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Recombinant Human Interferon-α2a Increases Hormone Receptor Level of a Human Breast Carcinoma Xenograft in Nude Mice and Enhances the Anti-proliferative Activity of Tamoxifen

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The effect of recombinant human interferon-α2a (rhIFN-α2a) on the hormone receptor level and antitumor activity of tamoxifen (TAM) was investigated in nude mice using ZR-75-1, an estrogen receptor (ER)-positive, and progesterone receptor (PgR)-negative human breast carcinoma xenograft. ER levels (maximum binding sites) of tumors treated with rhIFN-α2a at a dose of 6×10^5 U/mouse/day for 1 or 3 wk were not significantly different from the control, whereas those with rhIFN-α2a at a dose of 6×10^4 U/mouse/day for 1 or 3 wk were higher than the control (3.9- to 4.4-fold) with a significant difference at $P<0.01$. The increase of ER by rhIFN-α2a was investigated using a sucrose density gradient method. The peak was only seen at 8S in both rhIFN-α2a-treated tumor and control ER, and the sedimentation patterns were almost the same, suggesting that both ERs were essentially equivalent. On the other hand, PgR of all the treated groups could be detected, while that of the control group was undetectable. The antitumor effect of the combination treatment of rhIFN-α2a and TAM was compared with those of single treatments. While rhIFN-α2a at a dose of 6×10^5 U/mouse/day and TAM did not show a combination effect, rhIFN-α2a at a dose of 6×10^4 U/mouse/day and TAM showed a synergistic combination effect, and ER was decreased to the threshold of detection by the combination treatment. These findings indicated that a low dose of rhIFN-α2a increased the ER levels of ER-positive human breast cancer *in vivo* as well as *in vitro* and enhanced the anti-proliferative effect of TAM, and the newly synthesized ER was essentially the same as the original ER.

Key words: Recombinant human interferon α2a — ZR-75-1 — Hormone receptor level — Tamoxifen — Synergistic combination effect

When interferon (IFN[2]) was introduced for cancer therapy, this agent was expected to be effective against neoplastic cells not only by direct action but also by host-mediated mechanisms.[1–4] In breast carcinomas, however, although only IFN-α was examined in a Phase-II trial, the results were not satisfactory.[5] Nevertheless, IFN has recently been reported to increase the expression of estrogen receptor (ER) and progesterone receptor (PgR) of breast cancer cell lines *in vitro*[6–8] and to enhance the antitumor activity of anti-estrogenic agents.[8,9] If this synergistic action could be obtained *in vivo*, IFN might be a promising agent against human breast carcinoma in combination with anti-estrogenic agents such as tamoxifen. However, an increase of ER levels of breast cancer by IFN *in vivo* has only been reported by Pouillart *et al.*,[10] who found that human fibroblastoid interferon increased ER levels of metastatic breast cancer to the skin in 2 of 2 patients with an elevation of PgR in 5 of 6 patients.

In this study, we have observed the changes of hormone receptor levels of human breast carcinoma xenografts in nude mice treated with recombinant human interferon-α2a. The synergistic antitumor activity of IFN and tamoxifen was also investigated.

MATERIALS AND METHODS

Mice Athymic, BALB/c, *nu/nu* female nude mice were purchased from CLEA Co., Ltd., Tokyo and maintained under specific pathogen-free conditions using laminar air flow racks in our institute. Six- to eight-week-old mice weighing 20–25 g were used for the experiments.

Tumors ZR-75-1, an ER-positive and PgR-negative human breast carcinoma xenograft, was used for the experiments. ZR-75-1 was established as a cultured cell line at Flow Laboratories, Irvine, Scotland, and was successfully transplanted into nude mice treated with estrogen and progesterone in 1986 by the authors.[11]

Agents Recombinant human IFN-α2a (rhIFN-α2a) was kindly supplied by Nippon Roche K.K., Tokyo. Tamoxifen citrate (1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1-ene; TAM) was a gift from ICI-Pharma Manufacturing, Ltd., Osaka.

