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Increased proliferation of a human breast carcinoma cell line by recombinant interleukin-2

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Abstract. Two adenocarcinoma cell lines, Breast M25-SF and Breast M, were established from tumor tissue resected surgically from a patient with breast cancer. One, Breast M25-SF, expresses interleukin-2 receptor (IL-2R) on the cell surface and the other, Breast M, does not. The effects of recombinant interleukin-2 (rIL-2) on the proliferation of these cell lines were investigated. The growth of Breast M25-SF was significantly promoted by rIL-2 ranging from 1.25 U/ml to 640 U/ml. Anti-CD25 (Tac) antibody significantly blocked the growth enhancement of Breast M25-SF by rIL-2. Breast M, however, did not respond to rIL-2. To confirm more directly the promotion of Breast M25-SF growth by rIL-2, cloning of IL-2 responders from parent Breast M25-SF cells was carried out by limiting dilution without feeder cells in 96-well microplates. No colony formation was found in 24 wells without rIL-2. Eleven, 13 and 6 clones were established from groups of 24 wells containing rIL-2 at 200, 20 and 2 U/ml respectively. All of the clones expressed IL-2R and respond to rIL-2. By using a sensitive polymerase chain reaction technique, we demonstrated that Breast M25-SF but not Breast M expressed IL-2 mRNA, and IL-2 secretion from Breast M25-SF but not Breast M was also confirmed by radioimmunoassay. These findings suggest a role for IL-2 in autocrine support of Breast M25-SF growth. IL-2 may play an important role in the growth control of breast carcinoma cells.

Key words: Breast carcinoma cell line – Interleukin-2 – Interleukin-2 receptor – Autocrine growth factor – Interleukin-2 secretion

Introduction

Interleukin-2 (IL-2) is a low-molecular-mass glycoprotein necessary for activation and differentiation of several distinct lineages of normal hemopoietic cells [12, 17]. Large amounts of highly purified recombinant IL-2 (rIL-2) with full biological activity have become available for in vitro and in vivo studies through the cloning of the gene for IL-2 and its successful insertion and expression in *Escherichia coli* [18, 23]. It is known that recombinant IL-2 (rIL-2) is capable of efficiently generating lymphokine-activated killer (LAK) cells in vitro [20], and that high doses of rIL-2 alone, administered to mice, can mediate the regression of established pulmonary and hepatic metastases [10, 14]. For these reasons, systemic administration of rIL-2 to patients with inoperable metastatic tumors, aimed at generating LAK cells in vivo for mediation of tumor regression, is now in clinical trials [19].

The IL-2 receptor (IL-2R) has been observed on leukemic cells of myeloid, B, and T cell origin [2, 21], murine and human fibroblasts [4, 16], and human squamous-cell carcinoma cell lines and tumors [25]. Recently it was demonstrated that human corticotrophic adenoma and murine pituitary cells express IL-2 mRNA and IL-2R mRNA and that IL-2 might have an autocrine regulatory role within the pituitary [3]. In addition, several cancers have been shown to express other types of cytokine receptors and the specific corresponding cytokine functions in an autocrine loop [6, 9, 11]. These findings indicate that several types of cytokines may control tumor cell growth. In this paper, we demonstrated that some human breast carcinoma cells probably produce and use IL-2 in an autocrine fashion.

Materials and methods

Tumor cell lines. Two breast adenocarcinoma cell lines, Breast M and Breast M25-SF, were established at the same time from tumor tissue surgically resected from a patient with advanced breast cancer (mucinous adenocarcinoma). Briefly, the tissue was minced into pieces

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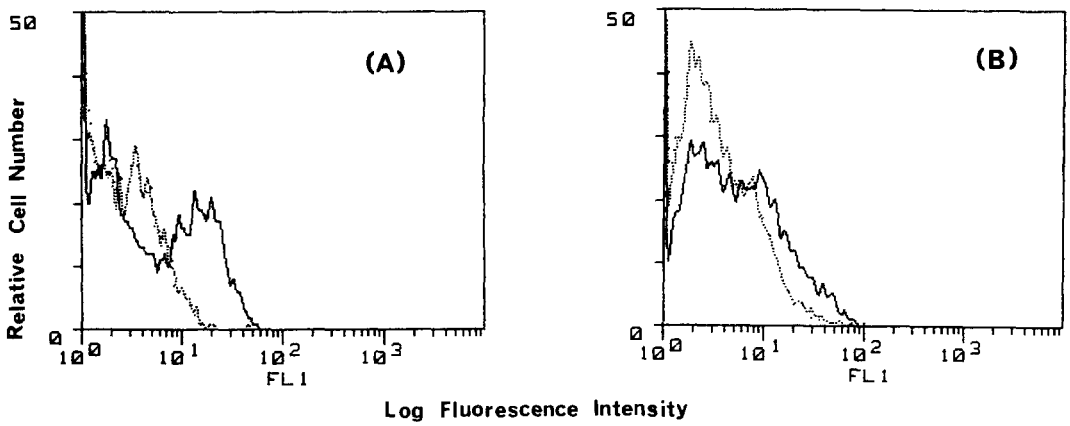

