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Progression of Weakly Malignant Clone Cells Derived from Rat Mammary Carcinoma by Host Cells Reactive to Plastic Plates

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Tumor progression is the process by which tumor cells acquire more malignant properties, such as invasiveness and metastasis, during tumor development. To elucidate mechanisms of tumor progression, we examined the role of interactions between the tumor and its host by using a cloned cell line, ER-1, which was derived from a rat mammary carcinoma. ER-1 is weakly tumorigenic and non-metastatic when s.c. injected into syngeneic hosts in single cell suspension. However, ER-1 cells show a high incidence of lethal growth when s.c. implanted (5×10^2 cells), being attached to a $10 \times 5 \times 1$ mm polystyrene plate. Tumor cell lines (PLT) obtained from tumors which had arisen from the plate-attached ER-1 cells no longer required plates for their growth in normal hosts, and had acquired metastatic ability to the lungs. The malignant phenotypes of PLT were stable under a usual culture condition for at least 6 months. Furthermore, the incidence of tumor development increased when small numbers of ER-1 cells were injected onto plates (or at their periphery) which had previously been implanted s.c. without tumor cells. The tumorigenicity of ER-1 cells increased after they were cocultivated for more than 30 days with host reactive cells obtained from the tissues surrounding the plates. These results suggest that host cells reactive to the foreign body (plastic plate) may not only promote the local growth of ER-1 cells but also convert them into much more malignant tumors.

Key words: Tumor progression — Host reactive cells — Rat mammary carcinoma — Plastic plate — Inflammation

The phenomenon that tumor cells inexorably acquire malignancy has been termed "tumor progression" by Foulds.[1] Therefore, it is important to examine how and why transformed cells acquire malignant phenotypes, because the major problem of cancer is tumor progression rather than carcinogenesis itself. Although several investigators have focused on the mechanisms of tumor progression,[2–6] there still remain unknown factors of both tumor cells and host cells which induce and/or enhance tumor progression.

It is also known that inflammation or surgical procedures may be associated with carcinogenesis in some clinical and experimental cases.[7,8] It has been reported that implantation of foreign bodies such as glass and plastic substrates induces sarcomas in several animal species[9] and that several normal cell lines show malignant tumor growth following s.c. implantation of cells attached to substrates such as plastic plates or glass beads.[10–13] Vasiliev and Moizhess have reported that growth of a small number of tumor cells was stimulated when they were inoculated on the surface of polymer film or close to it, and speculated that the local environment around the implanted film might promote tumor cell proliferation.[14] These findings suggest that host reaction such as inflammation or wound healing may cause or promote tumorigenesis or tumor growth, though the mechanisms are still unclear.

In the present study, we investigated the mechanisms of tumor progression by using an experimental model of a rat mammary carcinoma cell line (c-SST-2),[2] and succeeded in converting ER-1, a weakly tumorigenic cloned cell line of c-SST-2, into a highly tumorigenic and metastatic cell line by interaction with host cells reactive to plastic plates.

MATERIALS AND METHODS

Animals SHR-strain female rats, 7 to 10 weeks of age, were used throughout the experiments. They were purchased from Nippon Rat Co. (Urawa).

Tumor cell lines A cloned line, Cl-4, was isolated from a spontaneous rat mammary adenocarcinoma cell line, c-SST-2, by a single cell culture. Cl-4 is tumorigenic and

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[2] Abbreviations: c-SST-2, a rat mammary carcinoma; ER-1, a weakly tumorigenic and non-metastatic cell line derived from c-SST-2; PLT, a tumor cell line derived from the plate-attached ER-1 cells; GTG, 6-thioguanine; Oua, ouabain; EMS, ethyl methanesulfonate; DME, Dulbecco's modified medium, NCS, newborn calf serum; PBS, phosphate-buffered saline.