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[2] Abbreviations used are: IFN, interferon; rhIFN-α2a, recombinant human IFN-α2a; TAM, tamoxifen citrate; ER, estrogen receptor; PgR, progesterone receptor.

Tumor inoculation and measurement Two tissue fragments of ZR-75-1, each approximately 3×3×3 mm in size, were inoculated by means of a trocar needle into the subcutaneous tissues of the backs of nude mice under general anesthesia with diethyl ether. Five mg of 17β-estradiol dipropionate and 250 mg of 17α-progesterone caproate per kg (0.1 ml of EP Hormone Depot™ per mouse, Teikoku Zoki, Co. Ltd., Tokyo) were administered intramuscularly on the tumor inoculation day as reported previously.[11] Tumors were measured with a sliding caliper by the same observer three times a week and the tumor weight (W) in mg was calculated according to the method of Geran *et al.*[12] as follows: W=(width in mm)^2×(length in mm)/2.

Treatment When the tumor weight reached 100–300 mg and tumors started to grow exponentially, the mice were randomized into test groups consisting of 4 to 6 mice so that the average weights of all the groups were similar.

(1) rhIFN-α2a alone: RhIFN-α2a (6×10^5, 6×10^4 U/mouse) dissolved in 0.1 ml of distilled water was administered s.c. daily for 3 weeks using a #26 tuberculin needle.

(2) TAM alone: Five mg of TAM per kg dissolved in 0.1 ml of 10% dimethyl sulfoxide (DMSO) with 0.5% carboxymethyl cellulose (CMC) was administered p.o. daily for 3 weeks.

(3) Combination of rhIFN-α2a and TAM: the above-mentioned amounts of rhIFN-α2a and TAM were administered simultaneously for 3 weeks.

The combination effect of rhIFN-α2a and TAM was compared with the single treatments to elucidate the influence of induced ER upon anti-proliferative effect of TAM. ER and PgR values pre- and post-treatment were also assessed. Upon completion of each experiment, all the mice were killed, the tumors, uteri and spleens were weighed, and hormone receptors (ER and PgR) were measured by the dextran-coated charcoal (DCC) method and exchange assay.[13, 14]

Evaluation The relative mean tumor weight (RW=Wi/Wo) was calculated, where Wi is the mean tumor weight at any given time and Wo is the initial mean tumor weight. The growth curve was drawn by plotting RW against the number of days after initial treatment. The effect of each treatment was evaluated as positive when the T/C ratio [the lowest value of (RW of treated group)/(RW of control group)] was less than 50%. The combination effect was regarded as synergistic when the T/C obtained by the combination therapy was less than the estimated T/C calculated by multiplying the T/C ratios of the single agents according to the method of Berenbaum.[15] The average tumor weight in each group was compared by means of Student's *t* test.

The uterus and spleen weights were measured to evaluate the side effects of each treatment.

Sucrose density gradient centrifugation Each tumor was homogenized separately in TEMG buffer (20 m*M* Tris-HCl, 1.5 m*M* EDTA and monothioglycerol, pH 7.5), and centrifuged at 105,000*g* for 45 min at 2°C with a Hitachi SCP70H2 ultracentrifuge. Two ml of cytosol was incubated with 10 n*M* ^{3}H-E_2 for 2 h on ice and unbound ^{3}H-E_2 was removed using the DCC method. Two hundred μl of lysate and 5–20% sucrose gradients were incubated and centrifuged at 40,000*g* for 18 h at 2°C. Fractions were collected from the bottom of the tube and measured with a scintillation counter. Ovalbumin (3.6S) and γ-globulin (6.6S) were used as density markers.

RESULTS

The antitumor activity of tamoxifen and/or rhIFN-α2a at a dose of 6×10^4 is shown in Table I. The effect of rhIFN-α2a alone was limited to 90.8% in terms of T/C_{RW}, while TAM alone showed a moderate antitumor effect on this strain with a T/C_{RW} of 65.9%. This antitumor activity of TAM alone was enhanced in combination with rhIFN-α2a. The antitumor activity of TAM alone and TAM with rhIFN-α2a against ZR-75-1 is shown in Fig. 1, where the relative mean tumor weight is plotted against the number of days after treatment. It is clear that the antitumor activity of TAM was enhanced by simultaneous administration or rhIFN-α2a. No statistically significant differences were observed in the uterine and spleen weights of mice treated with rhIFN-α2a, TAM alone and their combination.