Fig. 1 A, B. Single-color histograms displaying log (mean fluorescence intensity) of interleukin-2 receptor (IL-2R; ———) on Breast M25-SF (**A**) or Breast M (**B**) cells. Negative controls were defined by labeling cells with a non-relevant antibody followed by fluorescein-isothiocyanate-labeled goat anti-(mouse IG) (···)

with surgical blades and then dissociated by mechanical stirring for 2 h at 37° C in a flask containing collagenase. The cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS) and cultured in 96-well flat-bottomed microplates. Seven weeks after the initiation of culture, two cell lines were established. Breast M proliferates in RPMI-1640 medium supplemented with 2%–10% FCS, and Breast M25-SF proliferates in both FCS-containing RPMI-1640 medium and in serum-free SF-02 medium (Sanko Junyaku Co., Tokyo, Japan) containing human insulin and transferrin. Doubling times of Breast M and Breast M25-SF were 18–22 h and 26–30 h respectively. Breast M and Breast M25-SF were maintained in RPMI-1640 medium containing 10% FCS, and cultures were transferred at 3- to 5-day intervals. Breast M does not express IL-2R. Expression of IL-2R on Breast M25-SF has been confirmed by flow cytometry.

Recombinant human IL-2. rIL-2 (TGP-3) was provided by Takeda Pharmaceutical Co. (Osaka, Japan) [8]. The specific activity of rIL-2 was 2.4×10^4 Japan Reference units (JRU)/mg protein. When the Biological Response Modifiers Program standard was used, it corresponded to 2.0×10^6 units/mg protein.

Monoclonal antibody to human IL-2R. Unlabeled antibodies (anti-CD25) were purchased from Immunotech (Luminy, France).

Immunofluorescence. Immunofluorescent staining of living tumor cells was performed as follows. Breast M cells were detached with EDTA. Breast M25-SF proliferated while forming floating clumps of cells. The cells were washed in phosphate-buffered saline and incubated for 30 min at 4° C with mouse monoclonal antibodies against IL-2R (Tac). Anti-IL-2R (CD25) was purchased from Coulter (Hialeah, Fla.). Cells were then washed twice and incubated for 30 min at 4° C with fluorescein-isothiocyanate(FITC)-coupled goat anti-(mouse Ig) antibodies. Negative controls were defined by labeling cells with a non-relevant antibody followed by FITC-labeled goat anti-(mouse Ig). The percentage of labeled cells and the relative mean fluorescence were determined by fluorescence-activated cell sorting (FACScan; Becton Dickinson Immunocytometry Systems, Calif.).

IL-2 determination in culture supernatants. Breast M25-SF and Breast M (10^5/ml) cells were cultured for 5 days. The supernatants were centrifuged and filtered through a Millipore filter of 0.22 µm pore size. IL-2 concentrations of culture supernatants were determined by radioimmunoassay using specific antibodies. IL-2 sensitivity was 0.8 U/ml.

Cell growth. Cellular growth was determined by a previously described 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) microculture tetrazolium assay [13]. Briefly, 50 µl tumor cells (10^4/ml) and 50 µl cytokine solution were plated in flat-bottomed microtiter wells and incubated for 2–5 days at 37° C in a humidified atmosphere of 5% CO_2 in air. Samples containing 10 µl MTT (5 mg/ml, Sigma, St. Louis, Mo.) were added to each of the microculture wells. After 2 h incubation at 37° C, 100 µl was removed from each well, to which 100 µl 100% dimethylsulfoxide was added to solubilize the MTT-formazan product. Absorbance at 570 nm was measured with a Dynatech Model MR 600 microplate reader.

