RAPID COMMUNICATION Jpn. J. Cancer Res. 80, 818–821, September 1989

In vitro Invasion of Endothelial Cell Monolayer by Rat Ascites Hepatoma Cells

Hiroaki Ohigashi,[1] Kiyoko Shinkai,[2] Mutsuko Mukai,[2] Osamu Ishikawa,[1] Shingi Imaoka,[1] Takeshi Iwanaga[1] and Hitoshi Akedo[2]

Departments of [1]Surgery and [2]Tumor Biochemistry, The Center for Adult Diseases, Osaka, 1 Nakamichi, Higashinari-ku, Osaka 537

To study the tissue preference of invasion, we developed an assay system for the invasion of endothelial cells as a modification of the previously established assay of tumor cell invasion of mesothelial cells. Rat ascites hepatoma cells (AH 130) that had been seeded on a monolayer of cultured endothelial cells penetrated and formed tumor cell colonies under the monolayer. The penetration was time-dependent and the number of penetrated tumor cells and colonies was proportional to the number of tumor cells seeded. Comparison of the *in vitro* tumor cell invasion of endothelial cell monolayer with that of cultured mesothelial cell layer showed that a clone from the tumor cells (Cl-30) which was highly penetrative into the mesothelial cell layer had only a limited ability to penetrate the endothelial cell layer.

Key words: *In vitro* invasion — Culture — Endothelial cell — Ascites hepatoma

The most characteristic process of peritoneal seeding and hematogeneous metastasis is the tumor cell invasion of mesothelial and endothelial cell layers, respectively. To understand the mechanism by which the invasion takes place, several investigators have developed models for tumor cell attachment and invasion using cultured endothelial cell monolayers.[1] Invasion models using artificial blood vessels, chorioallantoic membranes and organ tissue culture have also been developed.[2–4] We have reported a model for tumor invasion of mesothelial cell monolayer.[5] In this model, rat ascites hepatoma cells (AH 130) penetrated the cultured mesothelial cell layer (MCL) and formed tumor cell colonies under the monolayer. A subline of AH 130 cells with a high invasive potential *in vivo* formed a much larger number of penetrated tumor cell colonies *in vitro* than did cells with a low invasive potential.[5] By using this model, we found that interactions of AH 130 cells with host cells or host mediators potentiated both the *in vitro* and *in vivo* invasive capacities of the tumor cells, verifying the usefulness of our model in studying tumor cell invasion of the mesothelial cell layer.[5–9]

Many malignant tumors demonstrate a propensity for invasion and metastasis to specific tissues and organs. To understand the mechanism by which non-random invasion and metastasis take place, it is useful to develop models for invasion of a variety of host cells to compare invasive behaviors of tumor cells. In the present study we developed an *in vitro* model for the invasion of endothelial cell monolayers by AH 130 cells as a modification of our previous system,[10] with which the invasion of mesothelial cells was studied. By comparing the behaviors of tumor cells in the present and previous systems we found that a clone from AH 130 cells which was highly penetrative into the mesothelial cell layer showed only a limited ability to penetrate the endothelial cell monolayer.

Calf pulmonary arterial endothelial cells (CPAE) were the gift of the Japanese Cancer Research Resources Bank (JCRB). The cells (2×10^5) were seeded on a 35 mm plastic culture dish and cultured in a humidified atmosphere of 5% CO_2 and 95% air in Eagle's minimum essential medium (MEM) with 20% fetal calf serum. They grew forming a monolayer on the surface of the culture dish. The parent AH 130 cells were maintained by successive transplantation into the peritoneal cavity of rats (Donryu strain). The tumor cells obtained from the peritoneal cavity were cultured for a week in MEM containing two-fold concentrations of amino acids and vitamins supplemented with 10% calf serum. A subline (LC-AH) of AH 130 cells was obtained by successive passages in culture of the parent AH 130 cells. Cl-30 was cloned from LC-AH cells.