When the dose of rhIFN-α2a was increased to 6×10^5 U/mouse, the growth of ZR-75-1 was retarded in a comparison with rhIFN-α2a at a dose of 6×10^4 U/mouse (Table II). However, this antitumor activity by rhIFN-α2a at a dose of 6×10^5 U/mouse was evaluated as negative according to our criteria. Although TAM alone showed an antitumor activity on ZR-75-1 with a T/C_{RW} value of 62.9%, which was almost equivalent to the antitumor effect of TAM alone in the experiment shown in Table I, this effect was not enhanced in combination with rhIFN-α2a. No adverse effects were observed in terms of changes in the uterine and spleen weights.

ER levels of tumors treated with rhIFN-α2a at a dose of 6×10^5 U/mouse/day for 1 and 3 wk (Bmax: 154.2±36.9 and 197.6±36.3 fmol/mg protein, respectively) were not significantly different from the control (136.1±53.6 fmol/mg protein) (Table III). PgR levels of these treated tumors were 42.5±13.8 and 85.4±44.5 fmol/mg protein, with control PgRs being undetectable. On the other hand, ER levels of tumors treated with rhIFN-α2a at a dose of 6×10^4 U/mouse/day for 1 and 3 wk (536.7±36.4 and 601.3±294.1, respectively) were higher than control ER, with a significant difference at $P<0.01$. PgRs were also produced by IFN at a dose of 6×10^4

Table I. Antitumor Activity of Tamoxifen and/or Recombinant Human Interferon-α2a (60,000 U/mouse) on ZR-75-1

Group	n[a]	TW[b] (mg)	T/C[c] (TW)	T/C[d] (RW)	Uterus wt.[e] (mg)	Spleen wt.[f] (mg)
Control	8	380.1±135.2	—	—	148.2±15.8	157.9±21.6
IFN[g]	9	357.2±75.8	93.9	90.8	156.8±32.7	138.9±17.3
Control	7	195.5±38.4	—	—	119.5±16.9	168.0±29.3
TAM[h]	5	102.5±34.3*	52.4	65.9	127.3±13.3	162.7±22.5
IFN+TAM[i]	7	90.2±22.3**	46.1	49.8	139.5±27.5	145.3±18.7

a) Number of tumors.
b) Actual tumor weight in mg at the end of experiment (M±SD).
c) T/C ratio of the actual tumor weight (%).
d) The lowest T/C of relative mean tumor weight (%) during the experiment. Underlining indicates positive antitumor activity.
e) Actual uterine weight (M±SD).
f) Actual spleen weight (M±SD).
g) Recombinant human interferon-α2a at a dose of 6×10^4 U/mouse was administered s.c. daily for 3 wk.
h) Tamoxifen at a dose of 5 mg/kg was administered p.o. daily for 3 wk.
i) Combined treatment (*g*+*h*).
* $P<0.02$ relative to control.
** $P<0.01$ relative to control.

