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Serum Requirement for *in vitro* Invasion by Tumor Cells

Fumio Imamura,[1] Takeshi Horai,[2] Mutsuko Mukai,[3] Kiyoko Shinkai[3] and Hitoshi Akedo[3]

[1]Department of Internal Medicine, [2]Clinical Research for Lung Cancer, Research Institute, [3]Department of Tumor Biology, Research Institute, The Center for Adult Diseases, Osaka, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537

The effect of fetal calf serum (FCS) on *in vitro* invasion by rat ascites hepatoma cells (AH130) was studied by using the *in vitro* invasion assay. Although the coculture of the highly invasive clone (MM1) of AH130 cells and the mesothelial cell layer or endothelial cell layer in modified minimum essential medium supplemented with 10% FCS resulted in extensive penetration of the layer by the tumor cells, the omission of FCS resulted in an almost complete elimination of the *in vitro* invasion. The *in vitro* invasiveness by human small cell lung cancer cells (OC10) was also remarkably reduced by the omission of FCS from the assay medium, suggesting a requirement of serum for the *in vitro* tumor cell invasion. When 10% FCS was added to the medium 2 h after the tumor cell seeding in FCS-free invasion assay system, penetration by MM1 cells was observed within an hour. This rate of penetration was almost the same as that when 10% FCS was added at the time of tumor cell seeding. FCS was also required for the penetration of a mesothelial cell monolayer by MM1 cells in a defined growth medium (SFM-101), in which MM1 cells were well maintained. The invasion-inducing activity appears to be independent of the growth-stimulating activity in serum.

Key words: *In vitro* invasion — Fetal calf serum — Mesothelial cell — Ascites hepatoma cell

Malignant tumor cells have certain distinct characteristics, among which invasiveness is the most prominent.[1)] Since invasion is crucial to the malignancy of tumor cells, studies on invasion should provide valuable insights into tumor biology, especially tumor progression. We have established a cultured mesothelial cell (M-cell) monolayer system for studying invasion of tumor cells in suspension.[2)] In this system, rat ascites hepatoma cells (AH130 cells) that had been seeded on a M-cell monolayer penetrated and formed colonies under the monolayer. Since AH130 cells appeared in the form of an independent single-cell suspension in culture, each penetrated colony had developed from a single penetrated tumor cell; therefore the number of penetrated tumor cells and colonies under an M-cell monolayer per unit area can be considered to represent the invasive capacity of the tumor cells. This *in vitro* penetration of an M-cell monolayer by tumor cells mimics the *in vivo* invasion of rat peritoneum by i.p.-implanted AH130 cells. This system has the advantage that the *in vitro* invasiveness of tumor cells estimated in this system corresponds well to their *in vivo* invasive ability.[3,4)]

By using this system, we have found that the invasive capacity of AH130 cells was potentiated by the preculture of the tumor cells with activated platelets,[5)] activated macrophages,[3)] TGF-β,[4)] oxygen radicals,[6)] and adriamycin,[7)] whereas it was suppressed by the invasion-inhibiting factors (IIFs) from rat and bovine livers[8,9)] or transmethylation inhibitors.[10)] These results show that the invasive capacity of tumor cells is not only genetically determined, but is also greatly influenced by their interactions with host cells and mediators.[5)] Our experiments so far have employed the *in vitro* invasion assay in which the culture medium contained 10% fetal calf serum (FCS). Since serum is well-known to have a variety of biological activities,[11,12)] we considered that it might influence the *in vitro* tumor cell invasion. To examine this possibility, FCS was removed from the assay medium and the penetration by a highly invasive clone of AH130 cells (MM1) was studied in FCS-free medium.

The experimental procedures for the *in vitro* invasion were essentially the same as reported previously[2-8,10)]: briefly, primary cultured M-cells were prepared from rat mesentery by trypsin digestion each time for each series of experiments. They were cultured as a monolayer in Eagle's minimum essential medium with 2-fold concentrations of amino acids and vitamins (modified MEM) supplemented with 10% FCS (Cell Culture Laboratories) in an atmosphere of 95% air and 5% CO_2 at 37°C. When an M-cell monolayer became confluent, $1.0–2.0 \times 10^5$ tumor cells were overlaid on it in 2 ml of assay medium with or without FCS. After an appropriate time period the cells were fixed with 10% formalin. The number of penetrated tumor cells and colonies was counted under a phase-contrast microscope.

