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RAPID COMMUNICATION

TERATOCARCINOMA CELLS CULTURED ON EMBRYONIC SUBSTRATES SHOW ACCELERATED MIGRATING BEHAVIOR IN VITRO AND INCREASED METASTATIC ACTIVITY IN VIVO

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Teratocarcinoma cells (402AX) were grown on feeder layers of whole mouse embryos (Day 4) or mouse embryonic fibroblasts and then were either examined in vitro or transplanted in vivo. After twenty-four hours of coculture, teratocarcinoma cells demonstrate accelerated cell migration in vitro. Furthermore, transplantation of teratocarcinoma cells with embryonic substrates into syngeneic hosts produces grossly detectable lymph node metastases. These effects appear to be due to soluble factor(s) produced by embryonic substrates which enhance tumor cell proliferative/migratory activity. This suggests that tumor cell invasion and metastasis may be stimulated by soluble factors produced by host tissues.

The natural history of many neoplasms involves growth, invasion and metastasis. Each of these events select subpopulations of tumor cells which appear better adapted for autonomous growth (1,2,3). When certain tumor cells are propagated in vitro, their serum requirement correlates inversely with their invasive or metastatic potential (4). Tumor cell lines which require less serum demonstrate greater cell migration and invasiveness when propagated in vivo. This has been interpreted to mean that autonomous tumor cells have a diminished requirement for the low molecular weight peptide growth factors which are present in serum and consequently are better adapted for survival and growth in host environments where these factors are limiting (5). It has also been suggested that highly malignant tumor cells with low serum requirements, may have acquired an "autocrine" phenotype and are able to support their patterns of malignant growth by the production of endogenous tumor growth factors (6,7). In this report we present evidence for another mechanism by which tumor cells may acquire the characteristics associated with increased malignancy. We show that mouse teratocarcinoma cells, which have been cocultivated with whole mouse embryos or mouse embryo fibroblasts, exhibit accelerated migratory behaviour in vitro and metastasis in vivo. This suggests that tumor cell invasion and metastass may be stimulated by soluble factors produced by host tissues.

MATERIALS AND METHODS

Tumor Cell Line. The 402AX is a syngeneic, spontaneously derived teratocarcinoma of testicular origin. It was obtained from M.A.Isa, who maintained it in the ascites form in 129/J mice (8). The 402AX is a nullipotent tumor with some differentiation towards endoderm. However, the majority of cells are embryonal carcinoma cells.

Cell Migration Assay. The technique of Varani was used to measure in vitro migration (9). Teratocarcinoma cells at a concentration of 1×10^7/ml were pelleted in conical centrifuge tubes at 500 g. The cell pellets were (Marine Colloids, Inc., Rockland, MD) dissolved in test culture median

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(see serum free medium below) or control serum (10 % FBS). A two microliter drop was then placed in a microtiter dish and allowed to gel at 4°C. After cooling the drop was covered by 300 microliters of media and incubated for 18 hours in a CO2 incubator.

Preparation of Serum-Free Conditioned Medium. Serum-free medium, used for the growth of teratocarcinoma cells and embryonic cells, was composed of minimal essential medium supplemented with fetuin (500 ug/ml CalbioChem-Pedersen type), bovine insulin (10 ug/ml), transferrin (5 ug/ml) and 10-5 M beta-mercaptoethanol. This medium was added to subcultured teratocarcinoma cells, mouse embryonic fibroblasts, and early whole mouse embryo organ cultures (Day 4). Either teratocarcinoma cells or mouse embryonic fibroblasts were passaged into one ml 24well cluster plates at a cell density of 10,000/cm^2. The cultures were incubated in a 5% CO2 incubator kept at 37°C. Medium was added, then was removed after 5-7 days, pooled and kept at -20°C until ready for use.

Experimental Design For Metastasis Generation. The determination of metastases in this study was based on a spontaneous metastasis assay. Inguinal, para-aorta and hilar lymph nodes were examined macroscopically and microscopically for the presence of tumor. In vivo cloning of teratocarcinoma cells was achieved by the technique of Kleinsmith (10). Briefly capillary tubes containing single cells were attached to an orally controlled 18 inch length polyethylene tubing. Using and inverted microscope the cell was then placed on an embryonic feeder layer. Animals which produced tumors were sacrificed for autopsy examination. Controls consisted of tumor cells cloned on glass coverslips alone. All specimens were examined macroscopically and microscopically.