weakly metastatic as described previously.[15] The 6-thioguanine (6-TG)- and ouabain (Oua)-resistant mutant cell line, ER-1, was obtained by the following method. Cl-4 cells were treated with a mutagen, ethyl methanesulfonate (EMS, 250 μg/ml), for 24 h, and then washed and cultured for 5 days in Dulbecco's modified MEM (DME) supplemented with 7% newborn calf serum. The cells were harvested and seeded onto new culture dishes with 6-TG at a starting concentration of 0.3 μg/ml and final concentration of 10 μg/ml. Surviving cells were treated with Oua at a starting concentration of 0.3 m*M* and final concentration of 10 m*M*. One cell line obtained from 6-TG- and Oua-resistant mutants by cell cloning was designated ER-1. Cultured cell lines were established from s.c. growing tumors after inoculating ER-1 cells into syngeneic rats. The tumors were aseptically removed from the hosts and minced with scissors. The pieces of tumor tissue were placed on culture dishes, into which culture media were added. The cells which migrated from the tissue pieces and proliferated were detached with 0.2% trypsin plus 2 m*M* EDTA solution, and were seeded onto new culture dishes and maintained. Culture cell lines which were established from s.c. growing tumors by the inoculation of ER-1 cells attached to plastic plates into syngeneic rats were designated as PLT, and those established from s.c. growing tumors by the inoculation of a large number of ER-1 cells in single cell suspension were designated as Tsus. All the culture cell lines were maintained in DME supplemented with 7% NCS in 5% CO_2 and 95% air at 37°C.

Implantation of tumor cells Cultured tumor cells were detached from culture dishes with 2 m*M* EDTA solution, and washed twice with phosphate-buffered saline (PBS). Resuspended tumor cells in PBS (0.2 ml) were s.c. injected into SHR rats. Procedures for s.c. implantation of ER-1 cells attached to plastic plates were as follows. The bottom of a 100-mm culture dish (polystyrene, Corning, New York, NY) was cut into $1\times5\times10$ mm pieces (plastic plates), which were sterilized by UV-irradiation, and placed in 60-mm culture dishes. ER-1 cells suspended in the medium (50 μl) were seeded on each plate. Two to 3 h after the seeding, the medium was added to the culture of the ER-1 cells, and the cells were incubated for 18–24 h. The number of cells attached to each plate was counted in a hemocytometer after the tumor cells were detached from 10–20 plates with trypsin plus EDTA solution. A plate with ER-1 cells attached was implanted s.c. into a rat as follows. The rat was anesthetized with light ether, and an approximately 1-cm incision was made with scissors in the skin of its right back. The plate was inserted s.c. with the attached cells facing the epidermis, and the skin incision was closed immediately with two Muchell clips.

Coculture of ER-1 cells with host cells reactive to plastic plates Plastic plates alone were s.c. implanted into the back of rats in the same manner as above. The plates and reactive tissues surrounding the plates were aseptically removed 50–60 days after the plate implantation. Pieces of host reactive tissues were placed on culture dishes. When the reactive cells were nearly confluent, ER-1 cells (1×10^4 cells/ml) were seeded onto the cultures. Ten days after the seeding, the cells were treated with 2 m*M* EDTA solution for a few minutes. Although this treatment detached ER-1 cells, leaving most of the host reactive cells adhering to the dishes, the cell suspension contained approximately 5% of fibroblast-like cells other than ER-1 cells as observed under a phase-contrast microscope. The detached cells were passed through 4 layers of gauze, washed with DME and centrifuged at 1,200 rpm for 5 min. The cells suspended in DME (1×10^4 cells/ml) were seeded onto new reactive cell cultures and were maintained for at least 10 weeks (7 passages).

Conditioned medium from cultured cells reactive to plastic plates or fibroblasts of normal rat skin When cultured host cells reactive to the plates or the fibroblasts from rat skin reached confluency, the medium was discarded, and monolayer cultures were washed three times with DMEM medium. The cultures were incubated with DMEM medium for 24 h, then the medium was collected, filtered through 0.22 μm filters and stored at −20°C.