[1] To whom all correspondence should be addressed.

Table I. Effect of Fetal Calf Serum (FCS) on *in vitro* Invasion by MM1 Cells of Mesothelial Cell Monolayer and Endothelial Cell Monolayer

Monolayer	Addition to FCS-free medium[a]	Number of penetrated cells and colonies/cm²	
		4 h[b]	24 h
MCL[c]	10% FCS	466±48[d]	922±41
MCL	—	0.0±0.0	1.6±2.8
ECL[e]	10% FCS	312±13.4	NT[f]
ECL	—	0.0±0.0	NT

a) Eagle's minimum essential medium with 2-fold concentrations of amino acids and vitamins.
b) Time after tumor cell seeding.
c) Mesothelial cell monolayer.
d) Mean±SD of at least 2 determinations.
e) Endothelial cell monolayer.
f) Not tested.

MM1 cells hardly penetrated the M-cell monolayer (MCL) in the absence of FCS in the assay medium as shown by the number of penetrated tumor cells and colonies (Table I). In sharp contrast, appreciable penetration by tumor cells was seen in the presence of 10% FCS in the invasion assay medium.

The effect of FCS on the *in vitro* invasion by MM1 cells was also tested using an endothelial cell monolayer system reported previously.[13] Endothelial cells (E-cells, the gift of the Japanese Cancer Research Resources Bank), which were derived from calf pulmonary arteries, were cultured in modified MEM supplemented with 20% FCS. When they grew to confluency, they were used for the invasion assay; the assay procedure was essentially the same as reported elsewhere[13] and as described for the M-cell system. As shown in Table I, the removal of FCS from the assay medium again resulted in a complete elimination of the *in vitro* penetration of an E-cell monolayer (ECL) by MM1 cells.

The above results strongly suggest that the requirement of FCS for the *in vitro* invasion is independent of the kind of monolayer which the MM1 cells penetrate. Then, we tested the effect of FCS on the *in vitro* invasion of an M-cell monolayer by human small cell lung cancer cells (OC10 cells) to determine whether the FCS effect depends on the kind of overlaid tumor cells. OC10 cells were established from material obtained by fiberoptic transbronchial brushing of a pulmonary tumor of a 65-year-old male and cultured *in vitro* as loosely connected floating aggregates. The numbers of penetrated OC10 cells and colonies at 4 h were 7.2±3.5/cm² (mean±SD) and 238±44/cm² in FCS-free and FCS-containing medium, respectively (Table II). This result shows that the *in vitro* invasion of OC10 cells was also strongly influenced by the serum.

Table II. Effect of Fetal Calf Serum (FCS) on *in vitro* Invasion of Mesothelial Cell Monolayer by OC10 Cells

Addition to FCS-free medium	Number of penetrated cells and colonies/cm²	
	4 h	24 h
10% FCS	238±44[a]	802±190
—	7.2±3.5	77±0.0

a) Mean±SD of at least 2 determinations.

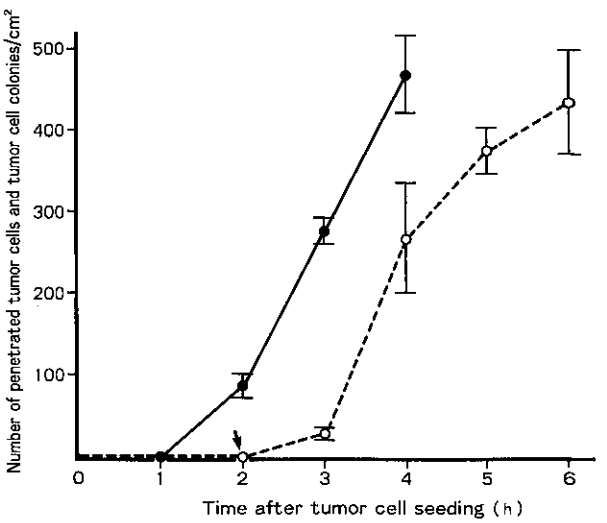

Fig. 1. Dependency of *in vitro* invasion on FCS addition. ●: control, 10% FCS was added to the assay medium at the time of tumor cell seeding (0 h). ○: FCS was omitted from the assay medium at the time of tumor cell seeding; 2 h after the tumor cell seeding FCS was added to give the final concentration of 10% (arrow). The numbers of penetrated tumor cells and colonies/cm² at 24 h were 998±20 and 922±41 for the delayed addition of FCS and for the control, respectively. Data points are mean±SD.

