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Enhancement of Tumorigenicity and Invasion Capacity of Rat Mammary Adenocarcinoma Cells by Epidermal Growth Factor and Transforming Growth Factor-β

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We have studied the effects of growth factors and cytokines on the tumorigenicity and invasion capacity of tumor cells by using regressor and progressor tumor cell lines (ER-1 and ERpP, respectively) derived from an SHR rat mammary adenocarcinoma. ER-1 cells regress spontaneously whereas ERpP cells show invasive growth and high metastasis to lung and other organs in syngeneic SHR rats. When ER-1 cells were pretreated with either epidermal growth factor (EGF) or transforming growth factor-β (TGF-β) for 24 h *in vitro*, and intraperitoneally transplanted into SHR rats, they grew and killed the host, whereas ER-1 cells pretreated with tumor necrosis factor-α did not. Tumorigenicity and invasion capacity of ERpP cells were also enhanced by treatment with EGF and TGF-β. The ER-1 cells pretreated with EGF, once grown *in vivo*, had acquired irreversible tumorigenicity and invasion capacity without requiring further EGF treatment, and the enhanced malignancy was irreversible. These findings suggest that growth factors play an important role in acquisition of malignancy of tumor cells.

Key words: Rat mammary tumor — Tumorigenicity — Invasion and metastasis — Regressor tumor cell — Growth factor

Cancer progression is the process by which tumor cells acquire malignant properties, such as progressive growth, invasion and metastasis, under the influence of genetic or epigenetic factors.[1] The process is regulated by the interactions between the tumor cells and their host reactive cells, which can regulate tumor growth and invasion ability directly or through various humoral factors within the body.[2–5] It is already known that various host cell-produced factors, such as growth factors, cytokines, metabolizing enzymes and oxygen radicals, are involved in the enhancement of tumor growth and invasion capacity; in particular, epidermal growth factor (EGF) is considered to be one of the most important factors, since EGF affects not only epidermal cells but also non-epidermal cells.[6–8]

Previously we succeeded in isolating regressor tumor cells from an SHR rat mammary adenocarcinoma.[9, 10] The regressor tumor cells, which we named ER-1, spontaneously regress in normal rats, although they grow progressively in immunosuppressd rats, or in normal rats if a large amount of them is inoculated.

In this study, we examined the role of growth factors, by which the regressor tumor cells were converted to progressor tumor cells with irreversible acquisition of tumorigenicity and metastatic ability.

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MATERIALS AND METHODS

Animals Spontaneously hypertensive rats (SHR strain) were purchased from Nippon Rat Co. (Urawa). Female SHR rats aged 7 to 10 weeks were used throughout the experiments.

Tumor cell lines A cloned cell line (ER-1) was isolated from a spontaneously developed rat mammary adenocarcinoma cell line, c-SST-2, which had been treated with ethylmethanesulfonate for 24 h.[8, 9] The ER-1 cell is a regressor tumor cell with no metastatic ability. When implanted s.c. (1×10^4) into normal syngeneic rats, ER-1 cells regress spontaneously, although they grow in, and ultimately kill, syngeneic rats immunosuppressed by exposure to ^{60}Co or by removal of helper T cells by monoclonal antibodies. They also grow in normal syngeneic rats if a larger number of cells (1×10^6) is implanted. There were no differences between ER-1 cells and the parental cell line SST-2 in *in vitro* characteristics such as growth rate, and serum requirement for colony formation in soft agar.

Each tumor cell line was maintained in culture through serial passages, and implanted s.c., being attached to a plastic plate, into SHR rats. The tumor which developed from the implanted ER-1 cells attached to the plastic plate was designated as ERpP (progressor ER-1 tumor cell). ERpP cells showed progressive proliferation; a small number (1×10^4) of ERpP cells im-

planted s.c. into syngeneic rats was lethal to the host and metastasized to the lung at high incidence.[11]

Culture of tumor cell lines ER-1 cells were cultured in Eagle's minimum essential medium (MEM) containing 7% fetal bovine serum (FBS, 56°C, inactivated for 30 min), and L-glutamine (292.9 mg/liter), at 37°C, in 95% air with 5% CO_2.

Detachment and culture of mesothelial and endothelial cells According to the method of Akedo *et al.*, mesenterium of SHR rats was aseptically removed.[12] The mesenterium was placed in 10 ml of phosphate-buffered saline containing 25% trypsin for 20 min, then the mixture was filtered through gauze, and the filtrate was centrifuged. Endothelial cells were cultured in MEM containing 10% FBS (56°C, inactivated for 30 min) and L-glutamine (292.9 mg/liter).