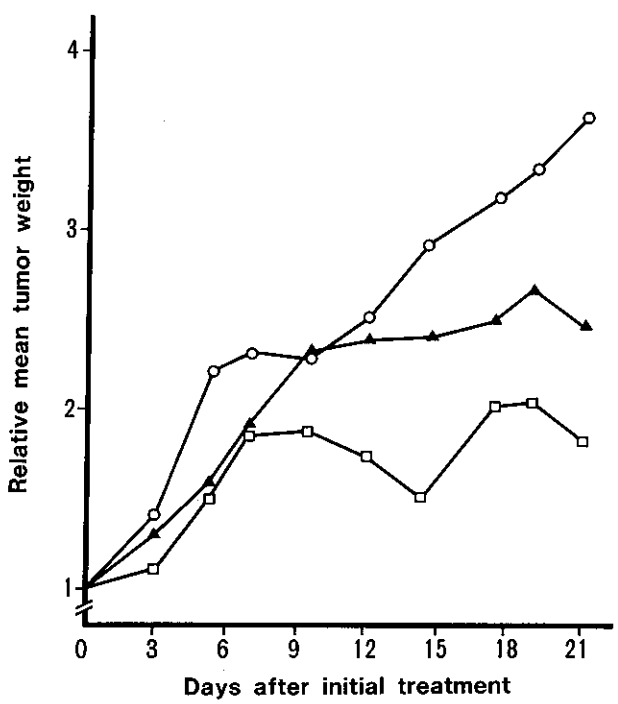

Fig. 1. Antitumor activity of tamoxifen (TAM) alone (▲) and TAM with rhIFN-α2a administered simultaneously (□), compared with the control (○). Five mg of TAM per kg dissolved in 0.1 ml of 10% dimethyl sulfoxide with 0.5% carboxymethyl cellulose was administered p.o. daily for 3 weeks. RhIFN-α2a (6×10^4 U/mouse) dissolved in 0.1 ml of distilled water was administered s.c. daily for 3 weeks using a #26 tuberculin needle. It is clear that the antitumor activity of TAM was enhanced by combined administration of rhIFN-α2a.

U/mouse/day (Table IV). ER levels decreased to the threshold of detection in both the TAM treatment group and the combination group. PgR showed a tendency to increase in each treatment group.

ERs newly synthesized after rhIFN-α2a treatment at a dose of 6×10^4 U/mouse/day for 3 wk were compared with control ERs using the dense amino acid labeling technique of sucrose density gradient centrifugation (Fig. 2). The peak of the treated group (B) was 4.2-fold higher than the control group (A), though they were both sedimented at approximately 8S. The 4S peak, which is another common peak of ER, was not seen in either group.

DISCUSSION

IFNs seem to be of some value as cytostatic antiproliferative agents for leukemia, renal cell carcinoma, melanoma and lymphoma, though clinical trials designed to assess the antitumor activity of IFN towards other solid tumors have yielded disappointing results.[5] On the other hand, much attention has been focused on the influences of IFN upon gene expression and cell growth, function and differentiation.[16-18]

It has been shown that IFN exerts its activity by binding with a specific cell-surface receptor, sharing common second-messenger pathways with other polypeptide hormones.[19,20] It is reported that IFN-α and IFN-β interact with the same receptor while IFN-γ interacts with another receptor. In any case, the binding of

Table II. Antitumor Activity of Tamoxifen and/or Recombinant Human Interferon-α2a (600,000 U/mouse) on ZR-75-1

Group	n[a]	TW[b] (mg)	T/C[c] (TW)	T/C[d] (RW)	Uterus wt.[e] (mg)	Spleen wt.[f] (mg)
Control	5	552.0±329.7	—	—	167.0±7.2	180.0±35.6
IFN[g]	5	352.0±80.7	63.8	78.3	167.0±61.4	163.8±14.0
Control	10	427.3±140.0	—	—	153.8±27.9	181.4±15.8
TAM[h]	10	286.1±90.0	67.0	62.9	161.4±19.0	176.4±26.3
IFN+TAM[i]	9	263.0±91.8	61.6	64.2	142.2±30.5	194.8±27.4

a) Number of tumors.
b) Actual tumor weight in mg at the end of experiment (M±SD).
c) T/C ratio of the actual tumor weight (%).
d) The lowest T/C of relative mean tumor weight (%) during the experiment.
e) Actual uterine weight (M±SD).
f) Actual spleen weight (M±SD).
g) Recombinant human interferon-α2a at a dose of 6×10^5 U/mouse was administered s.c. daily for 3 wk.
h) Tamoxifen at a dose of 5 mg/kg was administered p.o. daily for 3 wk.
i) Combined treatment (*g*+*h*).

