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CAPACITY OF ADIPOSE TISSUE TO PROMOTE GROWTH AND METASTASIS OF A MURINE MAMMARY CARCINOMA: EFFECT OF ESTROGEN AND PROGESTERONE

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Previously we have shown that a murine mammary carcinoma cell line, designated SP1, grows and metastasizes more efficiently in the mammary gland than in the subcutis. In this report, we examine the tissue specificity of this phenomenon. Our results show that SP1 cells grow best in the mesenteric and ovarian fat pads and well in the mammary gland, but very poorly in the subcutis or peritoneal cavity. Massive dissemination of tumors from the ovarian and mesenteric sites occurs to the liver, spleen and diaphragm. In contrast, metastases from the mammary site occur primarily in the lung. Co-transplantation of a threshold number of SP1 cells with mammary or ovarian fat fragments into the subcutis results in increased tumor growth, whereas very few tumors form in sham controls receiving no fat fragments. Removal of the ovaries of donor and recipient mice abrogates tumor growth in adipose tissue transplants. Estrogen can stimulate growth of SP1 in adipose tissue sites, whereas progesterone inhibits growth. In contrast, *in vivo* growth of a stable metastatic variant selected from SP1 cells was not inhibited by progesterone. SP1 cells growing in ovarian and mesenteric fat pads showed increased expression of estrogen receptors and progesterone receptors, as well as detectable levels of epidermal-growth-factor receptors, whereas receptor levels decreased to baseline on tumors in the subcutis. The levels of estrogen-receptor mRNA reflect the corresponding functional expression of receptors; this finding suggests that the regulation of estrogen-receptor expression in this system is, at least in part, at the mRNA level. Our results are consistent with the model that adipose tissue exerts an estrogen-dependent positive regulatory effect on primary SP1 tumor growth, and promotes the formation of metastases.

Recurrent breast cancer is responsible for the highest rate of cancer deaths among non-smoking women (Willett, 1989). Frequent sites of dissemination are bone, lung and liver. Mammary mesenchyme is important in the growth of normal, pre-malignant, and malignant mammary epithelial cells (Miller and McInerney, 1988), and can facilitate tissue-specific metastasis formation (Price *et al.,* 1990). However, the nature of the cellular interactions in the mammary stroma that promote growth and metastasis of breast-cancer cells is not known.

In normal mammary gland development, one of the first signals for proliferation of epithelial end bud cells is estrogen. Two important actions of estrogen are to stimulate the local production of peptide members of the epidermal growth factor (EGF) family (Snedeker *et al.,* 1991), and to induce expression of EGF receptors (EGFR) on epithelial cells (Haslam, 1988). Evidence suggests that the estrogen signal is delivered to epithelial end bud cells indirectly through neighboring stromal tunic cells (Haslam, 1988). Estrogen receptors (ER) are expressed early in development in stromal tunic cells adjacent to ducts and in luminal epithelial cells of the end bud, but are undetected in rapidly proliferating cap cells (Silberstein and Daniel, 1982). Stromal fibroblasts can facilitate estrogen-induced proliferation of mammary epithelial cells and expression of EGFR *in vitro* (Haslam, 1988). Adipocytes, another component of mammary stroma, have also been shown to stimulate mammary epithelial cells *in vitro* (Levine and Stockdale, 1984); however the role of estrogen in this response is not known. An understanding of the cellular and hormonal interactions which affect the growth and metastasis of breast-cancer cells would aid in the design of better treatment protocols.

We have developed a murine mammary carcinoma (SP1) model (Elliott *et al.,* 1988) to study hormonal-stromal cell interactions that stimulate tumor growth and metastasis. Previously, we have shown that SP1 cells grow slowly in the subcutaneous site and rarely metastasize. Cells from the same cell inoculum grow rapidly in the mammary fat pad, and frequently metastasize to the lung. In the present study, we have examined the influence of various adipose-tissue sites and of ovarian-derived sex hormones on the growth and metastasis of SP1 cells. SP1 cells grow rapidly in the ovarian and mesenteric fat pads, with massive dissemination to the liver, spleen and diaphragm. Co-transplantation of adipose tissue with SP1 cells can enhance tumor growth in subcutaneous sites. In ovariectomized animals, SP1 tumor growth was abrogated, but was enhanced when these animals were supplemented with 17-β-estradiol pellets; progesterone suppressed the estrogen effect. Interestingly, growth of a metastatic variant of SP1 cells, SP1-3M, was not inhibited by progesterone. To further assess the hormone-responsiveness of SP1 cells growing in various tissue sites, we examined the expression of receptors for estrogen, progesterone and epidermal growth factor. These hormone and growth-factor receptors are modulated by estrogen in normal epithelial cells, and are important indicators of early-stage breast-cancer cells (Lippman and Dickson, 1990). Our results suggest that adipose tissue can promote estrogen-dependent growth of SP1 cells, with a concomitant induction of hormone and growth factor receptor expression.