Treatment of ER-1 cells with growth factors and cytokines ER-1 cells and ERpP cells were treated with EGF (10 ng/ml), transforming growth factor-β (TGF-β) (10 ng/ml), or tumor necrosis factor-α (TNF-α) (100 U/ml) for 24 h, then either cultured on overlayered mesothelial cells, or injected i.p. into syngeneic SHR rats without removal of the growth factors.

Measurement of invasion capacity of ER-1 cells When the mesothelial cells reached confluency *in vitro*, ER-1 and ERpP cells (2×10^4 respectively) were cultured on overlayered mesothelial cells, and the invasion capacity of ER-1 cells was measured by counting the number of colonies per 1 cm^2 formed under the mesothelial cells. For *in vivo* study, ER-1 and ERpP cells (1×10^5 respectively) were injected i.p. into syngeneic SHR rats. After 15 days, the rats were killed for autopsy. Weights of tumors developed in the abdominal cavity were measured.

RESULTS

Enhancement of *in vitro* growth of the ER-1 and ERpP cells by growth factors As shown in Fig. 1, no difference was observed in *in vitro* growth ability between non-treated ER-1 cells and non-treated ERpP cells. However, treatment with EGF for 24 h significantly enhanced the growth ability of both tumor cells, starting on the 3rd day of culture ($P<0.05$).

Morphological study of the invasion of ER-1 cells When mesothelial cells which had previously been removed from the mesenterium of normal SHR rats became confluent in culture medium, ER-1 cells were cultured on monolayered mesothelial cells. Three days later, phase-contrast microscopic observation revealed that ER-1 cells had invaded the layer of mesothelial cells and proliferated. A vertical section of the invasion is shown in Fig. 2.

Enhancement of *in vitro* invasion capacity of ER-1 and ERpP cells by growth factors and cytokines Invasion capacity of ERpP cells was markedly enhanced by treatment with EGF (1 ng/ml), and the enhancement was at the same level as that seen with 10 ng/ml of EGF (Table I).

Table II shows the time-dependent enhancement of invasion capacity of ERpP cells in relation to the duration of EGF treatment. EGF treatment for longer than 24 h did not enhance the invasion capacity of ERpP cells any further.

We also examined changes of invasion capacities of ER-1 and ERpP cells treated with EGF, TGF-β or TNF-α for 24 h. As shown in Table III, both ER-1 cells and ERpP cells pretreated with EGF or TGF-β formed a

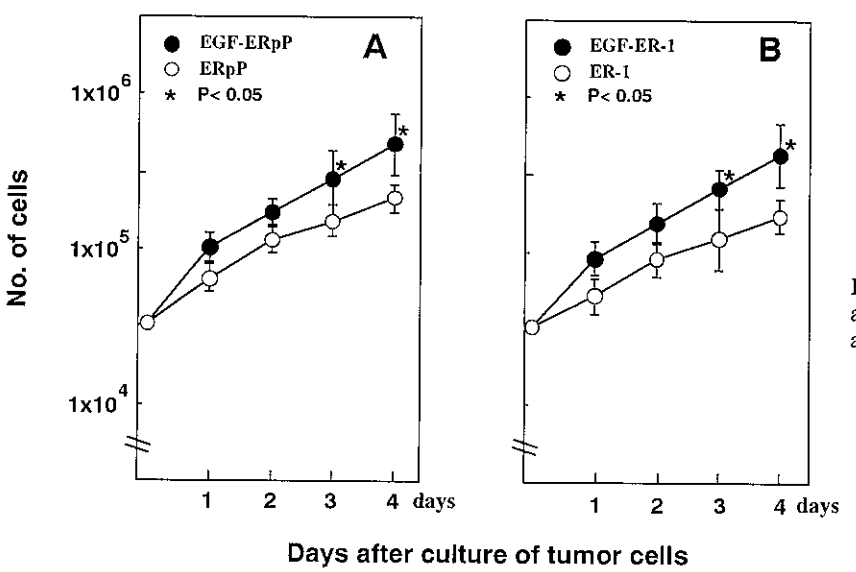

Fig. 1. *In vitro* growth curves of ERpP (A) and ER-1 (B) tumor cells in the presence and absence of EGF pretreatment (10 ng/ml).

Fig. 2. A vertical section of the invaded EGF-treated ER-1 cells (T) shows invasion and growth under the mesothelial cells (M).

greater number of colonies underneath mesothelial cells than did the non-treated cells after 3 days. On the other hand, TNF-α treatment showed little effect on the invasion capacity of ER-1 cells, and ERpP cells pretreated with TNF-α formed significantly fewer colonies compared to the non-treated cells.

