Factors on Tumor Cells

The effects of known purified mitogens such as EGF, IGF-I and IGF-II (2.5–40 ng/ml) and transferrin (2.5–100 μg/ml) were also assessed with respect to their influence on the growth of M3 cells. None showed a significant effect, either in the presence or in the absence of FCS [data not shown].

Effect of Conditioned Media on Cell Migration

The assay used allowed the measurement of the chemokinesis or random migration by M3 cells. M3 cells quickly adhered and spread on the bottom of the excavation in the agarose, and cell migration was observed as soon

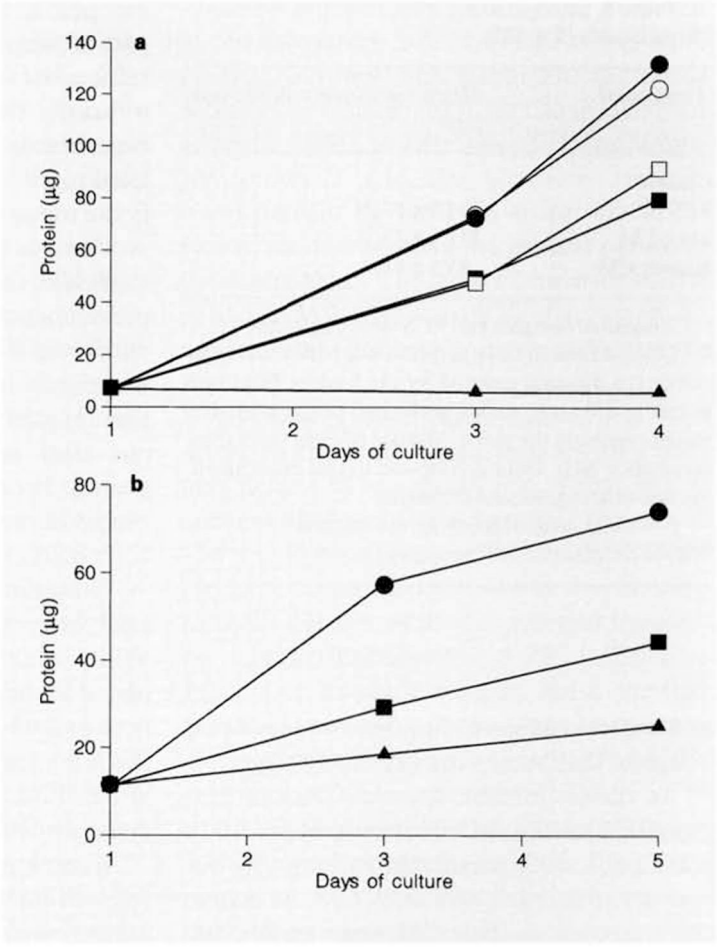

Fig. 1. Effect of organ conditioned media (20%, v/v) on the growth of tumor and normal cells in the presence of 4% serum. **a** M3 cells. **b** Murine embryo cells. ● = Lung CM; □ = kidney CM; ▲ = liver CM; ■ = control medium; ○ = medium added with 10% SFB. * $p < 0.05$ vs. control, n = 4. SD was usually less than 15% of the mean value.

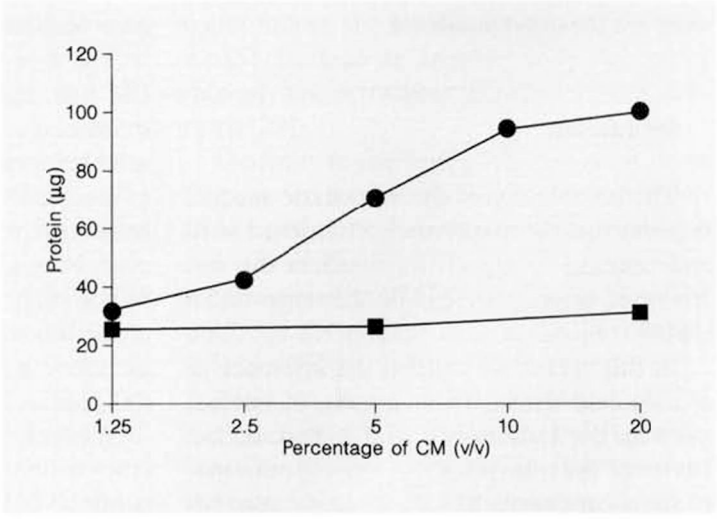

Fig. 2. Dose-response curve of the effect of lung conditioned media on the growth of M3 cells in serumless conditions. ● = Lung CM; ■ = control medium.

Table 4. Effect of organ conditioned media on M3 chemokinesis ($\bar{x} \pm SD$)

Treatment[a]	Migration distance of M3 cells μm[b]
Control	55.3 ± 11.9
FCS	134.9 ± 57.3[c]
Lung CM	111.1 ± 13.7[c]
Kidney CM	85.1 ± 11.9

[a] Undiluted samples and FCS were employed.

[b] Quantitation of cell migration was performed measuring the distance covered by the leading front in 8 points at 45° each, with a graduated ocular grid. The values represent the mean distance of eight wells measured after 48 h. Data correspond to one experiment, but two others gave similar results.

[c] $p < 0.05$ with respect to the control (ANOVA, Scheffé for planned comparisons).

as 6 h after seeding. Cells migrated as isolated 'tongues' that became progressively bigger.

As shown in table 4, serum factors promoted the motility of tumor cells. M3 motility was significantly stimulated by lung CM but was not stimulated by kidney CM. As migratory response to liver CM was variable, according to the batch employed, these data were not included in table 4.

Discussion

The complexity of the metastatic process requires that the metastatic cells interact with and respond to signals localized in the microenvironment provided by the target organ [16].