Table III. Effects of Recombinant Human Interferon-α2a on Hormone Receptors of ZR-75-1 in Nude Mouse

IFN[a]	w[b]	n[c]	Estrogen receptor[d]		Progesterone receptor[d]	
			Bmax[e]	K_d[f]	Bmax	K_d
Control	—	8	136.1±50.7	1.0±0.7	UD[g]	UD
6×10^4	1	6	536.7±36.4*	6.1±2.3	72.6±36.4	5.1±1.8
	3	8	601.3±294.1*	6.3±3.5	11.2±9.9	5.6±5.6
6×10^5	1	6	154.2±36.9	0.9±0.3	42.5±13.8	3.9±1.1
	3	8	197.6±36.3	0.9±0.3	85.4±44.5	5.5±3.3

a) Recombinant human interferon-α2a was administered daily s.c. for 1–3 weeks at doses of 6×10^4 and 6×10^5 U/mouse.
b) Weeks after initial treatment.
c) Number of tumors.
d) Hormone receptors were detected by the dextran-coated charcoal method.
e) Maximum binding site in fmol/mg protein.
f) Dissociation constant in 10^{-10} *M*.
g) Undetectable.
* $P<0.01$ relative to control.

IFN with its receptor is stable and resistant to proteolytic digestion.

Many studies have revealed that exposure of cells to IFN not only down-regulates IFN receptors but also influences those of other hormones. In particular, recent *in vitro* experiments revealed that IFNs increased ER expression of some breast cancer cells[6-8] and enhanced the anti-proliferative activity of tamoxifen.[8,9] The major points of these reports are: (1) the increase of ER was detected only in ER-positive cell lines, and (2) this effect had nothing to do with the anticancer effect and was inversely proportional to dose, and (3) the enhanced effect of tamoxifen was only seen at the dose at which IFN induced ER.

In the present study, 6×10^4 U of rhIFN-α2a per mouse, which showed no anti-proliferative effect on ER-positive ZR-75-1 in nude mice, increased ER expression of ZR-75-1 significantly, whereas 6×10^5 U of rhIFN-α2a per mouse, which showed some anti-proliferative effect, did not change the expression of ER. These results were compatible with the *in vitro* findings of van den Berg on the ZR-75-1 cell line.[7] On the other hand, IFN did not modulate the ER expression of ER-negative MX-1 at any dosage examined (data not shown). These ERs were detected by the DCC method and exchange assay as fmol/mg protein. In our previous study, the ER content of human breast carcinoma xenografts detected by the DCC method and exchange assay was stable for 17 days

Table IV. Effects of Tamoxifen Recombinant Human Interferon-α2a and on Hormone Receptors of ZR-75-1 in Nude Mouse

Treatment	n[a]	Estrogen receptor[b]		Progesterone receptor[b]	
		Bmax[c]	K_d[d]	Bmax	K_d
Control	8	136.1±50.7	1.0±0.7	UD[h]	UD
TAM[e]	7	5.6±0.4*	2.5±2.0	13.5±1.3	3.1±1.2
IFN[f]	8	601.3±294.1*	6.3±3.5*	11.2±9.98	5.6±5.6
TAM+IFN[g]	7	4.2±4.2*	2.9±2.7	16.8±0.3	2.9±0.1

a) Number of tumors.
b) Hormone receptors were detected by the dextran-coated charcoal method.
c) Maximum binding site in fmol/mg protein.
d) Dissociation constant in 10^{-10} *M*.
e) Tamoxifen citrate at a dose of 5 mg/kg was administered p.o. daily for 3 weeks except on Sundays.
f) Recombinant human interferon-α2a at a dose of 6×10^4 U/mouse was administered s.c. daily for 3 weeks except on Sundays.
g) Combined treatment (*e*+*f*).
h) Undetectable.
* $P<0.001$ relative to control.