Anchorage-independent growth assay The cells (1×10^3 cells/ml) were suspended in 0.3% agar in DMEM containing 1% NCF and conditioned medium at the indicated concentrations or DMEM alone, and seeded into 60-mm dishes over a 0.6% agar base layer. After 10 days' incubation, colonies were counted under a microscope.

Cell migration assay Transwell™ chambers (Costar, Cambridge, MA) with 6.5-mm diameter tissue culture-treated membranes of 8 μm pore size were used for the migration assay. Conditioned medium (600 μl) was placed in the lower compartment of a Transwell™ chamber. The cells (5×10^4 cells/ml) were suspended in DMEM supplemented with 0.1% BSA. The cell suspension (100 μl) were placed in the upper compartment. After an 8-h incubation, the membranes were fixed in 3% glutaraldehyde and stained in Giemsa solution. The cells attached to the upper side of the membranes were removed with cotton swabs. The membranes were dried and the cells attached to the lower side of the membranes were counted with a microscope.

Histologic procedures After the s.c. inoculation of ER-1 cells (1×10^4) attached to plastic plates into syngeneic rats, the plates and tissues surrounding the plates were periodically (12 h–50 days) removed from the rats. The specimens were immediately fixed in 10% neutral

buffered formalin, and then dehydrated and embedded in paraffin by standard methods. Tissue sections were stained with hematoxylin and eosin.

RESULTS

Tumorigenicity and metastatic ability of ER-1 cells attached to plastic plates In Table I, we compare the tumorigenicity of ER-1 cells in saline suspension with that of the cells attached to plastic plates. The ER-1 cells were weakly tumorigenic in normal syngeneic rats when less than 1×10^6 cells were injected s.c. in suspensions, whereas small numbers of the cells showed tumorigenicity in immunosuppressed rats. The ER-1 cells were also highly tumorigenic in normal rats when implanted attached to plates (5×10^2 cells/plate).

Acquisition of malignant properties of tumor cells (PLT) derived from ER-1 cells attached to plastic plates We established culture cell lines from tumors grown 50 days after s.c. implantation of ER-1 cells attached to plastic plates (1×10^4 cells/plate). These tumor cell lines (PLT-D50) grew sufficiently to kill the hosts without any further requirement for plates, and also possessed metastatic ability to the lungs (Table II). The increased tumorigenicity and acquired metastatic ability of PLT-D50 cells were stable under a usual tissue culture condition for at least 6 months, but tended to decrease when the cells were cultured for a longer period (9 months). The tumorigenicity and metastatic ability of PLT lines correlated closely with the period since s.c. implantation of ER-1 cells attached to the plates (Table III).

In order to determine whether the increased malignancy of ER-1 cells was caused simply by *in vivo* growth or whether it required attachment to plates, we compared the malignant properties of PLT lines with those of cultured lines (Tsus) obtained from the tumors developed after the injection of large numbers of ER-1 cells.

Table II. Stability of Tumorigenicity and Metastatic Ability of PLT Cell Lines Obtained from the Tumors Arisen from ER-1 Cells Attached to Plates

Culture line[a]	Culture period[b] (months)	Incidence[c]	
		Tumor	Pulmonary metastasis (Number of colonies)
PLT-D50-1	0	5/5	3/5 (5, 19, 20)
PLT-D50-2	0	5/5	4/5 (1, 21, 30, 33)
PLT-D50-3	0	5/5	4/5 (3, 7, 19, 33)
PLT-D50-1	3	5/5	4/5 (3, 6, 20, 21)
PLT-D50-2	3	5/5	4/5 (7, 11, 29, 33)
PLT-D50-3	3	5/5	4/5 (5, 6, 18, 29)
PLT-D50-1	6	5/5	3/5 (2, 13, 18)
PLT-D50-2	6	5/5	3/5 (20, 29, 36)
PLT-D50-3	6	5/5	5/5 (3, 3, 18, 20, 31)
PLT-D50-1	9	4/5	2/5 (3, 10)
PLT-D50-2	9	1/5	0/5
PLT-D50-3	9	2/5	0/5

a) ER-1 cells attached to plates (1×10^4 cells/plate) were s.c. implanted into three normal syngeneic rats. Fifty days after the implantation, tissue culture cell lines (PLT) were separately established from tumors which had arisen in each rat.
b) PLT cell lines were cultivated for the indicated periods.
c) Tumor cells (1×10^4 cells/0.2 ml PBS) were s.c. injected into syngeneic rats, which were observed for 3 months.

