Isolation and characterization of subpopulations of rat ascites hepatoma cell line of AH109A with different metastatic potentials

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Abstract

Rat ascites hepatoma cell line of AH109A proved to be divided into two subpopulations with different invasive and metastatic potentials, when cultured in the medium containing allogeneic rat sera. One population adheres to the culture dish, actively extending pseudopodia, and the other remains in a floating state. Utilizing this character, we have separated these two populations. After three successive separation steps, adhesive AH109A cells and floating AH109A cells were obtained. Adhesive AH109A cells proliferated more rapidly and invaded more actively than did floating AH109A cells. Adhesive AH109A cells metastasized mainly to lung, while floating AH109A cells to mesentery, when intravenously injected into tail veins. Histological studies revealed that adhesive AH109A cells showed lymphatic metastases to lung. These results suggest that the two populations separated from parental AH109A cells provide good models for the study of tumor invasion and tissue-specific metastasis and that adhesive AH109A cells can be used for the creation of lymphatic metastasis model of rats.

Introduction

Metastasis is a complex and sequential cascade of events that leads to the dissemination of tumor cells to distant tissues (Liotta et al. 1988). In many cases, metastasis may be lethal in the course of clinical treatment of cancer. There are three modes for tumor metastasis, that is, hematogenous metastasis, lymphatic metastasis and disseminative metastasis (Poste and Fidler 1980). Although all of them are important in tumor diagnosis, especially patients with lymphatic metastases often have poor prognoses. Intensive studies have been done by many researchers to prevent tumor metastasis. In the course of such

studies, animal models for tumor metastasis have been developed (Nicolson 1982). However, almost of these models are to mimic hematogenous metastases. There are few animal models for lymphatic or disseminative metastases (Schackert and Fidler 1989; Nicolson 1988). In addition, some tumor cells show tissue-specific metastases, for instance, colon cancer cells often metastasize to liver or lung, and breast cancer cells to bone marrow. Although these tissue-specific metastases were reported more than a century ago (Paget 1889), little is known about the mechanisms for these phenomena.

We have been investigating the effects of food factors on tumor cell proliferation and invasion,

the most important step of metastasis, using rat ascites hepatoma cell line of AH109A cells as a model tumor cell (Miura et al. 1997a, b) and already found that many food factors with antioxidative activities could suppress tumor cell invasion (Kozuki et al. 2001a, b). In these reports, we examined bioavailabilities of food factors that show anti-proliferative and anti-invasive activities *in vitro*, that is, the effects of sera from rats to which food factors were administered were evaluated *in vitro*. In the course of these studies, we have noticed that there are two populations in AH109A cells. When cultured in the medium containing rat sera, some AH109A cells adhere to culture dishes and other cells remain in a floating state. In this report, we describe the isolation of subpopulation of AH109A cells and characterization of these populations. We also found that adhesive AH109A cells metastasize lymphatically to lung and floating AH109A cells to mesentery, when injected intravenously and these cells have a potential to give a new animal model for lymphatic metastases and for study of the mechanisms for tissue-specific metastases.

Materials and methods

Animals and culture of AH109A cells

Male Donryu rats were purchased from NRC Haruna (Gunma, Japan). The animals were treated in accordance with the guidelines established by the Animal Care and Use Committee of Tokyo Noko University. AH109A cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan and maintained in the peritoneal cavity of Donryu rats as described previously (Miura et al. 1997a). AH109A cells cultured in MEM (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% calf serum (JRH, Lenexa, KS, USA) (10% CS/MEM) for at least 2 weeks after preparing from accumulated ascites were used for the following experiments.

Isolation of two populations of AH109A cells

Adhesive AH109A cells and floating AH109A cells were isolated from parental AH109A cells by

culturing in MEM containing 10% rat serum prepared from Donryu rats (10% RS/MEM). Rat bloods were collected by decapitation, clotted at room temperature and sera were prepared by centrifugation (1500g, 15 min). When cultured in 10% RS/MEM, some AH109A cells adhere to the culture dish, actively extending pseudopodia, and other cells remain in the floating state (Figure 1). These two populations were easily separated because one adhered to the dish and the other did not. During three successive cultures in the presence of RS, cells which continued adhering to the culture dish were designated as adhesive AH109A cells (AH109A-Ad) and cells which remained at the floating state as floating AH109A cells (AH109A-Fl).

Proliferation and invasion assays

Proliferative activities of these two populations of AH109A cells were assessed by an MTT method as described previously (Miura et al. 1997a). Invasive activities of them were examined by co-culturing them with primary cultured mesentery-derived mesothelial cells as described previously (Miura et al. 1997a).