Blastocyst Culture Techniques. Female 4-5 week old 129/J mice were superovulated according to the method of Runner (11). Briefly, 2.0 I.U. of Gestyl (Organon, Inc., West Orange NJ) were given i.p. After 48 hours, 8.0 I.U. of chorex-10 (Hyrex, Pharm., Memphis, TN) were given i.p., and the animals were mated. On the 4th day, females are sacrificed, and both uterine horns were flushed with phosphate buffered saline (PSB) under a dissecting microscope.

Three and one half day old embryo (blastocysts) were visually transferred with micropipettes into individual 96 well flat bottom tissue culture plates (Falcon Plastics, Oxnard, CA). Prior to transfer, a small 3 $mm.^2$ glass coverslip (Bellco Glass, Inc., Vineland, NJ) was placed in each well. Several drops of mineral oil were placed on each air/media interface. These embryos were then cultured for 5-7 days.

Preparation of Mouse Embryonic Fibroblast Monolayers. Embryos were obtained from pregnant 12-14 day old 129/J females. The embryos were removed from the uterus and dissected free of extraembryonic membranes. Collected embryos were teased apart, trypsinized (0.125%), washed, and placed (1 x 10^6) in 75 $cm.^2$ flasks (Falcon Plastics, Oxnard, CA) containing MEM 5% FCS. The following day, they were subcultured (2 x 10^4) into 96 well flat-bottom plates containing 3 mm^2 coverslips. After a 12-18 hour incubation, monolayers had formed.

RESULTS

In Vitro Migrating Activity of Teratocarcinoma Cells. Tumor cell migration was assessed in vitro in response to SFM (serum free media) conditioned by 402AX teratocarcinoma cells (SFM & TER), mouse embryonic fibroblasts (SFM & MEF) or whole mouse embryos (SFM & ME). Controls consisted of comparing migration responses to MEM 10% FBS and in MEM alone. Figure 1A&B shows the results of incubating 402AX teratocarcinoma cells with various test substances for 1-5 days. Only SFM conditioned by whole mouse embryos or mouse embryonic fibroblasts contained the most migrating activity. In addition, teratocarcinoma cells responsed to a migratory factor(s) in fetal bovine serum. SFM alone or SFM conditioned by teratocarcinoma cells had much less activity.

Production of Metastases by the Growth of Tumor Cells on Embryonic Substrates in Vivo. When single or few teratocarcinoma cells were cloned onto organ cultures of mouse embryos

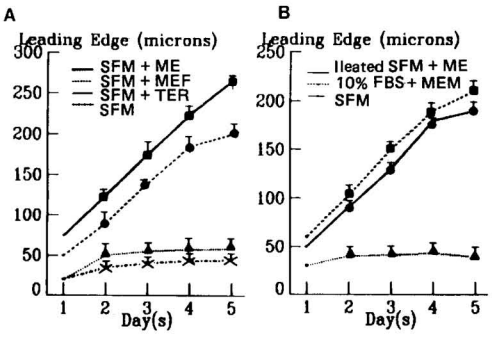

Figure 1A&B. Migration of teratocarcinoma cells. The distance migrated by 402A10 cells out of Agarose drops was determined from a total of four readings on each of four agarose droplets.

attached to glass discs and were then transferred to the peritoneal cavity, they produced ascites tumors in 29% (36/124) and metastases in 75% (27/36) [p less than .005]) of animals (Table 1). Similar experiments were carried out using tumor cells cloned onto monolayer substrates of mouse embryonic fibroblasts (attached to glass discs). In these experiments, metastases also occurred, although the incidence of regional lymph node metastases was only 23% (7/33) (p less than .05 when compared to controls). Single cells without substrates produced 37% (12/32) tumors but did not metastasize. In order to determine the effect of glass discs on the metastatic incidence, single teratocarcinoma cells were cultured with glass (3 $mm.^2$) discs for 24 hours. They were then cotransferred to the peritoneal cavity. Tumors were produced in 20% (8/40) mice, however, metastases occurred in only 13% (1/8). Figure 2A shows the histological appearance of primary tumor obtained from the abdominal cavity. The tumor metastases were histologically embryonal carcinoma with the characteristic epithelial cells and some bizarre cells (Figure 2B).