MATERIAL AND METHODS

Mice

Female or male CBA/J mice between 8 and 12 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME. Female BALB/c nude mice of the same age range were obtained from Sprague Dawley.

Media

The medium used for all manipulations was RPMI-1640 (GIBCO, Grand Island, NY, 430-1800) supplemented with 5 to 7% FCS (Flow, McLean, VA), 2 mM L-glutamine (GIBCO 320-5030), 36 mg/L-asparagine, 116 mg/L-arginine HCl, 10 mg/L folic acid, and 100 mg/L sodium pyruvate (Sigma, St. Louis, MO).

Tumors

SP1 is a mammary adenocarcinoma which arose spontaneously in an 18-month-old CBA/J female retired breeder obtained from Jackson Laboratories. The histological and ultra-structural characterization of the original SP1 tumor as

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an infiltrating ductal carcinoma and its growth properties have been described by Elliott *et al.* (1988). A cell line was established *in vitro* and frozen down to maintain stock. A metastatic variant cell line, SP1-3M, was isolated from a metastatic nodule which had undergone 3 cycles of selection in the mammary gland; the SP1-3M cell line has been described (Elliott *et al.*, 1988). Cells were kept in culture for no more than 3 months before thawing out fresh stocks. All tumors were tested periodically for Mycoplasma; only Mycoplasma-free lines were used.

Separation of transplanted SP1 tumor cells

Tumors were excised and cell suspensions were prepared by 2 or 3 enzymatic digestions for 40 min at 37°C. The enzymes used were a mixture of 0.02% collagenase III (Sigma) and 0.01% DNAase (Sigma) in Ca^{2+}/Mg^{2+}-free PBS. After each treatment, cells were pooled and washed 3 times; the fatty layer was carefully pipetted off the top. Cells were then introduced onto a discontinuous gradient of Percoll (Pharmacia, Arlington Heights, IL; stock solution with 10 parts Percoll to 1 part 10 × PBS (final osmolarity = 285 mosm per kg H_2O)). The discontinuous gradient was composed of the following concentrations of stock Percoll: 30% (5 ml), 20% (2 ml), 15% (2 ml), 10% (2 ml). Cells were centrifuged at 150 *g* for 10 min. In these conditions, tumor cells, which carry twice the amount of normal cellular DNA (data not shown), move through the Percoll to form a tight pellet devoid of host tissue, which remains in the supernatant.

Transplantation of tumor cells

Animals were anesthetized with sodium pentobarbitol and were positioned on an operating board. Fat-pad transplantation was carried out as described (Elliott *et al.*, 1988). For ovarian-fat-pad injections, a minor incision was made in the dorsal abdominal wall at the base of the rib cage and the ovarian fat pad was exposed. Tumor cells were injected in a 10-μl volume with a Hamilton syringe. The abdominal wall was closed and the wound was clamped with stainless steel clips. A similar procedure was carried out for injection of SP1 cells into mesenteric and epididymis fat pads, except that the incision was made via the ventral side. Transplants into the kidney sub-capsule were carried out according to the procedure of Cunha *et al.* (1983). The region of s.c. injection was the mid-dorsal side of the animal.

Ovariectomy and steroid hormone implants

In some experiments, animals were ovariectomized (OVX) by surgical removal of the ovaries. High luteinizing hormone (LH) levels (determined by radioimmunoassay) and low uterine weight indicated that systemic estrogen was depleted. Administration of hormones was carried out by s.c. implantation of pellets containing 17-β-estradiol (0.72 mg/pellet) or progesterone (5 mg/pellet). 17-β-estradiol and progesterone pellets in a 60-day release matrix of cholesterol:methyl cellulose: hormone [or cholesterol:methyl cellulose:lactose (placebo control)] were purchased from Innovative Research (Rockville, MD). The dose of 17-β-estradiol used has previously been shown to yield approximately 5-times the physiological blood level of estrogen in 8- to 10-week-old female mice (Hitselberger *et al.*, 1988). Two- to fourfold increased uterine wet weight and 10-fold lower LH levels occurred in OVX animals whose systems had been reconstituted with estrogen, compared with OVX mice receiving placebo control pellets.