Table I. Tumorigenicity and Metastatic Ability of ER-1 Cells Inoculated in Suspension or Attached to Plastic Plates in Normal SHR Rats

Cells in PBS suspension[a]			Cells attached to plates[a]		
No. of cells inoculated	Incidence		No. of cells Inoculated	Incidence	
	Tumor	Pulmonary metastasis (No. of colonies)		Tumor	Pulmonary metastasis (No. of colonies)
1×10^7	4/5	1/5 (3)	5×10^4	6/6	5/6 (8, 10, 23, 23, 40)
1×10^6	2/8	0/8	1×10^4	5/5	5/5 (3, 7, 26, 34, 35)
1×10^5	1/10	0/10	1×10^3	5/5	5/5 (5, 5, 15, 24, 28)
1×10^4	0/15	0/15	5×10^2	5/5	4/5 (10, 18, 20, 23)

a) Tumor cells in PBS suspension or attached to plates were s.c. inoculated into 7- to 10-week-old SHR rats. The rats were observed for 3 months. When plates were s.c. implanted without any attached cells, none of the rats (n=10) developed tumors during 6 months of observation.

Table III. Tumorigenicity and Metastatic Ability of PLT Cell Lines Derived from the Tumors Arisen from ER-1 Cells Attached to Plastic Plates

Cell lines[a]	Days after inoculation	Incidence[b]	
		Tumor	Pulmonary metastasis (No. of colonies)
PLT-D10-1	10	0/5	0/5
PLT-D10-2	10	1/5	0/5
PLT-D10-3	10	2/5	0/5
PLT-D30-1	30	4/5	1/5 (13)
PLT-D30-2	30	4/5	0/5
PLT-D30-3	30	2/5	2/5 (10, 21)
PLT-D50-1	50	5/5	3/5 (5, 19, 20)
PLT-D50-2	50	5/5	4/5 (1, 21, 30, 33)
PLT-D50-3	50	5/5	4/5 (3, 7, 19, 33)
ER-1		0/5	0/5

a) ER-1 cells attached to plastic plates (1×10^4 cells/plate) were s.c. implanted into 9 normal rats. After the implantation, tumors developing in 3 rats were excised and three types of cultured cell lines were established.
b) Tumor cells (1×10^4 cells/0.2 ml PBS) were s.c. injected into 8-week-old SHR rats, which were observed for 70 days.

Table IV. Comparison of Tumorigenicity and Metastatic Ability between the Tumors Arisen from ER-1 Cells Attached to Plates and the Tumors Developed by Injection of Large Numbers of ER-1 Cells in PBS Suspension

Tumor cell lines cell	Days after tumor inoculation	Incidence[a]	
		Tumor	Pulmonary metastasis (No. of colonies)
Tsus-1[b]	50	2/5	0/5
Tsus-2	55	1/5	1/5 (8)
Tsus-3	57	0/5	0/5
PLT-1[c]	48	5/5	4/5 (11, 19, 31, 33)
PLT-2	55	5/5	5/5 (9, 13, 18, 27, 51)
PLT-3	50	5/5	4/5 (14, 27, 33, 41)

a) Each Tsus and PLT cell line was s.c. injected as suspensions (1×10^4 cells/0.2 ml PBS) into SHR rats. The rats were observed for 3 months.
b) Tsus cell lines were established from the tumors developed by injections of large numbers of ER-1 cells. ER-1 cells ($5–10\times10^6$ cells/0.2 ml) in PBS suspension were injected into SHR rats. At the indicated days after the tumor injection, the tumor-bearing rats were killed, and the developed tumors were removed and cultured.
c) PLT cell lines were established from the tumors arisen from ER-1 cells attached to plates. ER-1 cells attached to plates (1×10^4 cells/plate) were s.c. implanted into SHR rats. At the indicated days after the implantation, the tumor-bearing rats were killed and the developed tumors were removed and cultured.