In vivo metastatic analyses

To investigate *in vivo* metastatic characteristics of these two populations, 5×10^5 cells of them were respectively, injected intravenously into the tail veins of male Donryu rats. After 3 weeks, rats were sacrificed and dissected. Metastatic foci were macroscopically checked. Lungs were collected, fixed in 4% paraformaldehyde, dehydrated with graded ethanol, embedded in paraffin, sectioned at 5 μ m, stained with hematoxylin and eosin, and observed under a light microscope.

Statistical analyses

Data were indicated as means $\pm$ SEM. They were analyzed by one-way analysis of variance followed by Tukey's Q test or Student's *t* test. *p* value less than 0.05 is considered significant.

(a)
Parental AH109A (10% CS/MEM)

(b)
Adhesive AH109A (10% RS/MEM)

(c)
Floating AH109A (10% RS/MEM)

Results

Isolation of adhesive AH109A and floating AH109A cells

As shown in Figure 1, AH109A cells grow well as floating cells when cultured in 10% CS/MEM (Figure 1a). However, when cultured in 10% RS/MEM, some AH109A cells adhere to the culture dish, actively extending the pseudopodia (Figure 1b) and other cells remain at the floating state (Figure 1c). We separated these two populations by three successive culture in 10% RS/MEM. Interestingly, adhesive AH109A cells returned to the floating state when cultured again in 10% CS/MEM (data not shown). To the extent we have examined, not only sera from Donryu rats, but also sera from Wistar rats and ICR mice have the ability to cause these morphological changes to AH109A cells. Human, rabbit and horse sera did not cause such morphological changes to AH109A cells (data not shown). At present, we do not know why only rodent sera have such ability. Nevertheless, this result clearly shows that there are two populations in AH109A cells, which respond differently to rat serum.

In vitro proliferative and invasive activities of these two populations

In vitro proliferative activities of these two populations were compared using an MTT method. Proliferative activity of adhesive AH109A cells was 120% of parental AH109A cell, while that of floating AH109A cells was 65% when they were cultured in 10% CS/MEM (Figure 2a). Furthermore, adhesive AH109A cells showed about two or threefold higher invasive activity than floating AH109A cells in a co-culture system between AH109A cells and mesentery-derived mesothelial cells cultured in 10% CS/MEM (Figure 2b).

These results clearly show that adhesive AH109A cells proliferate more rapidly and invade more actively than do floating AH109A cells.

Figure 1. Phase contrast microscopic photos of AH109A cells. (a) AH109A cells cultured in 10% CS/MEM. (b) Adhesive AH109A cells and (c) floating AH109A cells cultured in 10% RS/MEM. Bar indicates 50 μ m.

Figure 2. Proliferative and invasive activities of adhesive and floating AH109A cells. (a) Parental, adhesive and floating AH109A cells (5×10^4 cells) were seeded in 24-well culture plate and cultured in 10% CS/MEM for 20 h. After adding MTT reagent to each well, cells were further cultured for 4 h and proliferative activities were determined spectrophotometrically. Data are the means $\pm$ SEM for 6 wells. (b) Adhesive and floating AH109A cells (4×10^5 cells) were overlaid onto confluent cultured mesentery-derived mesothelial cell layer in 10% CS/MEM. After 24-h culture, invaded cells and colonies were counted under phase contrast microscope. Data are the means $\pm$ SEM for 10 areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey's Q test (a) and Students' t test (b).

In vivo metastatic potentials of two populations

To investigate *in vivo* metastatic potentials of these two populations, 5×10^5 cells were respectively injected into tail veins of male Donryu rats. After 3 weeks, rats were dissected. All of rats injected

with adhesive AH109A cells had aggressive lung metastatic foci (Figure 3 and Table 1). Only one rat had metastatic foci in mesentery, into which AH109A cells invade during their growth in peritoneal cavity. While all of rats injected with floating AH109A cells metastasized to mesentery,

Figure 3. Metastases of lung and mesentery of rats injected with adhesive and floating AH109A cells. (a) Lung metastases of adhesive AH109A cells. (b) Mesentery metastases of floating AH109A cells.

Table 1. *In vivo* metastatic profile of adhesive AH109A and floating AH109A cells

Cell population	Metastasis	
	Lung	Mesentery
Adhesive AH109A	5/5	1/5
Floating AH109A	0/5	5/5

5×10^5 cells of each population were intravenously injected into tail vein respectively. After 3 weeks, rats were dissected and metastatic foci were macroscopically inspected.