Examination of tumors

For s.c. and intramammary tumors, mean tumor diameter was measured at various times after injection and data were expressed as the number of days required for tumors to reach 10 mm in diameter, calculated by linear regression analysis (Miller, 1985). For tumors in ovarian and mesenteric fat pads, tumor take and tumor size were evaluated at death. Animals were killed and examined for gross metastases at 36 or 63 days following transplantation. Primary tumors were resected and portions of the tumor were formalin-fixed and paraffin-embedded. Sections were then cut and stained with hematoxylin, phloxine and safranin (HPS) for histological studies. Statistical significance between groups was assessed by Fisher's exact probability test, using a Biosoft III Software programme (Rief, 1982).

Assays for estrogen receptor (ER), progesterone receptor (PR) and epidermal-growth-factor receptor (EGFR)

Ligand binding assays were performed on viable SP1 tumor cells separated by Percoll-gradient centrifugation to remove host cells. Specific binding of ^{3}H-labelled 17-β-estradiol to nuclear type-I and type-II binding sites was carried out on charcoal-stripped, 0.4 M KCl extracts as described by Haché *et al.* (1987), with the following modifications: reducing agents were omitted from all stages of nucleus isolation and salt extraction. The liberation of type-II sites was accomplished by subjecting all nucleus preparations to at least 2 freeze-thaw cycles in extraction buffer (400 mM KCl, 10 mM EDTA, 10 mM Tris HCl, pH 7.4). Levels of type-II receptors were determined by measuring the difference in estrogen-specific binding at 37°C with or without 0.1 mM dithiothreitol (Lyttle *et al.*, 1989). Progesterone binding was determined by the method of Horwitz *et al.* (1981). An assay for nuclear receptors was used, since ER has previously been shown to be exclusively localized to the nucleus (King and Greene, 1984). We have analyzed cytosolic fractions for ER and PR and were unable to detect any significant amount of either receptors using the described protocols (data not shown). Nuclear ER receptor expression is also unaffected by enzymatic treatment (Berthois *et al.*, 1986).

EGF binding was determined by the method of Roos *et al.* (1986). Briefly, tumor cells were dispersed by collagenase digestion and grown in tissue-culture medium for 24 to 40 hr at 37°C to a density of 1 to 5 × 10^6 cells per dish. This procedure allowed cell membranes to recover from enzyme treatment; however, the observed EGFR numbers may still be an underestimate of the true receptor number *in situ*. For the binding assay, adherent cells were washed twice with 1 ml of PBS containing 2% BSA (PBS/BSA). The cells were incubated for 3 hr at 25°C with 0.5 ml of PBS/BSA containing 0.2 ng of ^{125}I-EGF per ml (specific activity, 178 μCi/μg obtained from NEN, Boston, MA). Non-specific binding was assessed by adding a 50-fold excess of unlabelled mouse maxillary EGF (Sigma). After incubation, the medium was removed and the dishes were washed 3 times with 1 ml of ice-cold PBS/BSA. To detach the cells, a 0.25% solution of trypsin in PBS (2 ml per dish) was added and the cultures were incubated at 37°C for 10 min. A 0.2-ml portion of the cell suspension was used for counting viable cells (determined by Trypan-blue exclusion). The remaining cells were transferred to vials and the radioactivity was measured with a Beckman gamma counter. The number of receptors per tumor cell was determined by Scatchard analysis.

RNA preparation and Northern blotting

Total RNA was extracted from Percoll-separated tumor cells, and poly(A)-containing RNA was selected using an oligo(dT) column as described by Sambrook *et al.* (1989). For Northern blotting analysis, RNA was subjected to electrophoresis on formaldehyde-agarose gels in 1 × MOPS buffer and transferred to Hybond-N filters in 20 × SSC for hybridization as described by Sambrook *et al.* (1989). cDNA probes for estrogen receptor [HEO (Green and Chambon, 1990)] and β-actin (Ueyama *et al.*, 1984) were provided by Dr. P. Chambon and Dr. G. Jay respectively.

RESULTS

Site specificity of tumor growth and metastasis in different fat pads

To determine the influence of different tissue microenvironments on SP1 growth and metastasis, we compared the growth properties of SP1 in various tissue sites. The rate of primary tumor growth was greatest in the ovarian fat pad, less in the mesenteric and mammary fat pads, and least in the subcutis and peritoneal cavity (Table I, Fig. 1*a,b*). The most profound difference was observed at a tumor-cell dose of 3×10^3 cells per mouse. At this cell dose, no tumors arose in the peritoneal cavity and only 3/17 tumor takes occurred in the subcutis, whereas 100% tumor takes were observed in the mesenteric and ovarian fat pads. Interestingly, the same dose grew aggressively in the kidney sub-capsule and in the epididymis fat pad of male mice (Table I, Fig. 1*d*). Animals with tumors in the subcutis exhibited a significantly prolonged survival compared with animals with tumors in adipose-tissue sites. Massive dissemination of tumors to liver, spleen and diaphragm occurred from tumors growing in the mesenteric and ovarian fat pads. Tumors from the mammary gland spread primarily to the lung (Fig. 1*c*). No metastases from tumors in the subcutis were observed.