In this report we studied the influence of conditioned media from organs of normal mice on the tumorigenic and metastatic behavior of the transplantable murine mammary adenocarcinoma M3. First, we injected tumor cells in the presence of CM into the footpad of syngeneic mice. Only soluble factors released by the lungs were able to enhance significantly the local tumor growth. In these experiments a single dose of lung CM inoculated together with the cells was able to modify the tumorigenic pattern.

Then, we assayed the ability of lung CM to modulate the metastatic process. A 1-hour preincubation and coinjection of M3 cells with lung CM produced a 30- to 60-fold increase in the number of pulmonary colonies. However, when the cells were washed out after preincubation this effect disappeared. It cannot be ruled out that a longer period of incubation before washing out lung CM could induce the same effect.

Thus, lung CM was also inoculated separately from M3 cells. A significant enhancing ability was observed when lung CM was inoculated via intraperitoneal route simultaneously, 3 or 24 h after the cells were injected into the tail vein, suggesting that lung CM could also facilitate the growth of lung colonies via a systemic pathway.

When lung CM was administered 3 h before cell injection, it did not promote growth suggesting a rapid clearance and almost excluding the possibility of an indirect phenomenon such as immune suppression.

The fact that cells responded when lung CM was injected 24 h after the intravenous inoculation of M3 cells suggested that lung soluble factors were not modifying the primary mechanisms of tumor cell adhesion to the lung microvessel endothelial cells [17]. However, lung CM might alter the retention of tumor cells in the lung or might stimulate properties related to secondary invasion such as tumor motility or enzyme activation, as was previously demonstrated [11].

Western blotting of lung CM with a specific antibody that recognizes the laminin peptide SIKVAV showed three specific bands

of 110, 70 and 38 kDa [unpubl. results]. As Sweeney et al. [18] demonstrated, this peptide is able to enhance tumor and metastatic growth in in vivo assays. It would be interesting to study whether laminin fragments are responsible for the effects elicited by lung CM.

Another interesting aspect is that preincubation and coinjection of tumor cells with lung CM enhanced the size of lung colonies. We cannot discriminate yet whether these results are due to a difference in the blood concentration of lung CM or whether preincubation primed M3 cells in a special way.

According to the in vivo results, we studied whether the lung CM was able to modify some in vitro properties that are thought to be critical determinants in the invasive and metastatic process, such as cell proliferation, cell motility or lytic action.

Several reports [7, 9, 19] have demonstrated that the organ microenvironment contains multiple soluble molecules able to stimulate and/or inhibit growth in tissue culture. Recently, it was shown that CM prepared from parenchymal or endothelial cells or fibroblasts from the target organ stimulated the growth of metastatic sublines that specifically metastasize to these organs [20–22]. In our model CM from lung, the target metastasizing organ, enhanced the proliferation of M3 cells, even in serum-free culture, indicating the presence of mitogenic factor(s). Some authors [23, 24] have found that lung factors preferentially enhanced the in vitro proliferation of cells with high metastatic ability. We found that M3 cells and a highly lung-metastatic variant (MM3) were stimulated by lung CM in a similar way [25].

In contrast to the lung-derived growth factor isolated by Cavanaugh and Nicolson [23], our lung CM preparations also had proliferative activity on normal cells.

The characterization of lung CM prepared in our laboratory indicated that the factor/s are relatively heat-/acid-stable. The mitogenic activity was resistant after exposure to trypsin or protease but, as the molecule/s could be a glycoprotein/s and the glycoside residues could prevent the catalytic action of the enzymes, we cannot deny the protein nature of the components. The main stimulating activity had an MW higher than 30 kDa, but we do not know whether one or more molecules are involved.

Nicolson et al. [26] have isolated and characterized the main paracrine mitogen from lung tissue CM as a transferrin. Although it is probable that transferrin is present in our lung CM, this factor alone could not be responsible for the mitogenic activities observed because M3 cells did not respond to purified transferrin. It is also improbable that IGF-I, IGF-II or EGF were involved because these purified growth factors did not stimulate M3 cells to grow, though these cells presented specific receptors as shown previously [27]. Lung CM could regulate proliferation through the induction of tumor autocrine cell factors. In our model, lung CM stimulated the activation of secreted urokinase-type plasminogen activator [11]. It was proposed that this enzyme could induce the proliferation of tumor cells directly through its 'growth factor' domain or through the activation of latent growth factor(s) [28].

Contrary to the results with lung CM, liver CM exerted a cytostatic, reversible inhibition of M3 and normal embryo cell growth but it did not inhibit tumor growth in vivo. Also, other authors [7, 10] have found that liver CM inhibited the proliferation of tumor cells unable to metastasize to the liver.

Then, we evaluated the influence of CM from normal organs on the tumor cell chemokinesis. Factors influencing the passage through a filter (such as cell deformability in

the Boyden chamber assay) are probably involved to a lesser extent in the agarose plate assay employed. Conflicting results have been obtained in studies attempting to correlate in vitro locomotion of neoplastic cells with their capacity for metastasis [8, 29, 30]. We observed that M3 cells presented a high spontaneous migration activity and lung CM contained factors able to significantly stimulate the movement of the metastatic cells.

In conclusion, we demonstrated that lung, the target organ for M3 cells, produced factors with abilities to enhance the tumorigenic and metastatic behavior in vivo. Though these effects could be attributed to many different causes, our experimental data from in vitro studies suggest that lung CM presented mitogenic and migration enhancing activities as well as factor(s) able to increase the enzyme activity secreted by the tumor cells, stressing the significance of the organ environments in the metastatic outcome. Now we are attempting to isolate and characterize the factor(s) responsible for each of these biological activities.

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