Figure 4. Hematoxylin-eosin staining of lung metastatic foci of adhesive AH109A cells. (a) around lung arteries and tracheae ($\times 40$), (b) higher magnitude of (a) ($\times 100$). A: artery, Tr: trachea, T: tumor, A Bar in each figure represents $100 \mu\text{m}$.

no metastatic foci was observed in their lungs (Figure 3 and Table 1). These results suggested that the two populations we have isolated had different tissue-specific metastatic potentials.

Lung metastases of adhesive AH109A cells were histologically analyzed and AH109A cells proved to exist mainly in small vessels around lung arteries and tracheae (Figure 4). Lymphatic vessels are known to anatomically exist around arteries and

tracheae in lung. Therefore, adhesive AH109A cells seem to metastasize to lymphatic vessels in lung.

Discussion

Some reports suggested that allogeneic, syngenic or autologous serum often affected the proliferative activities and morphology of cultured animal cells. Korotzer et al. (1980) reported that fresh human serum induced the proliferation of human diploid fibroblasts. Cramer et al. (1986) reported that autologous serum caused an increase in human neutrophil adherence to and migration across epithelial cells. Both these two groups suggested the involvement of complements in the effect of autologous serum on cell proliferation, adhesion or migration. However, rat sera still induced morphological changes in adhesive AH109A cells when they were heat-inactivated at 65°C for 30 min (data not shown). This result suggests that complements in rat sera are not involved in the phenomenon observed by us. Although we have not characterized the substance(s) in rat sera that induce the morphological changes in AH109A cells, we have already observed that boiling of rat serum abolishes its effect on morphology of AH109A cells (unpublished results), suggesting the involvement of some proteinous factor(s) other than complements.

Adhesive AH109A cells showed higher proliferative and invasive activities than floating AH109A cells when they were cultured in the presence of calf sera. The results also indicate that two subpopulations with different proliferative and invasive activities were induced from parental AH109A cells by the treatment with allogeneic rat sera. There are many reports about establishment of highly invasive subline from parental tumor cells by using various *in vitro* and *in vivo* selection methods (Miner et al. 1982; Brodt 1986). However, some sublines are often unstable because of genetic instability of tumor cells. On the other hand, the proliferative and invasive characters of adhesive and floating AH109A cells remained stable after at least 15 passages (data not shown), suggesting that different sets of genes were induced by the treatment with rat sera and these changes were irreversible for 15-passages periods. AH109A cells are originally isolated from accumulated ascites of rats implanted with carcinogen (diaminobenzidine)-

induced hepatic tumor and registered as an established cell line (Abe et al. 1981). Our results clearly show that parental AH109A cells have the potential to differentiate into two subpopulations with different characters, when cultured in the presence of allogeneic rat sera.

Adhesive AH109A cells and floating AH109A cells are expected to show different metastatic characteristics *in vivo*, because the former may easily adhere or embolize in vascular endothelium or tissues of rats and the latter may not. In fact, adhesive AH109A cells metastasize mainly to lung, into which tumor cells flow at first, when injected intravenously (Figure 3 and Table 1). They may adhere to lung vascular endothelium and form embolization there, resulting in lung metastatic foci. However, histological studies revealed adhesive AH109A cells existed in lymphatic vessels in lung, suggesting they moved to lymphatic vessels at hilar region after adhering to vascular endothelium and embolize in lymphatic vessels. On the other hand, floating AH109A cells unexpectedly metastasize mainly to mesentery. Although we have not observed microscopically, the metastatic foci formed in mesentery macroscopically seemed to be in mesenteric lymph nodes (Figure 3). These results suggest that AH109A cells have higher affinity to lymphatic vessels than to vascular endothelium. Suzuki and Pantazia (1993) reported that ascites-form of murine testicular teratocarcinoma (402AX) showed spontaneous metastasis from ascites to lungs and hilar lymph nodes in 129/J strain mice. We also have often experienced that parental AH109A cells metastasize spontaneously from accumulated ascites to mesentery and lung. There are few animal models for spontaneous metastasis originated from ascites. Our model using adhesive and floating AH109A cells may offer a useful animal model for spontaneous lymphatic metastasis.

In addition, adhesive and floating AH109A cells seem to be the same when cultured in medium containing calf serum, but the former metastasize mainly to lung and the latter exclusively to mesentery. Tumor cells have been known to metastasize tissue specifically. However little is known about the mechanisms for this phenomenon. Adhesive and floating AH109A cells isolated in this study may be useful for the study of the mechanism for tissue-specific metastasis by comparing transcriptome or proteome of these

cells and such study is now in progress in our laboratory.

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