Histological comparison of tumor growth in the subcutis, and the ovarian and mammary fat pads

The morphology of tumors in fat-pad sites was different from those in the subcutis. A clear connective-tissue sheath was evident around SP1 tumors in the subcutis (Fig. 2*a*). In contrast, tumors growing in the mammary, ovarian (Fig. 2*c,d*) and mesenteric fat pads (data not shown) showed direct interdigitation with surrounding adipose tissue; no connective-tissue sheath was evident.

Enhancement of SP1 growth in the subcutis by adipose-tissue transplants

To directly test the effect of adipose tissue on SP1 growth, we co-transplanted adipose tissue and tumor cells into the subcutis. Enhanced tumor growth occurred in the subcutis in groups co-injected with a threshold dose of SP1 cells (3×10^3) and adipose tissue from either ovarian or mammary fat pads (Table II, Fig. 2*b*). Histological examination of the tumor mass revealed the presence of tumor cells immediately adjacent to adipocytes (Fig. 2*b*, insert). Transplanted fat tissue could be distinguished by the presence of anucleate necrotic adipocytes with ill-defined membranes; no host-cell infiltrate was evident. In control animals receiving sham surgery without a fat-tissue transplant, an injection of the same cell inoculum into the subcutis yielded very little or no tumor growth. These results confirm that adipose tissue can support primary growth of SP1 cells.

Estrogen-dependence of SP1 growth in adipose-tissue sites

To investigate the estrogen dependence of SP1 growth in adipose-tissue sites, we examined tumor growth in ovariectomized animals supplemented with hormone-containing pellets. Two approaches were taken. First, we co-transplanted adipose-tissue fragments with SP1 cells into the subcutis of ovariectomized mice (Table II). Ovariectomy both of donor and of recipient mice abrogated tumor growth in adipose-tissue transplants. This observation suggests that adipose-tissue-dependent growth of SP1 cells requires ovarian-derived growth substances, and argues against the possibility that the observed enhanced SP1 tumor growth is an artifact of the transplantation procedure. Ovariectomy of the ovary-fat-pad donor mice, but not the recipient mice, yielded partial reduction of tumor takes in Experiment 1 and no reduction in Experiment 2; this finding suggests that sufficient ovarian-derived factors were present in normal recipients. Ovariectomized recipient mice that received 17-β-estradiol showed at least 2-fold increased tumor takes and faster tumor growth rates compared with placebo controls (Table II, Experiment 3). 17-β-estradiol therefore restored growth of SP1 cells in ovariectomized mice.

Second, we injected SP1 cells into the mammary fat pad of untreated or ovariectomized animals. The ductal epithelium of the mammary gland of recipient mice was removed by cauterization of the mammary bud to exclude any role of epithelial cells in SP1 growth; this treatment was previously shown to have no effect on SP1 tumor growth and metastasis (Elliott *et al.*, 1988). SP1 tumor takes grew in 100% of sham-treated control animals (*i.e.*, those with intact mammary glands) (Table III). In ovariectomized recipients, SP1 tumors grew in only 45% of animals injected, and the rate of tumor growth was slower. When animals were supplemented with 17-β-estradiol pellets, SP1 tumors grew in 90% of animals injected. Administration of progesterone pellets failed to restore tumor growth in ovariectomized animals and suppressed the 17-β-estradiol effect. These results indicate that estrogen stimulates, and progesterone inhibits, growth of the SP1 tumor in adipose-tissue sites. Interestingly, no inhibitory effect of progesterone was observed in the estrogen-dependent growth of a metastatic variant, SP1-3M. No effect of estrogen or progesterone on metastasis formation of SP1 or SP1-3M cells was demonstrated.