Limiting-dilution analysis of IL-2 responders. Cloning of IL-2 responders from parent Breast M25-SF was carried out by limiting dilution without feeder cells, using 96-well flat-bottomed microplates. For limiting-dilution analysis, Breast M25-SF cells (50th passage), which had been subcultured serially for 8 months after the initiation of culture, were used. Breast M25-SF cells were seeded at 1–2 cells/well in a volume of 100 µl culture medium containing various concentrations of rIL-2. After 4 weeks, positive wells were visually scored by the presence of more than 100 viable cells.

Polymerase chain reaction (PCR). Total RNA was extracted from logarithmically growing cells by the thiocyanate/guanidium method as described previously [5]. cDNA was generated from total RNA essentially as described previously [1]. Briefly, 1 µg total RNA was incubated at 70° C for 10 min, chilled on ice, and reverse-transcribed in a final volume of 20 µl containing 5 µl of 5× reversetranscriptase (RT) buffer (Gibco-BRL, Gaithersburg, Md.), 1 µl 10 mM dithiothreitol, 1 µl 10 mM (each) dATP, dCTP, dGTP, and dTTP, 1 µl 500 ng random hexamer primers, and 200 units Superscript RT II reverse transcriptase (Gibco-BRL). The mixture was incubated at 37° C for 1 h, heated to 92° C for 10 min, and stored at –80° C. Amplification was performed as described previously [24]. Briefly, cDNA was heated to 92° C for 2 min, and then 3 µl was added to a reaction mixture containing 5 µl 10× PCR reaction buffer (0.5 M KCl, 0.1 M TRIS, pH 8.0, 15 mM MgCl, 0.01% gelatin), 1 µl 10 mM (each) dATP, dCTP, dGTP, and dTTP, 100 ng each priming oligomer, 1.0 unit Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) and H_2O (total volume 50 µl). A 50 µl layer of mineral oil was introduced over the aqueous phase to prevent evaporation. Amplification was performed using a DNA thermal cycler (Program temperature-control system PC-700, ASTEC, Fukuoka, Japan) for 35 cycles. A cycle profile consisted of 1 min at 92° C for denaturation, 1 min at 57° C for annealing, and 2 min at 72° C for primer extension. Electrophoresis of 8.5 µl reaction mixture on a 1.5% agarose gel containing ethidium bromide was performed to evaluate amplification and the size of the fragment generated. Gene sequences used to construct oligonucleotide primers were from published sources [23]. Primer sequences of IL-2 were as follows: sense, CAGGATGCAACTCCTGTCTTGC, antisense, CAGTTCTGTGGCCTTCTTGGGC.

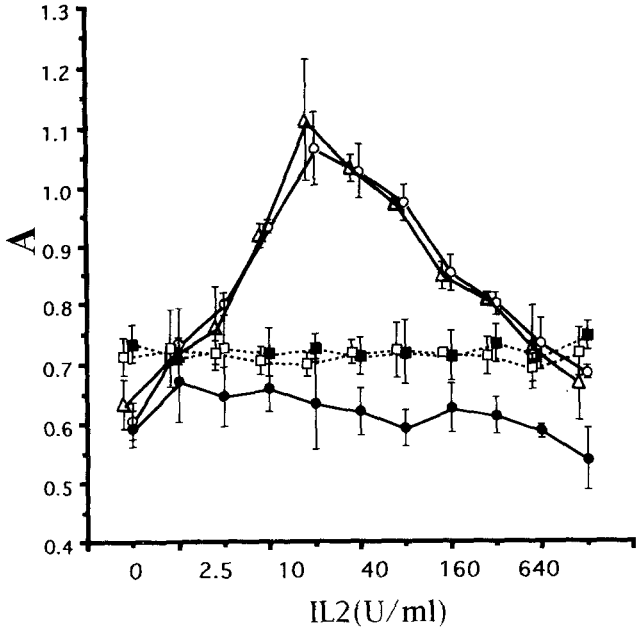

Fig. 2. Effects of recombinant IL-2 (rIL-2) on growth of IL-2R-positive Breast M25-SF and IL-2R-negative Breast M cells cultured in the presence of anti-CD25 antibody. Both types of cell were incubated in the presence of increasing concentrations of rIL-2 alone or rIL-2 plus a fixed concentration of anti-CD25 (50 μg/ml) or isotype antibody for 3 days. Proliferation was measured in MTT colorimetric assays as described in Materials and methods; *A*, absorbance. The data represent means ± SD of three independent wells. ○, Breast M25-SF + rIL-2; △, Breast M25-SF + rIL-2 + isotype control; ●, Breast M25-SF + rIL-2 + anti-CD25; □, Breast M + rIL-2;; ■, Breast M + rIL-2 + anti-CD25

Results

IL-2R expression on breast carcinoma cell lines

By flow-cytometric analysis we investigated the staining patterns of the anti-IL-2R on a suspension of Breast M and Breast M25-SF cells. Figure 1 shows that Breast M25-SF expresses IL-2R. No significant staining was observed in Breast M.