Physical Separation of Embryonic Substrates an Tumor Cells In Vivo

Since tumor cells used in this assay were placed on cultured mouse embryos, it was important to determine whether or not physical contact between tumor cells and substrates were essential for metastasis. Twenty-seven animals were inoculated with single 402AX cells and a mouse embyro organ culture was put in widely separate parts of each abdominal cavity. Ascites tumors developed in 27% (10/37) of animals. Of these, 60% (6/10) developed metastases to axillary and hilar lymph nodes. This incidence of metastases resembled that observed when tumor

Table 1. Effect of Embryonic Substrate on Tumor Behavior in Vivo

Tumor Cocultures	Tumor Incidence	Metastasis Incidence	
Organ Culture Feeder Layer	36/124 (29) [a]	27/36 (75) [b]	[5.3]
Monolayer Feeder Layer	33/104 (31)	7/33 (21) [c]	[3.3]
Without Feeder Layer	12/32 (37)	0/12	[1.3]

Cocultures of single teratocarcinoma cells attached to a feeder layer of mouse embryonic cells were surgically placed i.p. into 129/J mice. Four to six weeks later animals with ascites tumors were sacrificed and examined for lymph node metastases. Lymph nodes were examined visually by size and histologically for tumor content.

[a] Numbers in parentheses, percentages

[b] Significant numbers of metastases; $p<.005$

[c] Smaller than incidence in organ culture substrates by chi-square analysis.

Numbers in brackets, mean lymph size(mm)