TABLE I – INFLUENCE OF TISSUE SITE ON SP1 GROWTH AND METASTASIS

Experiment number	Injection		Tumor takes[2]	Time of killing (days)[3]	Metastases	
	Site[1]	Cells			Lung	Regional[4]
1	Subcutis	10^4	8/18	52 ± 8	0/8	0/8
	Mammary fat pad	10^4	13/17*	42 ± 2	5/13	0/13
	Mesenteric fat	10^4	17/18*	31 ± 3	0/17	8/17
	Ovarian fat	10^4	16/18*	30 ± 6	0/16	16/16
2	Subcutis	3×10^3	3/17	>60	0/3	0/3
	Mammary fat pad	3×10^3	10/12*	>55	3/10	0/10
	Mesenteric fat	3×10^3	18/18*	27 ± 3	0/18	13/18
	Ovarian fat	3×10^3	18/18*	28 ± 2	0/18	12/18
	Peritoneal cavity	3×10^3	0/7	>55	0	0
3	Subcutis (F)	3×10^3	0/5	>45	0	0
	Ovarian fat (F)	3×10^3	8/8*	27	0/8	4/8
	Kidney sub-capsule (F)	3×10^3	6/7*	27	0/6	0/6
	Subcutis (M)	3×10^3	0/8	>45	0	0
	Epididymis (M)	3×10^3	7/8*	27	0/7	1/7

[1]Cells were injected according to methods described in text. All recipients were female 10-week-old CBA mice, except in Experiment 3, where male mice were used. F, female; M, male.–[2]Significant increase in tumor takes compared with s.c. site in each group is indicated by asterisk (Experiment 1, $p < 0.023$; Experiment 2, $p < 0.002$; Experiment 3, $p < 0.008$).–[3]Time of animal death or killings, mean ± sem.–[4]Regional metastases in liver, spleen and diaphragm.