Effect of rIL-2 and anti-IL-2R antibody on proliferation of breast carcinoma cell lines

Both cell lines, Breast M25-SF and Breast M, were incubated for 3 days with or without supplementation with various concentrations of rIL-2. As shown in Fig. 2, Breast M25-SF responded with increased proliferation to rIL-2 over a wide dose range (1.25–640 U/ml), reaching a plateau response at the 10 U/ml dose. On the other hand, Breast M did not significantly respond to rIL-2 in this dose range.

To evaluate the specificity of the observed growth-promoting effects of IL-2, the ability of anti-IL-2R (anti-CD25) antibody to reduce the effect of IL-2 on the growth of Breast M25-SF was evaluated. Anti-CD25, but not the isotype control antibody, added to wells containing tumor cells in suspension plus rIL-2, significantly blocked the growth enhancement of Breast M25-SF by rIL-2.

Table 1. Cloning of interleukin-2 (IL-2) responders from Breast M25-SF

rIL-2 (U/ml)	Positive wells/total wells
0	0/24
200	11/24*
20	13/24*
2	6/24**

Cloning of IL-2 responders from parent Breast M25-SF cells was carried out by limiting dilution without feeder layers in 96-well microplates. Breast M25-SF cells (50th passage) were seeded at 1–2 cells/well in 100 μl culture medium containing various concentrations of rIL-2. After 4 weeks, positive wells were visually scored by the presence of more than 100 viable cells

* $P < 0.01$ (χ^2-test)

** $P < 0.05$ (χ^2-test)

Table 2. IL-2 secretion by Breast M25-SF

Subclones	IL-2 (U/ml)
Breast M25-SF	1.46 ± 0.45
Breast M	< 0.8

Breast M25-SF and Breast M (10^5/ml) cells were cultured for 5 days at 37° C in a humidified atmosphere of 5% CO_2 in air. IL-2 concentrations of the cell-free supernatants were determined by radioimmunoassay using specific antibodies. IL-2 sensitivity was 0.8 U/ml. The data represent means ± SD of four independent experiments

Cloning of IL-2 responders from Breast M25-SF

To identify the proliferative response of Breast M25-SF to rIL-2 more directly, cloning of IL-2 responders from parent Breast M25-SF cells was carried out using the limiting-dilution technique described in Materials and methods. The numbers of proliferating wells out of the 24 were scored and the results are shown in Table 1. When the wells contained rIL-2 at 200 U/ml, clone-like proliferation was found in 11 out of the 24 wells tested. rIL-2 at 20 U/ml and 2 U/ml induced clonal proliferation in 13 and 6 out of each group of 24 wells respectively. All of the 30 clones expressed IL-2R and responded to rIL-2 by increased proliferation (data not shown). But no proliferation was found in the 24 wells without rIL-2.

IL-2 secretion by Breast M25-SF

Breast M25-SF and Breast M cells were cultured in RPMI-1640 medium supplemented with 10% FCS for 5 days, and IL-2 production in culture supernatant was measured by radioimmunoassay as described in Materials and methods. Only low concentrations of IL-2 were detected in the supernatant from Breast M25-SF (Table 2), but supernatant from Breast M cells contained no detectable IL-2.

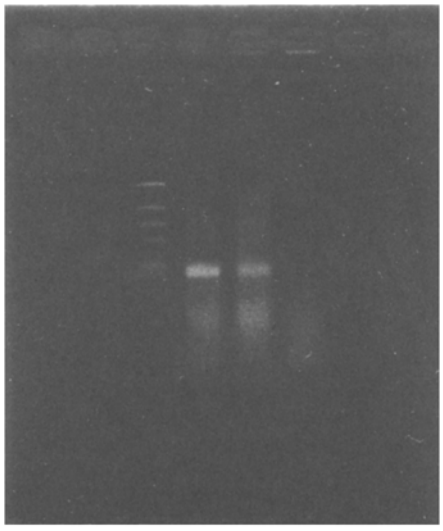

Fig. 3. Polymerase-chain-reaction(PCR)-assisted mRNA amplification of Breast M25-SF using specific primers to IL-2. Total RNA of cells was extracted and run in a PCR as described in Materials and methods. Lane *A*, molecular markers; lane *B*, phytohemagglutinin-stimulated lymphocytes; lane *C*, Breast M25-SF; lane *D*, Breast M

Expression of IL-2 mRNA on Breast M25-SF

To confirm the expression of IL-2 mRNA on Breast M25-SF, PCR analysis was carried out using specific IL-2 primers. As a positive control for IL-2 mRNA expression, mRNA from phytohemagglutinin-activated lymphocytes was analyzed. Breast M25-SF but not Breast M expressed IL-2 mRNA (Fig. 3). All of the 30 clones established from parent Breast M25-SF cells expressed IL-2 mRNA (unpublished data).