Fig. 3 shows the reversibility of the invasion capacity of ER-1 cells which were first treated with EGF for 24 h and then cultured without EGF. Colony-formation of the ER cells was found to decrease, in a time-dependent manner, reaching the level of non-treated ER-1 cells on the 4th day.

Enhancement of *in vivo* tumorigenicity and invasive capacity of ER-1 cells by EGF treatment ER-1 cells and ERpP cells (1×10^5 respectively) were implanted i.p. in syngeneic SHR rats. Fifteen days after, the rats were killed for the examination of tumor development in the peritoneum. The ER-1 cells were found to have regressed spontaneously, whereas ERpP cells showed invasive growth. In the case of ER-1 cells pretreated with EGF for 24 h, tumors developed in the peritoneum, and blood-containing ascites was observed in the abdominal cavity (Table IV).

Table V shows the survival periods of the tumor-bearing rats which were not killed on the 15th day. All the non-EGF-treated ER-1 cells spontaneously regressed, whereas all the EGF-treated ER-1 cells were ultimately lethal to the host. Survival periods of the rats transplanted with EGF-treated ERpP cells were significantly shorter than those of control rats.

Stability of *in vivo* tumorigenicity and invasion capacity of ER-1 cells acquired by EGF treatment The tumors induced by the i.p. implantation of EGF-treated ER-1 cells were cultured for 30 days without EGF, and the same number of tumor cells (1×10^5) was again implanted i.p. into SHR rats. The tumor cells eventually killed all the hosts (Table VI).

Table I. Dose-dependent Enhancement of Invasion Capacity of ERpP Tumor Cells by EGF

EGF/dose (ng/ml)	No. of penetrated colonies/cm^2±SD[a]
0	33.6±1.8
0.1	54.2±7.4
1.0	198.4±11.6[b]
5.0	176.3±25.7[b]
10.0	185.2±20.1[b]

a) ERpP cells (2×10^4/well) were pretreated for 24 h in the presence of various concentrations of EGF in a 24-well plate, washed and cultured on overlayered mesothelial cells for 4 days.
b) $P<0.01$ as compared with the non-treated group, by Student's *t* test.

Table II. Time-dependent Enhancement of the Invasion Capacity of ERpP Tumor Cells by EGF

Incubation time (h)[a]	No. of penetrated colonies/cm^2±SD
0	10.2±1.7
6	12.3±5.8
12	68.1±11.3
24	166.8±22.6[b]
48	170.1±36.2[b]
72	169.6±19.3[b]

a) ERpP tumor cells (2×10^4/well) were pretreated with EGF (1 ng/ml), washed and cultured on overlayered mesothelial cells for 4 days.
b) $P<0.01$ as compared with the non-treated group, by Student's *t* test.

Table III. Effects of EGF, TGF-β and TNF-α on the Capacities of ERpP and ER-1 Tumor Cells to Invade Mesothelial Cell Layer

Mesothelial cells[a] cocultured with	Treated with[b]	No. of penetrated colonies/cm^2±SD
ERpP	EGF	103.2±3.8[c]
	TGF-β	95.3±8.8[d]
	TNF-α	12.1±1.1[c]
	none	52.8±5.8
ER-1	EGF	78.5±20.3[e]
	TGF-β	61.2±13.5[e]
	TNF-α	2.8±2.0[e]
	none	1.5±1.0

a) Mesothelial cells derived from the mesenterium of SHR rats.
b) ERpP and ER-1 tumor cells (1×10^5/ml) were treated with 1 ng/ml EGF or 10 ng/ml TGF-β or 100 U/ml TNF-α for 24 h, and 3 days later their *in vitro* invasive capacities were tested.
c) $P<0.001$ compared with the ERpP non-treated group.
d) $P<0.01$ compared with ERpP non-treated group ("none").
e) $P<0.01$ compared with ER-1 non-treated group ("none").
(*c, d, e* vs. non-treated group: by Student's *t* test)

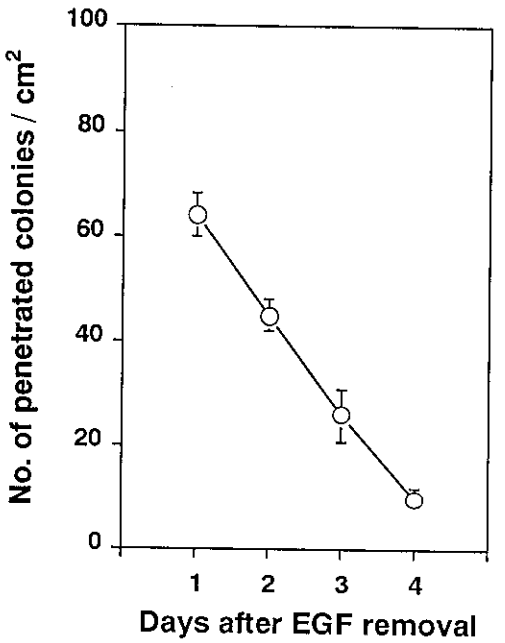

Fig. 3. Reversibility of the effect of EGF pretreatment (10 ng/ml) on ERpP and ER-1 tumor cells.