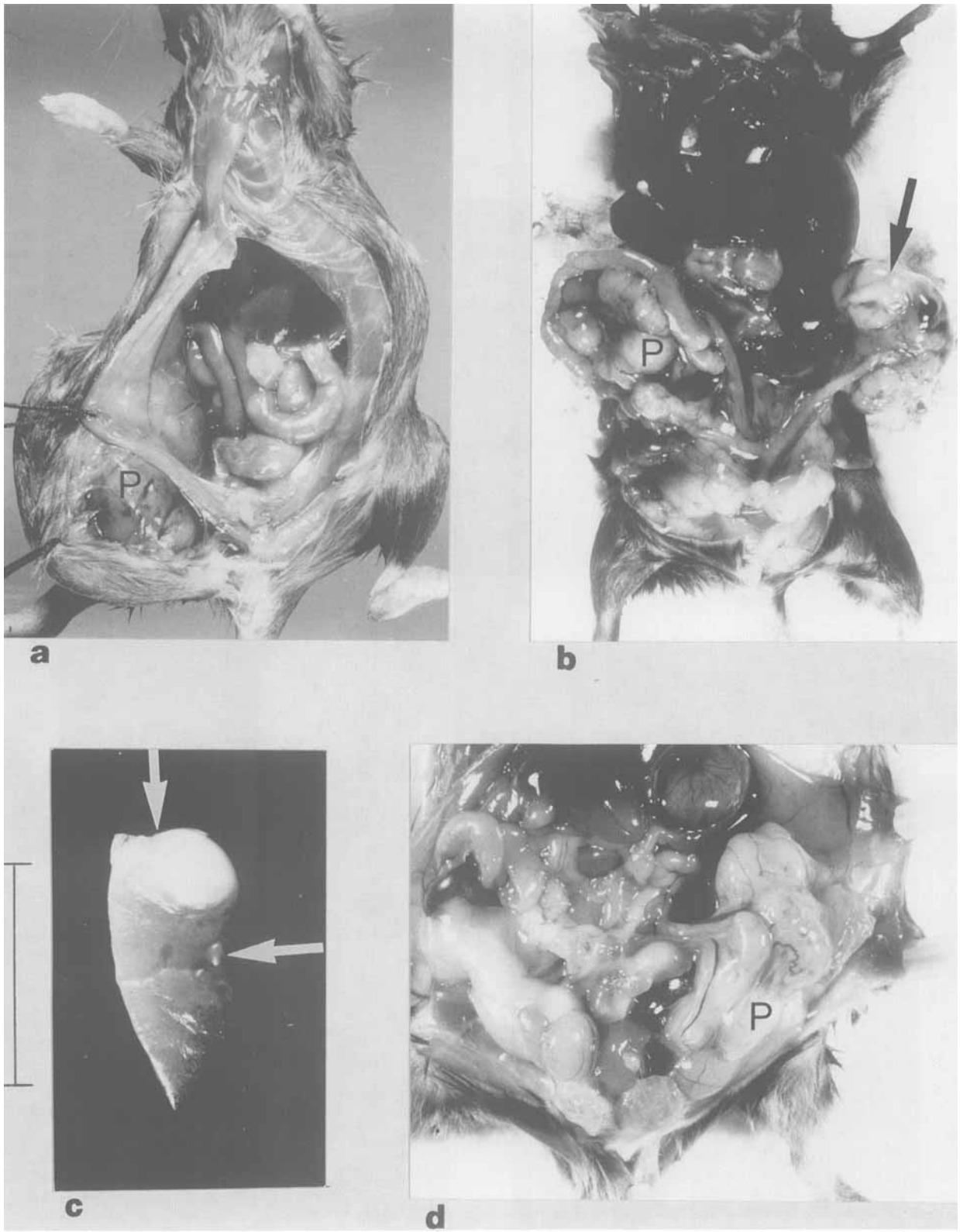

FIGURE 1 – Pathology of SP1 tumors in different adipose tissue sites. SP1 tumor cells were injected into the mammary gland *(a)*, the ovarian fat pad *(b)* and the epididymis fat pad *(d)*. After 5 weeks, animals were killed and examined for tumors. Lung metastases (indicated by arrow) from a tumor in the mammary gland tumor site is shown in *(c)* (scale bar indicates 0.5 cm). Primary tumors are indicated (P). Regional metastases from a tumor in the ovarian fat pad to the contralateral ovarian fat pad are indicated by arrows *(b)*.