Changes of IL-2R expression and IL-2 responsiveness during long-term cultures

IL-2R expression and response to rIL-2 were frequently examined during the continuous long-term culture. Figure 4 shows that, following serial subcultures in vitro, both IL-2R expression and IL-2 responsiveness gradually decreased and were lost 12 months (76th passage) after the initial culture.

A similar phenomenon was also found in Breast M25-SF-derived clones. Both IL-2R expression and IL-2 responsiveness were lost at almost the same time in all of the 5 clones examined 4 months after (30th passage) the establishment of transferrable clones (unpublished data).

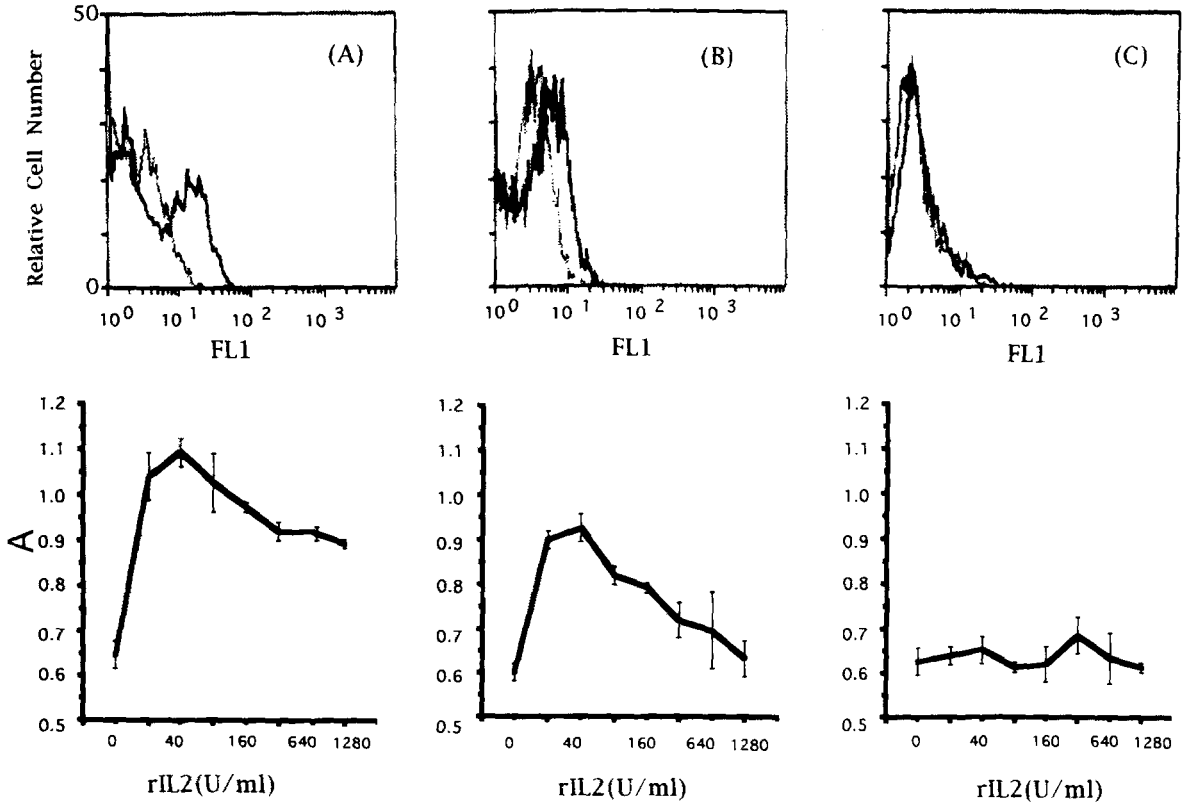

Fig. 4A–C. Changes of IL-2R expression and IL-2 responsiveness of Breast M25-SF cells during continuous long-term culture. IL-2R expression and response to rIL-2 were frequently examined during long-term continuous cultures: **A** 12th passage; **B** 50th passage; **C** 72nd passage. *Upper curves:* single-color histograms displaying log(mean fluorescence intensity) of IL-2R (———) on Breast M25-SF cells. *Lower curve:* growth curve of Breast M25-SF cells in 3-day culture in the presence of various concentrations of rIL-2. Proliferation was measured by MTT assay. Data represent means ± SD of three independent wells