Endothelial cells were grown almost to confluency on a culture dish (35 mm in diameter; culture medium, 2 ml), the endothelial cell monolayer (ECL) was rinsed once with the culture medium and the tumor cells (1×10^3–10^5 cells/dish) were seeded on it. The interaction of the tumor cells with ECL was observed under a phase contrast microscope. After a given period of coculture, the medium was removed and the remaining cell layers were fixed in 10% formalin in phosphate-buffered saline (PBS). The number of penetrated single tumor cells and tumor cell colonies formed under ECL (one colony con-

sisted of more than two cells) in 60 different visual fields (1.13 mm^2 each) was counted under a phase contrast microscope and expressed as the penetrated single cells and colonies/cm^2, which was defined as the *in vitro* invasive capacity of the tumor cells. In some experiments, endothelial cells were grown on collagen gel-coated plastic culture dishes and the tumor cells were seeded on the monolayer. After 48 h of coculture, the culture medium was removed and the remaining cell layers were rinsed with PBS and fixed in Zamboney's solution. The fixed cell layers with the underlying collagen gel were sectioned perpendicularly to the cell layer. The sections were stained with hematoxylin and eosin. *In vitro* invasion of MCL by the tumor cells was examined by the method reported previously,[7] except that the number of tumor cells seeded was 1×10^5 cells/dish.

About 3 h after the tumor cell seeding on ECL, the individual tumor cells adhered to and started to penetrate into ECL. About 6 h after the seeding, we observed flattened configurations of the tumor cells, which spread parallel to ECL, indicating the completion of the penetration. The flattened tumor cells then divided and formed

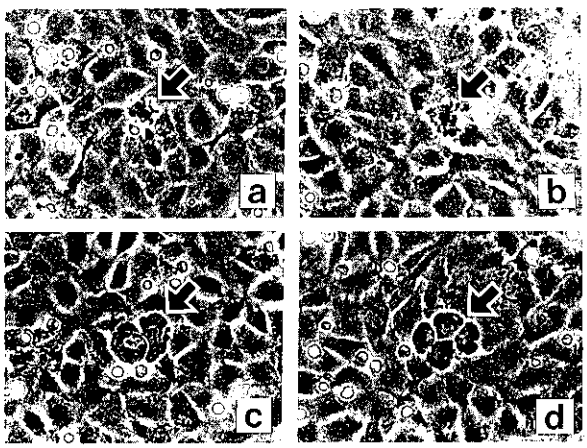

Fig. 1. Time course of the penetration of ECL by LC-AH cells. a) 3 h after the tumor cell seeding; the arrow indicates the pseudopod-like process developed on the tumor cell, b) 6 h after the seeding; the penetration of the tumor cell completed (arrow), c) 24 h after the seeding; division of the penetrated tumor cell (arrow), d) 48 h after the seeding. Phase-contrast microscopy, ×110.

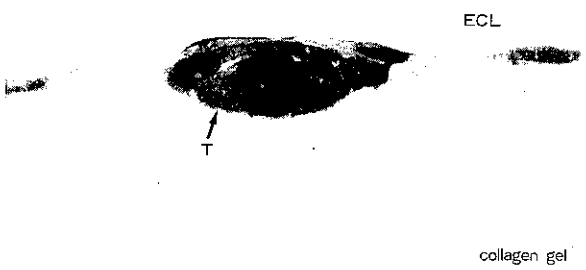

Fig. 2. Perpendicular section of a penetrated tumor cell colony. LC-AH cells were seeded on ECL that had been formed on collagen gel. The resultant penetrated colony was fixed and cut perpendicularly to ECL. Hematoxylin-eosin stain, ×825. T: LC-AH cells.

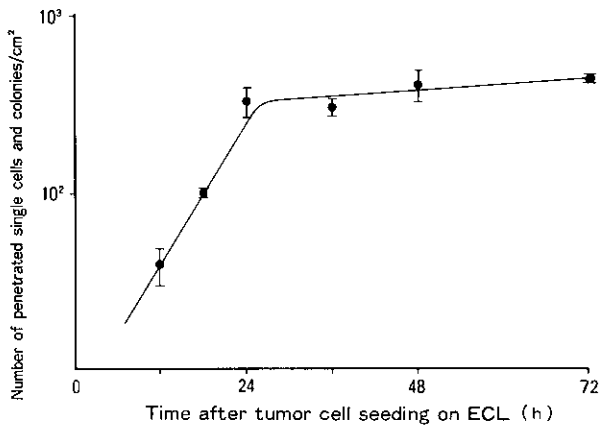

Fig. 3. Time-dependent increase in the number of penetrated single cells and colonies/cm^2. LC-AH cells (1×10^4 cells/dish) were seeded on ECL.