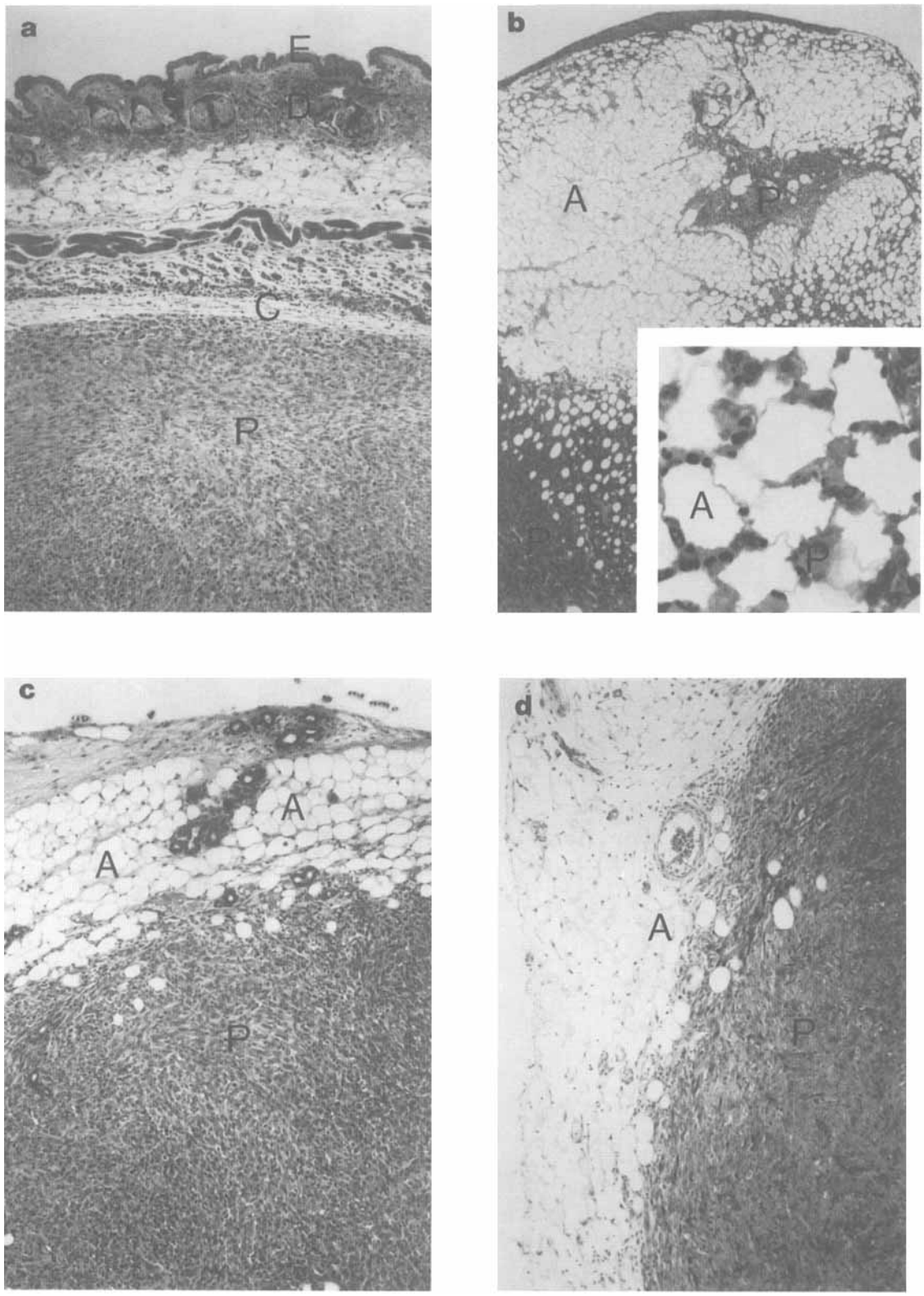

FIGURE 2 – Histology of SP1 growing in various primary sites. SP1 cells were injected into *(a)* the subcutis, *(c)* the mammary fat pad, and *(d)* the ovarian fat pad, and tumors at 21 days were resected and stained with hematoxylin, phloxine and safranin (96×). Tumor tissue is shown by (P). In one group *(b)*, SP1 cells were co-transplanted with ovarian adipose tissue fragments into the subcutis. The inset (enlarged 394×) shows the pattern of growth of SP1 cells in the fat tissue. E, epidermis; D, dermis; C, connective tissue sheath; A, adipocytes.

TABLE II – ADIPOSE TISSUE SUPPORTS GROWTH OF SP1 IN THE SUBCUTANEOUS SITE; INFLUENCE OF OVARIECTOMY

Experiment number	Donor fat[1]	Recipient	Injection site[2]	Tumor takes[3]	Day of 10-mm tumor diameter[4]	Day of killing (days)	Metastases	
							Lung	Regional[5]
1	Sham	Normal	SC	1/7	35	60	0/1	0/1
	None	Normal	OF	6/6*	NT	30 ± 2	0/6	6/6
	Normal-OF	Normal	SC	5/6*	28 ± 5	29	0/5	0/5
	Normal-OF	OVX	SC	2/8	NT	42	0/2	0/2
	OVX-OF	Normal	SC	2/6	33	42	0/2	0/2
	OVX-OF	OVX	SC	0/8	NT	42	0	0
2	Sham	Normal	SC	0/8	—	>60	0	0
	Normal-OF	Normal	SC	5/8*	28 ± 5	49	1/5	0/5
	OVX-OF	Normal	SC	5/8*	30 ± 5	49	0/5	0/5
	OVX-OF	OVX	SC	0/6	—	49	0	0
	Normal-MF	Normal	SC	5/8*	36 ± 6	49	0/5	0/5
	OVX-MF	OVX	SC	0/5	—	49	0	0
3	Sham	Normal	SC	3/8	24 ± 8	>60	0/3	0/3
	Normal-MF	Normal	SC	9/10*	30 ± 4	45	0/9	0/9
	OVX-MF	OVX + placebo	SC	4/8	27 ± 7	45	0/4	0/4
	OVX-MF	OVX + estrogen	SC	5/5*	13 ± 7	45	0/5	0/5

[1]Fragments of ovarian fat (OF) or mammary fat (MF) from 12-week-old female normal or ovariectomized donors were transplanted into the subcutis (SC) or ovarian fat pad (OF) of 12-week-old mice. Where indicated, animals were ovariectomized (OVX) at 5 weeks. An incision was made on the dorsal side to expose the underside of the dermis. Pieces of adipose tissue were transplanted with forceps under the skin. Sham controls consisted of a s.c. incision and injection without fat fragments.–[2]SP1 cells (3×10^3 per mouse) were injected with a 27 g needle into the site of tissue transplant at the time of surgery.–[3]Significant increase in tumor takes compared with sham controls in each experiment is indicated by asterisk (Experiment 1, $p < .025$; Experiment 2, $p < .012$; Experiment 3, $p < .045$).–[4]Number of days required to reach 10-mm tumor diameter was calculated by linear regression analysis (Miller, 1985).–[5]Regional metastases in liver, spleen and diaphragm.