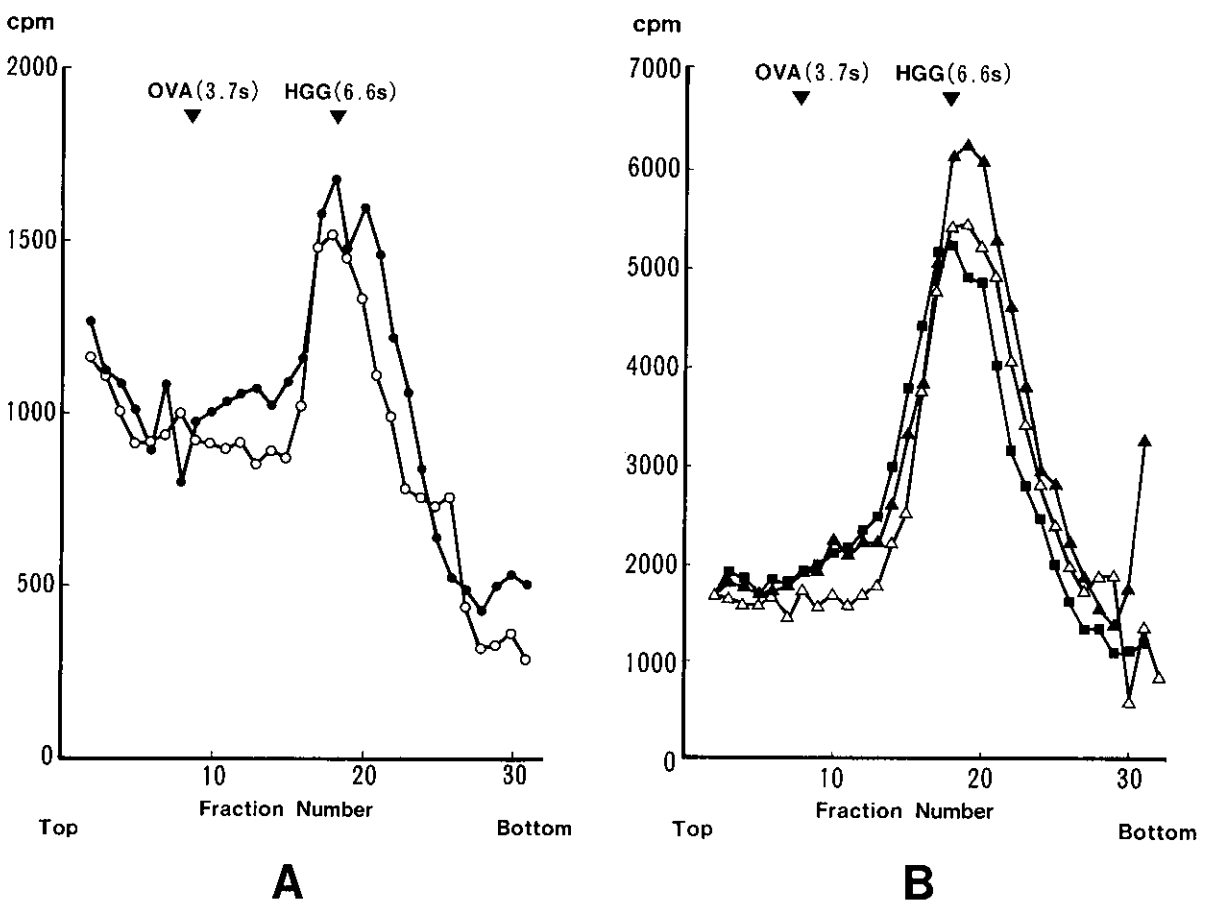

Fig. 2. ERs newly synthesized by rhIFN-α2a (6×10^4 U/mouse) (B) were compared with control ERs (A) by means of sucrose density gradient centrifugation. Both groups had the same peak at 8S, while 4S, which is another common peak of ER, was not seen in either group. Each tumor was homogenized separately. ○ and ● indicate the control tumors, and △, ▲ and ■ indicate the tumors treated with rhIFN-α2a.

after treatment with mitomycin C or cyclophosphamide, though the tumors regressed considerably.[21] The histological change of human breast carcinoma xenograft induced by TAM is inconspicuous compared with the effect of a cytotoxic agent.[22] These results suggested that the ER assayed in the group treated with tamoxifen and/or interferon-α2a would not be affected by the decrease of the tumor cell proportion.