Table IV. Effects of EGF on the Growth and Invasion Capacity of ERpP and ER-1 Tumor Cells in SHR Rats

Tumor cells[a)]	Tumor weight (g±SD)	Involved organs
EGF-treated ERpP	1.8±0.7	omentum, mesentery
ERpP alone	0.5±0.3	mesentery
EGF-treated ER-1	1.3±0.5	omentum, mesentery
ER-1 alone	0±0	

a) ERpP and ER-1 tumor cells (1×10^5/rat) pretreated with 1 ng/ml EGF for 24 h or untreated were implanted i.p. into SHR rats, which were killed 15 days later.

Table V. Effect of EGF on the Survival Period of SHR Rats Transplanted with ERpP and ER-1 Tumor Cells

Tumor cells[a)]	No. of rats dead/No. used	Tumor weight (g±SD)	MSD[b)]±SD (days)
EGF-treated ERpP	5/5	3.6±1.1	23.8±2.5
ERpP alone	5/5	3.2±0.8	30.8±2.8
EGF-treated ER-1	5/5	3.3±0.6	31.0±3.1
ER-1 alone	0/5	0±0	—

a) ERpP and ER-1 tumor cells (1×10^5/rat) pretreated with 10 ng/ml EGF for 24 h or untreated were implanted i.p. into SHR rats.
b) MSD: mean survival days.

Table VI. Tumorigenicity of EGF-treated ER-1 Tumor Cells in SHR Rats

Tumor cells[a)]	No. of rats with tumor/No. used	Tumor weight (g±SD)
EGF-treated ER-1[b)]	5/5	0.82±0.38
ER-1 alone	0/5	0±0

a) The tumor cells (1×10^5/rat) were implanted i.p. into SHR rats, which were killed 15 days later.
b) ER-1 tumor cells were treated with 1 ng/ml of EGF for a month.

DISCUSSION

Our results showed that growth factors, such as EGF and TGF-β, enhance not only the tumorigenicity but also the invasion capacity of ER-1 and ERpP cells, and that the ER-1 cells, once treated with EGF *in vitro*, no longer require further EGF treatment for invasion and lethal growth.

Although the mechanism by which ER-1 cells acquired tumorigenicity and invasion capacity upon EGF treatment is still under investigation, we suggest on the basis of the following evidence that growth factors are involved in the acquisition of malignant tumor growth: 1) the regressor tumor cell line, ER-1, acquired malignancy via host cells reactive to the implanted plastic plate,[13] 2) mRNA expression of EGF is markedly increased in ERpP cells compared to that in ER-1 cells, and mRNA expression of EGF receptors was strongly positive in ERpP cells compared to that in ER-1 cells (unpublished data).

Ren *et al.*[14] reported that the expression of erbB-2 on the surface of ERpP cells was marked. It is known that the product of erbB-2 is particularly correlated with EGF receptors. In general, growth factors produced not only by normal host cells but also by tumor cells themselves are considered to play a role, either autocrine or paracrine, in the proliferation of the tumor cells.[15] *In vitro* invasion capacity of ER-1 cells pretreated with EGF for 24 h was reversible, whereas the ER-1 cells acquired lethal tumorigenicity *in vivo*. The mechanism of this difference between *in vitro* and *in vivo* outcome probably depends on the environment of the tumor cells. ER-1 cells transplanted into syngeneic rats are continuously stimulated by growth factors including EGF and TGF-β released from host cells. However, *in vitro*, without the influence of growth factors, the invasive capacity of ER-1 cells will regress.

Based on these findings, we suggest that ER-1 cells transiently acquire invasion capacity through the action of growth factors *in vitro*, and also that through *in vitro*

or *in vivo* passages in the presence of growth factors ER-1 cells acquire malignancy, and the acquired malignancy becomes irreversible. Extrinsic factors such as carcinogens or antitumor drugs are known to be involved in malignant tumor progression.[16-20] In the present study we have shown that intrinsic factors such as growth factors are also important in the acquisition of malignancy of tumor cells.

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