Discussion

Two subclones, Breast M25-SF and Breast M, from the same breast tumor tissue of one patient were established. Breast M25-SF expressed IL-2R on their cell surface and their growth was significantly promoted by rIL-2. On the other hand, Breast M did not express IL-2R and did not responded to rIL-2. The receptor for IL-2 was first identified on the surface of human activated T cells [17]. The distribution of IL-2R on hematopoietic cells may be wider than previously thought, and their presence was recently reported on nonhematopoietic cells including murine and human fibroblasts [4, 16], human corticotrophic adenoma [3], murine pituitary cells [3], and human squamous-cell carcinoma cell lines [25]. The present study is the first to demonstrate functional IL-2R in human breast carcinomas. IL-2 interaction with IL-2R usually leads to the enhancement of proliferation of target cells as well as Breast M25-SF. But it has been reported that IL-2/IL-2R interaction leads to the inhibition of proliferation of cells such as oligodendrocytes [22] and human head and neck squamous carcinoma cell lines [25]. IL-2 has been reported to inhibit the growth of hormone-dependent human breast cancer cells [15] directly. At this time, it is not known what determines whether IL-2 interaction with IL-2R in different cells will result in positive or negative signals for growth.

A functional IL-2R is composed of at least three different IL-2-binding peptides: IL-2Rα, IL-2Rβ, and IL-2Rγ. It is usually considered that IL-2Rα is unable to transduce a biological signal alone, and that IL-2Rβ expression is necessarily constitutive for IL-2-mediated signaling and functional activation. In our experiments, IL-2Rβ expression on Breast M25-SF was not confirmed by flow cytometry. However, expression of IL-2Rβ mRNA was confirmed by reverse transcriptase/PCR (unpublished data). This result strongly indicates that a small amount of IL-2R is expressed on Breast M25-SF.

The IL-2 autocrine mechanism in Breast M25-SF is strongly suspect, in part because of the poor growth of Breast M25-SF when cultured at very low densities without rIL-2. We theorized that an effect of exogenous IL-2 on the growth of Breast M25-SF would be most apparent on cultures initiated at low density, because suboptimal amounts of endogenously produced IL-2 might be present under these conditions. In fact, many clones were successfully established only when rIL-2 was added to cultures initiated at low density (Table 1). Next, we examined if Breast M25-SF produces IL-2, and we have demonstrated that IL-2 is certainly produced in Breast M25-SF but at a low level (Table 2). In addition, expression of IL-2 mRNA in Breast M25-SF was confirmed even at the clonal level (unpublished data). In addition, the growth of Breast M25-SF and their clones was blocked by anti-CD25 antibody (Fig. 1). These results indicate that IL-2 may be an autocrine growth factor for Breast M25-SF.

It is unknown why Breast M, established from the same breast tumor tissue, neither expresses IL-2R nor responds to IL-2 (Figs. 1, 2). Following long-term culture in vitro, both the IL-2R antigen and responsiveness to rIL-2 were lost in Breast M25-SF (Fig. 4). Similar behaviour has recently been reported in peritoneal murine fibroblasts [7]. In that paper it was demonstrated that, following serial subcultures in vitro, the IL-2R was lost. Plaisarce et al. [16] have also investigated the expression of IL-2R at the membrane during the life span of human embrionic fibroblasts. They conclude that, during the aging process, normal human fibroblasts lose the IL-2R. These findings suggest that IL-2R expression on breast carcinoma cells may be transient in nature and that Breast M may already have lost the ability to express IL-2R. Breast M may have other autocrine mechanisms, as previously reported in other types of tumors [9, 11, 16]. We now hypothesize that tentative expression of IL-2R on the breast tumor cell surface may not be such a rare phenomenon. To test this hypothesis, the expression of mRNA for IL-2R and IL-2 on breast tumors resected surgically is now under investigation by in situ hybridization techniques. Our novel observations of the expression of IL-2 mRNA and IL-2R on Breast M25-SF may provide considerable new implications for the immunotherapy of cancer in general.

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