The serum estrogen levels of nude mice treated with estradiol are reported to be equivalent to that of untreated mice at 3 weeks after the estradiol treatment and ER levels in the tumor are considered to be indicative of the tumor's ability to synthesize ER. Thus, ER induction by IFN was regarded as evidence that IFN stimulated the ER-synthesizing ability of the tumor. This was also supported by the findings that the newly synthesized ER was essentially identical with the original ER, and the 8S peak of the treated group was 4.2-fold higher than the control, when ER synthesis induced by rhIFN-α2a at a dose of 6×10^4 U/mouse/day for 3 wk was compared with control ERs using the dense amino acid labeling technique of sucrose density gradient centrifugation. PgR of ZR-75-1 was also observed to be significantly increased by the rhIFN-α2a treatment. PgR is generally regarded as one of the proteins synthesized in response to the combination of ER with ER-agonist, so the ER induced by IFN treatment was again suggested to be true ER with the native functions. van den Berg reported that the effect of IFN on ER was prevented in the presence of cycloheximide and suggested that protein synthesis was required for ER induction by IFN.[7]

In the present study, the increase of ER was only observed at the low dose of IFN, which showed little anti-proliferative effect on ZR-75-1, suggesting that a sufficient dose of IFN to increase ER can be administered *in vivo* as well as *in vitro*. IFNs are generally considered to enhance cellular differentiation,[16] and a significant relation has been observed between the presence of ER and cell differentiation in primary breast cancers. This suggested that ER induction by IFN might be closely associated with differentiation of tumor cells. The mechanism involved in the augmentation of ER activity by IFN is not clear; the properties of the induced ER and the mechanisms of ER induction need to be further clarified.

TAM is an anti-estrogenic agent that exerts its antitumor activity by binding with the ER and is effective on tumors with high ER content. In the present study, the combination of IFN and TAM was used to evaluate the combination effect on tumor growth and hormone receptors. In ZR-75-1, 6×10^5 U of rhIFN-α2a per mouse, which did not affect ER expression, showed no combination effect with TAM, whereas 6×10^4 U of rhIFN-α2a per mouse increased ER expression and significantly enhanced the antitumor effect of TAM. Since 6×10^4 U of IFN per mouse alone showed no anti-proliferative effect on ZR-75-1, it was suggested that the enhancing effect on TAM action was closely related to the higher ER expression.

TAM is regarded as a partial agonist of estrogen as well as an estrogen antagonist. It is reported that the conjugation of TAM to ER inhibits the conjugation of E2 to ER, though TAM also suppresses the ER level and synthesizes PgR. This suppression of ER is accounted for by the loss of free ER, as the conjugate of TAM to ER binds strongly to the acceptor unit of DNA over an extended period of time. The present study revealed that the ER level of ZR-75-1 decreased from 136 fmol/mg protein to the threshold of detection with the combination treatment of rhIFN-α2a at a dosage of 6×10^4 U/mouse and TAM, as well as with TAM alone. The suppression by the TAM combination treatment of the IFN-increased ER level suggested that the antitumor effect of TAM was enhanced through the conjugation of TAM to the IFN-α-increased ERs.

In the present study, TAM and rhIFN-α2a were given simultaneously. If the combined antitumor effect of TAM and rhIFN-α2a depended only on the increment of intra-tumoral ER levels followed by TAM treatment, the administration of TAM after administration of rhIFN-α2a would have a greater antitumor effect than the simultaneous administration of both drugs. However, because TAM has an antitumor effect by itself on breast carcinomas with low toxicity, the simultaneous administration of IFN and TAM should be acceptable clinically. Since the dose of IFN tested in this study seems to be feasible in clinical treatment, the application of this combination therapy of IFN and TAM might be promising for ER-positive human breast carcinomas.

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