As shown in Table IV, the tumorigenicity and metastatic ability of Tsus lines did not increase as markedly as those of PLT lines.

To examine further whether ER-1 cells had to be directly attached to plates before the inoculation for enhancing their malignancy, small cell numbers of the ER-1 cell suspension were injected onto plates (between epidermis and plate) which had previously been implanted s.c. without tumor cells. It was found that the growth of ER-1 cells was promoted by the plates even though the cells were not anchored to the plates before being implanted into hosts (Table V).

Effects of host reactive cells on acquisition of malignant properties of ER-1 cells We examined the effect of host cells reactive to the plates on the malignancy of

Table V. Tumorigenicity and Metastatic Ability of ER-1 Cells Injected into the Plastic Plate-implanted Site

Days after plate implantation[a]	Incidence[b]	
	Tumor	Pulmonary metastasis (No. of colonies)
25	3/5	2/5 (10, 21)
35	3/5	2/5 (13, 23)
45	4/5	3/5 (18, 23, 29)
–[c]	0/4	0/4

a) Period between the plate implantation and the injection of ER-1 cells.
b) ER-1 cells (5×10^4 cells/0.2 ml) in PBS suspension were injected onto a plastic plate (between epidermis and the plate) which had previously been implanted s.c. without tumor cells. The rats were observed for 3 months.
c) ER-1 cells (5×10^4 cells/0.2 ml) in PBS suspension were injected into rats which had had no plate implanted.

Table VI. Increase in Tumorigenicity and Metastatic Ability of ER-1 Cells by Coculture with Reactive Cells Obtained from Tissues Surrounding Plastic Plates

No. of cells inoculated	Cocultured with host reactive cells[a]	Incidence[b]	
		Tumors	Pulmonary metastasis (No. of colonies)
1×10^4	–	0/7	0/7
	+	4/9	1/9 (15)
1×10^5	–	0/7	0/7
	+	5/9	2/9 (1, 19)

a) ER-1 cells were cocultured for 10 weeks (7 passages) with reactive cells obtained from the tissues surrounding the plates.
b) Tumor cells cocultured with or without reactive cells were s.c. inoculated into 8-week-old SHR rats. The rats were observed for 3 months.

ER-1 cells. ER-1 cells were cocultured for 10 weeks (7 passages) with host reactive cells obtained from the tissues surrounding the plates. After coculturing for 10 weeks, ER-1 cell suspension containing approximately 5% of fibroblast-like host cells was s.c. injected into syngeneic rats. The ER-1 cells cocultured with host reactive cells grew and killed about 50% of the rats, and pulmonary metastatic colonies were observed in a few cases (Table VI).

Effects of conditioned medium from cultured cells reactive to plastic plates (PL-CM) on *in vitro* growth and migration properties of ER-1 cells PL-CM stimulated anchorage-independent growth and migration of ER-1 cells compared to conditioned media from rat skin fibroblast cultures (Sk-CM) (Tables VII and VIII), whereas it did not affect plating efficiency, saturation density or doubling time.

Histological examination Twelve hours to 2 days after the implantation of ER-1 cells attached to plastic plates, neutrophil and macrophage infiltrations were observed at the periphery of the plates. Between 5 and 10 days, proliferation of fibroblasts with slight infiltration of lymphoid cells, in places with abundant vascularization, was observed around the tumor tissues (Fig. 1). After 20 days, the tumor mass on a plate was surrounded by massive proliferation of connective tissues such as fibroblasts, collagen fibers and inflammatory cells (Fig. 2).