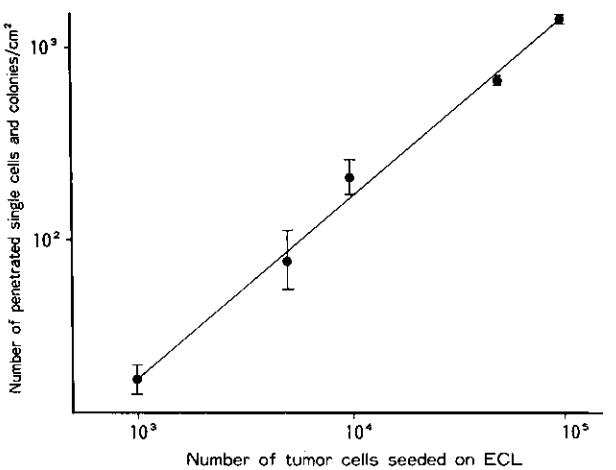

Fig. 4. Correlation of the number of penetrated single cells and colonies with that of LC-AH cells seeded. The number of penetrated single cells and colonies/cm^2 was counted 48 h after the tumor cell seeding.

Table I. *In vitro* Invasion of ECL and MCL by AH 130 Sublines

Tumor cells	Number of penetrated single cells and colonies/cm^2 [a]	
	Monolayer cells	
	Endothelial	Mesothelial
AH 130	62±10[b]	49±10[b]
LC-AH	1150±37	521±19
Cl-30	61±7[b]	381±37

Tumor cells (1×10^5 cells/dish) were seeded on either ECL or MCL, and cultured for 48 h. The number of penetrated single cells and colonies was counted.
a) Mean±SD from at least 6 determinations.
b) Not significant. Others are significantly ($P<0.05$) defferent from each other by the *t* test.

colonies. A typical process of the formation of a tumor cell colony is shown in Fig. 1. Because the tumor cells were in a form of single cell suspension, each tumor cell colony had developed from a single tumor cell.

To ascertain whether or not the flattened tumor cell colonies were formed under ECL, ECL was developed on a collagen gel matrix, then the tumor cells were seeded on ECL and cultured for 2 days. At the end of the culture, the cells on the collagen gel were fixed and sectioned perpendicularly to the cell layer. The flattened tumor cell colonies were seen in between ECL and the collagen gel surface (Fig. 2). Figure 3 shows that the number of penetrated single cells and colonies/cm^2 increased up to 24 h after the tumor cell seeding and thereafter stayed almost constant. The number of penetrated single cells and colonies/cm^2 measured at 48 h increased with the number of tumor cells seeded (Fig. 4).

We have reported that AH 130 cells, when seeded on MCL, penetrated and formed colonies under the monolayer.[7, 10] So we compared the *in vitro* invasion of ECL with that of MCL by AH 130 sublines (Table I). The parent AH 130 cells penetrated poorly into both ECL and MCL. In contrast, LC-AH cells were highly invasive into both of the layers. Cl-30 cells, the cell line cloned from LC-AH, were highly invasive into MCL, but penetrated poorly into ECL. To examine whether or not the preferential invasion of ECL by LC-AH cells could also be demonstrated *in vivo*, we tried to compare the lung colonizing ability of LC-AH with that of Cl-30 cells. However, AH cell lines including LC-AH and Cl-30 did not form lung metastasis, when iv, ip or sc injected. Metastasis develops by a series of steps including invasion and local growth. Obviously, the invasive ability is not the only determinant for successful metastasis.

Kramer and Nicolson,[11] and Nicolson[12] have reported an *in vitro* model for tumor cell invasion and investigated the interactions of tumorigenic and non-tumorigenic cells with cultured endothelial cell layer. However, they did not quantitate the invasive capabilities of the tumor cells, though they measured cell attachment to the endothelial cell monolayer and solubilization of the basal lamina. In the model system described here, the number of penetrated colonies increased linearly with the number of tumor cells seeded. Application of this system together with our previous model for the invasion of mesothelial cells[10] appears to provide a rapid determination of the invasive potential of tumor cells and to make it easy to screen substances which modify the invasion and metastasis.