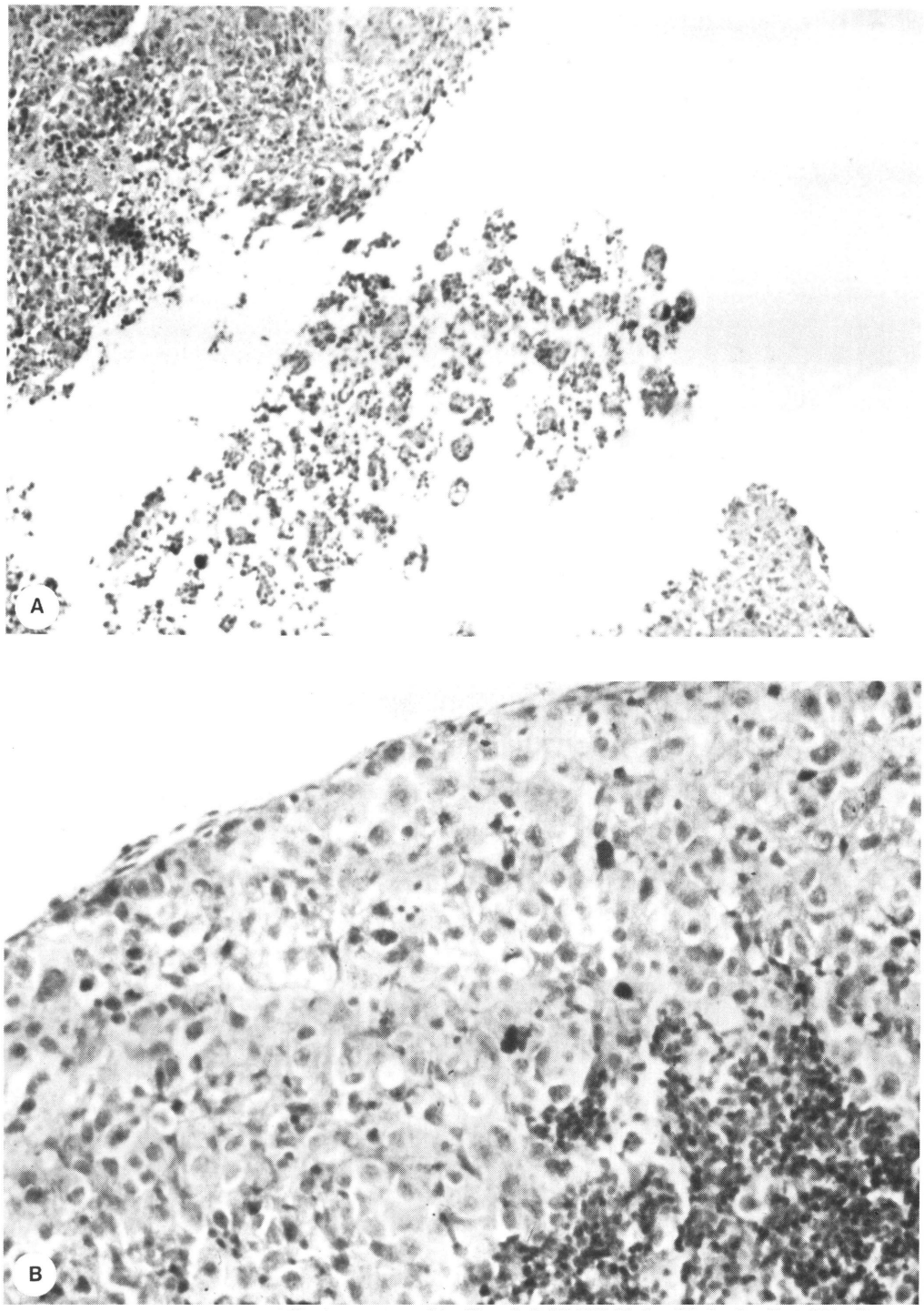

Figure 2A. Histology of primary tumor taken from the abdominal cavity. There is proliferation of tumor cells composed primarily of embryonal carcinoma. There is also present invasion into adjacent tissue (lower right). Hematoxylin and eosin, x 125.

Figure 2B. High power view of lymph made tissue removed from the axilla of 129/J mouse. Sections contain almost entirely embryonal carcinoma. Hematoxylin and eosin x 500.

Table 2. Effect of Metastatic Tumor Cells in Metastasis Assay

Tumor Cocultures	Tumor Incidence	Metastasis Incidence
Organ Culture Feeder Layer	14/19 (73)[a]	12/14 (85)
Without Feeder Layer	7/24 (29)	1/7 (14)

Teratocarcinoma cells metastatic to lymph nodes were disaggregated and cocultured with embryonic feeder layers. These mixtures were then examined in the metastasis assay.

[a] Numbers in parentheses, percentages

cells are in physical contact with mouse embryos (see Table 1), suggesting that physical contact of tumor cell and embryo was not required.

Effect of Metastastic Tumor Cells in Metastasis Assay. Cloned metastatic 402AX cells, cultured on embryonic substrates as described above, were then transplanted i.p. into syngeneic strains as shown in Table 2. In contrast to the parent stock tumor, metastatic 402AX cells produced a greater number of tumors, 73% as compared to 29%. In addition, they metastasized on retransplantation with greater frequency, 85%.

DISCUSSION

Teratocarcinoma cells (402AX) when grown *in vitro* or *in vivo* demonstrate characteristics of a low grade/non-metastasizing tumor. Their growth on plastic depends on high levels of fetal bovine serum. During our investigations, we observed that their serum dependence could be reduced if 402AX cells were grown on feeder layers of mouse embryonic fibroblasts or whole mouse embryos (unpublished observation). This suggested to us that the 402AX teratocarcinoma is partially growth factor-dependent (paraendocrine). We decided to examine the effects of mouse embryonic feeder layers on migratory activity *in vitro*. We found that cultures of either early whole mouse embryos or mouse embryonic fibroblasts contained an activity which, when grown in serum-free media, enhanced teratocarcinoma migration. Furthermore, this activity was not abolished by heat treatments. In order to determine if proliferative and migratory activity associated with embryonic feeder layers could be correlated *in vivo*, we transplanted cloned teratocarcinoma cells in association with embryonic substrates. When transplanted with embryonic substrates, tumor cell metastasized were observed in 75% of animals coimplanted with whole mouse embryo substrates and in 21% of animals containing mouse embryonic fibroblast substrates. In addition, this effect on tumor grown *in vivo* was not affected by physical contact between tumor cells and substrate. If inoculated separately into the peritoneal cavity, tumor cells still produced metastases. When treated in a similar fashion cloned metastatic 402AX cells produced 73% tumors and 85% metastases. Taken together, these data suggest that tumor cell invasion and metastases may be stimulated by soluble activities produced by host tissues. The activity reported here might be a macromolecular growth factor elaborated by mouse embryonic cells. Such factor(s) appear to be present in anaplastic highly metastatic tumor cell lines (autocrine) which are serum independent (6,7). Finally, these studies suggest that biological classes of neoplasms exist which can be defined by their differential dependence on host tissues.

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