Table VII. Influence of Conditioned Medium (CM) from the Tissues Surrounding Implanted Plastic Plates on Cloning Efficiency of ER-1 Cells

CM from	Concentration of CM	% Cloning efficiency[a] (No. of colonies)
–	–	0.4 (3.5±0.7)
PL-t[b]	12.5%	0.9 (8.5±4.9)
PL-t	25.0%	2.1 (21.0±4.6)
Sk-t[c]	12.5%	0.7 (6.5±3.5)
Sk-t	25.0%	0.5 (4.5±2.4)

a) Tumor cells (1×10^3) were seeded into 0.3% soft agar medium (60-mm dish). Colonies were counted under a microscope 10 days after the seeding.
b) Reactive cells obtained from tissues surrounding implanted plates.
c) Cells obtained from the skin of normal adult SHR rats.

Table VIII. Migration-stimulating Activity to ER-1 Cells of Conditioned Medium (CM) from the Implanted-plate-surrounding Tissues

Concentration of CM in lower chamber	No. of penetrated cells/filter[a] (mean±SD)
–	4.3±5.1
50%	214.7±36.2
100%	219.0±25.4

a) Tumor cells (5×10^3/0.1 ml) were seeded into the upper chamber of a Transwell. Eight hours after the incubation, the number of cells that had penetrated through pores of the Nuclepore membrane was counted under a microscope.

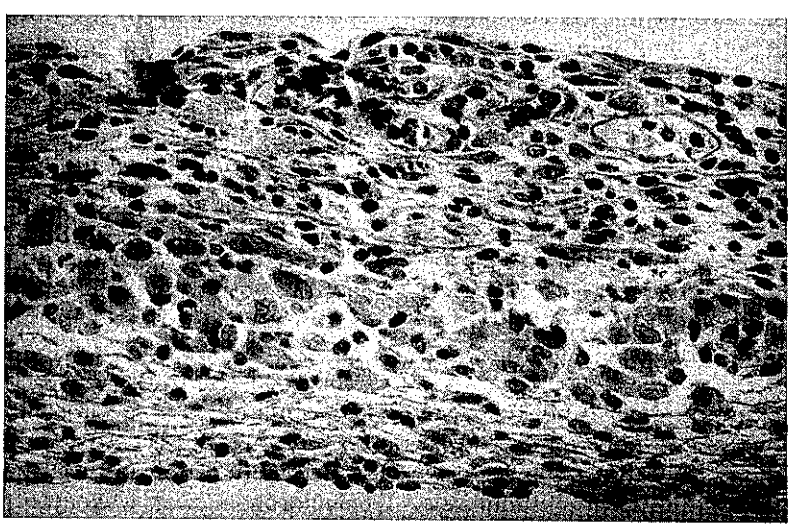

Fig. 1. A histological section of ER-1 cells on a plate 5 days after implantation into an SHR rat. Proliferation of fibroblasts with infiltration of lymphoid cells was observed around the tumor tissues. H & E, ×160.

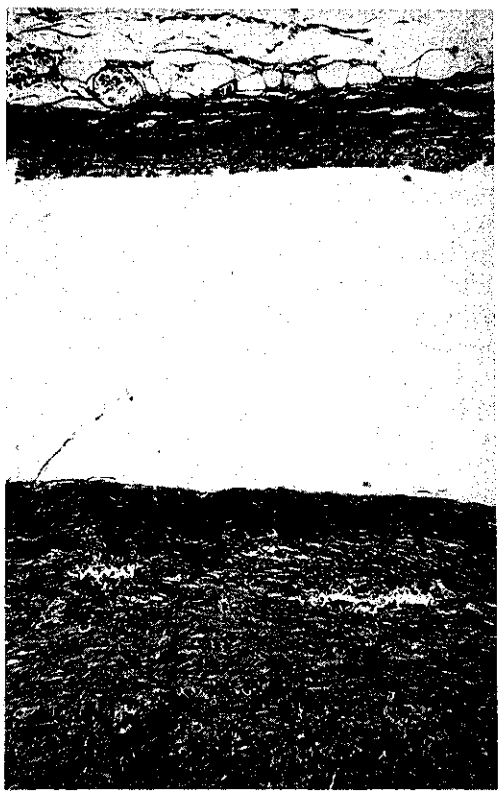

Fig. 2. A histological section of ER-1 cells on a plate 20 days after implantation. Abundant collagen fibers are apparent around the plate. Azan, ×60.