It is interesting to note that Cl-30 cells were poorly invasive into ECL, in spite of the fact that they were highly invasive into MCL. The reason for their selective invasion is not clear at present. However, such selectivity strongly suggests that the tissue preference of invasion is not solely determined by the unique properties of particular tumor cells, but also depends on the different characteristics of each normal cell layer. LC-AH cells, from which Cl-30 was cloned, demonstrated an extensive invasion into both ECL and MCL. The LC-AH cell population might contain cells preferentially penetrative into ECL or into both ECL and MCL, and these possibilities are now under investigation. Tsuruo *et al.* reported the growth factor(s) from lung extract as one of the determinants for successful lung colonization.[13] Several other determinants for nonrandom distribution of metastasis have also been suggested by Nicolson.[14] The selective invasiveness demonstrated in the present paper may give us a new approach to investigate the tissue- and organ-specificity of invasion and metastasis.

This work was supported in part by a grant from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan and by grants from the Research Foundation for Cancer and Cardiovascular Diseases and from Osaka Cancer Association.

(Received April 25, 1989/Accepted July 6, 1989)

REFERENCES

1) Schirrmacher, V. Cancer metastasis: experimental approaches, theoretical concepts, and impacts for treatment strategies. *Adv. Cancer Res.*, **43**, 1–73 (1985).
2) Jones, P. A., Neustein, H. B., Gonzales, F. and Bogenmann, E. Invasion of an artificial blood vessel wall by human fibrosarcoma cells. *Cancer Res.*, **41**, 4613–4620 (1981).
3) Nicolson, G. L., Dulski, K., Basson, C. and Welch, D. R.

Preferential organ attachment and invasion *in vitro* by B16 melanoma cells selected for differing metastatic colonization and invasive properties. *Invasion Metastasis*, **5**, 144–158 (1985).
4) Easty, D. M. and Easty, G. C. Measurement of the ability of cells to infiltrate normal tissues *in vitro*. *Br. J. Cancer*, **29**, 36–49 (1974).
5) Akedo, H., Shinkai, K., Mukai, M. and Komatsu, K. Potentiation and inhibition of tumor cell invasion by host cells and mediators. *Invasion Metastasis*, **9**, 134–148 (1989).
6) Mukai, M., Shinkai, K., Tateishi, R., Mori, Y. and Akedo, H. Macrophage potentiation of invasive capacity of rat ascites hepatoma cells. *Cancer Res.*, **47**, 2167–2171 (1987).
7) Shinkai, K., Mukai, M. and Akedo, H. Superoxide radical potentiates invasive capacity of rat ascites hepatoma cells *in vitro*. *Cancer Lett.*, **32**, 7–13 (1986).
8) Mukai, M., Shinkai, K., Komatsu, K. and Akedo, H. Potentiation of invasive capacity of rat ascites hepatoma cells by transforming growth factor-β. *Jpn. J. Cancer Res.*, **80**, 107–110 (1989).
9) Shinkai, K., Mukai, M., Komatsu, K. and Akedo, H. Factor from rat liver with antiinvasive potential on rat ascites hepatoma cells. *Cancer Res.*, **48**, 3760–3764 (1988).
10) Akedo, H., Shinkai, K., Mukai, M., Mori, M., Tateishi, R., Tanaka, K., Yamamoto, Y. and Morishita, T. Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. *Cancer Res.*, **46**, 2416–2422 (1986).
11) Kramer, R. H. and Nicolson, G. L. Interactions of tumor cells with vascular endothelial cell monolayers: a model for metastatic invasion. *Proc. Natl. Acad. Sci. USA*, **76**, 5704–5708 (1979).
12) Nicolson, G. L. Metastatic tumor cell attachment and invasion assay utilizing vascular endothelial cell monolayers. *J. Histochem. Cytochem.*, **30**, 214–220 (1982).
13) Tsuruo, T. Tumor-induced platelet aggregation and growth-promoting factors as determinants of probable tumor metastasis. *Jpn. J. Cancer Chemother.*, **14**, 2033–2039 (1987).
14) Nicolson, G. L. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev.*, **7**, 143–188 (1988).