Fig. 1 shows the time course of the penetration of an M-cell monolayer by MM1 cells. When 10% FCS was present at the time of tumor cell seeding, the number of penetrated tumor cells and colonies increased with time after a 1 h lag time period (possibly reflecting the time required for tumor cell adhesion to the M-cell monolayer). In contrast, the tumor cells hardly penetrated in the absence of FCS. However, when FCS was added 2 h after the tumor cell seeding in FCS-free assay medium (Fig. 1, arrow), penetrated cells were observed 1 h thereafter. These results suggest that the machinery for invasion was well preserved under FCS-free conditions.

Table III. *In vitro* Invasion of Mesothelial Cell Monolayer by MM1 Cells in SFM-101

Medium	Addition to medium	Number of penetrated cells and colonies/cm$^{2a)}$
MEM$^{b)}$	10% FCS	667±71$^{c)}$
SFM-101	—	0±5
SFM-101	10% FCS	902±28

a) Numbers at 7 h after tumor cell seeding.
b) Modified MEM.
c) Mean±SD of 3 determinations.

The proliferative ability of MM1 cells cultured in FCS-free modified MEM for 2 h was well preserved. MM1 cells which had been cultured with an M-cell monolayer in the absence of FCS for 2 h were harvested by gentle pipetting and subsequently cultured in modified MEM supplemented with 10% FCS. The generation time of these cells was 31.2 h, which is not prolonged from that (34.8 h) of MM1 cells cultured continuously in 10% FCS.

MM1 cells, however, could not be maintained in the serum-free modified MEM for a prolonged period of time. To ascertain that the reduced penetration in the case of omission of FCS was not due to cellular damage, we tested the penetrative ability of MM1 cells in a defined growth medium, SFM-101 (Nissui Pharmaceutical Co. Ltd., Tokyo), which is supplemented with 10 mg/liter transferrin, 10 mg/liter insulin, 0.002 mg/liter sodium selenite and 0.013 mg/liter putrescine. Although the growth rate of MM1 cells in SFM-101 was slower than that in MEM supplemented with 10% FCS, the cells could be maintained in SFM-101 which was not supplemented with FCS. In SFM-101, the penetration of an M-cell monolayer by MM1 cells was also remarkably reduced under FCS-free conditions (Table III). The requirement of FCS was consistent, and FCS could not be replaced by transferrin, insulin, sodium selenite or putrescine (contained in SFM-101). The number of penetrated tumor cells and colonies in SFM- 101 supplemented with 10% FCS was about 1.4 times that in modified MEM supplemented with 10% FCS. This may imply that SFM-101 gives a better environment for *in vitro* tumor cell invasion probably by improving cellular viability.

The purification of the corresponding factor is in progress. The activity in FCS was recovered in a fraction obtained by 35–50% ammonium sulfate fractionation. The further purification of the activity in the ammonium sulfate fraction on a Mono Q column by fast protein liquid chromatography showed that the main activity was eluted at about 0.1 *M* NaCl in 20 m*M* Tris-HCl (pH 8.0) (unpublished result). This partially purified factor had almost no effect on the proliferation of MM1 cells in SFM-101.

In summary, we showed that some serum factor was required for the *in vitro* invasion of cellular monolayers by tumor cells. The mechanism of the effect of the factor is unknown at present. It is also not clear whether FCS acts on tumor cells or monolayer cells or both. However, because FCS can be replaced by either calf serum or human serum in inducing tumor cell penetration, it is reasonable to assume that the invasion is supported by a factor in the body fluid.

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