DISCUSSION

In the present study, we have demonstrated that the microenvironment around the s.c. implanted plastic plate not only stimulated the local growth of ER-1 regressor cells but also induced more aggressive tumor cells.

Boone and his colleagues reported that s.c. implantation of BALB/3T3 or C3H10T1/2 cells attached to glass beads or polycarbonate platelets into syngeneic mice produced tumors, and suggested that their anchorage dependence was thus accommodated *in vivo* during the initial period of tumor development.[10-13] However, the mechanisms for neoplastic transformation of these normal cell lines by co-implantation with solid substrata remain unclear.

The ER-1 cell line used in this study is not a normal cell line since it can grow both in immuno-suppressed hosts and in normal hosts if large cell numbers are inoculated. It is possible that host reactive cells to plastic plates may play an important role in stimulating the growth of ER-1 cells. This possibility is supported by the evidence that incidences of tumor development increased when ER-1 cells were injected at the periphery of plates which had previously been s.c. implanted. It is very likely that persistent inflammatory reaction and wound healing were induced in the local site of plastic plate implantation and skin incision. In the early stage of the plate implantation, infiltranions of neutrophils and macrophages, and fibroblast proliferations were observed around the plates by histological examination. Macrophages or fibroblasts are known to enhance the tumorigenic invasive or metastatic capacity of some tumor cell lines.[16-18]

Recent studies have shown that some growth factors, such as platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), transforming growth factor-β (TGF-β) and interleukin 1 (IL-1), may participate in the inflammatory reaction and wound healing.[19-24] In particular, IL-1 and TGF-β have been reported to enhance the growth or invasiveness of certain tumor cell lines.[25,26] We also observed that the culture supernatant obtained from host reactive cells which had surrounded the s.c. implanted plates enhanced the anchorage-independent growth and migration of ER-1 cells. These findings suggest that the growth factors produced by the reactive cells to plastic plates may have some effects on ER-1 cells in a paracrine mode, and may promote both local growth and metastatic ability of ER-1 cells. The conditioned medium from host reactive cells contained prostaglandin E_2 (PGE_2) though this was not detected in the conditioned medium from the skin fibroblasts of normal adult rats (data not shown). PGE_2 is known to inhibit cytolytic activity of macrophages and natural killer cells against tumor cells and production of IL-2[27-31]; therefore, we consider that local immunosuppression at the site of plate implantation may induce the local tumor growth.

The implantation of ER-1 cells with plastic plates not only allowed them to grow lethally but also generated highly tumorigenic and metastatic tumor cell populations, as was proved by the fact that PLT lines were more malignant than Tsus lines. Furthermore, cocultivation of ER-1 cells with host cells reactive to the plastic plates brought about enhancement of the malignant properties. These data lead us to speculate that host cells reactive to the plates, rather than the growth itself of ER-1 cells, may promote their malignancy, although it is still not clear whether the development of malignant tumor cells was due to induction or selection of malignant populations since 2 of 3 Tsus cell lines became slightly malignant without the plates in comparison with the parental ER-1 cell line. Recently, several investigators demonstrated that inflammatory cells such as macrophages and polymorphonuclear leukocytes enhanced the mutation rate of tumor cells or transformation of normal cells.[32,33] Likewise, the host inflammatory cells may help the tumor cells to generate more malignant phenotypes. The malig-

nancy of PLT lines was low at the early stage of the implantation of ER-1 cells attached to the plate, but gradually increased with the passage of time after the implantation. These findings also indicate that the reactive cells to the plastic plate may provide a suitable environment for tumor progression, which might result from increased genetic instability and selection pressure.

The phenomenon which we obseved in the present study was "tumor progression," which converted non-malignant (weakly tumorigenic) tumor cells to highly malignant tumor cells. From our findings, we suggest that the experimental system using ER-1 cells and the plastic plate may be useful as a model for the study of tumor progression.

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