TABLE III – EFFECT OF OVARIECTOMY AND IMPLANTS OF 17-β-EXTRADIOL AND PROGESTERONE ON SP1 GROWTH IN THE MAMMARY GLAND AND ON METASTASIS FORMATION

Cell line	Cleared mammary fat pad[1]	OVX[2]	Implant[3]	Tumor takes	Time of 10-mm tumor diameter[4]	Lung metastases
SP1	–	–	Placebo	5/5[5]	27 ± 2	0/5
SP1	+	+	Placebo	4/9	32 ± 3	0/4
SP1	+	+	17-β-estradiol	9/10[5]	26 ± 3	3/9
SP1	+	+	Progesterone	2/9	30 ± 2	0/2
SP1	+	+	17-β-estradiol + progesterone	3/10	24 ± 2	0/3
SP1-3M	–	–	Placebo	6/8	23 ± 1	3/6
SP1-3M	+	+	Placebo	3/6	23 ± 3	2/3
SP1-3M	+	+	17-β-estradiol	7/7[5]	24 ± 2	3/7
SP1-3M	+	+	Progesterone	3/6	24 ± 5	1/3
SP1-3M	+	+	17-β-estradiol + progesterone	9/9[5]	25 ± 3	3/9

[1]SP1 or SP1-3M (a metastatic derivative) cells were injected (10^4 per mouse) into the mammary fat pad of 12-week-old animals. The mammary fat pad was cleared of normal ductal epithelial cells by cauterization of the mammary bud at 3 weeks.–[2]OVX, ovariectomy at 5 weeks.–[3]Implants were in the form of 60-day-release tablets containing 17-β-estradiol (0.72 mg/pellet) or progesterone (5 mg/pellet) or both.–[4]Calculated by linear regression analysis (Miller, 1985).–[5]Significant increase in tumor takes compared with ovariectomized placebo control (SP1, $p < .025$; SP1-3M, $p < .06$).

Adipose-tissue-dependent expression of receptors for estrogen, progesterone and epidermal growth factor on SP1 cells

Expression of type-I and type-II estrogen receptors (ER), progesterone receptors (PR) and epidermal-growth-factor receptors (EGFR) on human breast cancer have been used as prognostic indicators of estrogen responsiveness (Lippman and Dickson, 1990). We examined the expression of these receptors on SP1 cells *in vitro* and *in vivo* in different tissue sites. *In vitro,* SP1 cells growing on plastic with FCS expressed significant amounts of type-I ER, but not type-II ER (Table IV). *In vivo,* in s.c. tumors, the level of type-I ER was reduced to background within 20 days after injection. In contrast, type-I ER expression was maintained in SP1 tumors growing in the ovarian fat. During later stages (*i.e.,* >20 days) of tumor growth in ovarian fat, the level of type-II ER increased markedly, and was highest in tumors from mice with regional metastases. Low numbers of PR and EGFR compared with ER were present on SP1 cells growing in culture and on tumors in adipose-tissue sites, but were not detected on tumors in the subcutis (Fig. 3).

Increased expression of estrogen-receptor mRNA in SP1 tumors in adipose-tissue sites

The results in Table IV suggest that adipose tissue stimulates the expression of ER, PR and EGFR, as determined by ligand-binding assays. However, these findings do not exclude the possibility that reduced receptor expression in the s.c. tumors represents a non-specific suppression of metabolic activity. We therefore examined the level of expression of ER mRNA and an unrelated mRNA, β-actin. The results in Figure 4 showed that SP1 tumors growing in the mammary gland and ovarian fat pad expressed significant levels of a 6.5-Kb ER mRNA, whereas no ER mRNA was detected in the s.c. tumors. In contrast, β-actin mRNA (Fig. 4) and glyceroaldehyde transferase mRNA (data not shown) were detectable in all tumor samples examined; the variations in β-actin

TABLE IV – EXPRESSION OF ESTROGEN RECEPTORS ON SP1 TUMORS GROWING IN OVARIAN FAT AND SUBCUTIS

Experiment number	Injection			Number of tumors assayed	Tumor size[2]	Receptor sites per cell[3]		Regional metastases[4]
	Site[1]	Time *in vivo*	Number of cells			ER I	ER II	
1	—	—	—	—	—	7507[5]	<150	—
	SC	2 weeks	10^5	2	24×10^6	8122	<150	0/2
	SC	3 weeks	10^5	2	30×10^6	<150	<150	0/2
	OF	2 weeks	10^4	2	56×10^6	1788	<150	0/2
	OF	3 weeks	10^4	3	101×10^6	9555	3937	3/3
	MESF	2 weeks	10^4	2	16×10^6	2487	<150	0/2
	MESF	3 weeks	10^4	2	47×10^6	2700	<150	0/2
2	—	—	—	—	—	7430[5]	<150	—
	SC	2 weeks	10^5	4	9.7×10^6	250	<150	0/4
	SC	3 weeks	10^5	2	14×10^6	<150	<150	0/2
	SC	4 weeks	10^5	2	12×10^6	<150	<150	0/2
	OF	2 weeks	3×10^3	4	5.4×10^6	2329	866	0/4
	OF	3 weeks	3×10^3	2	17.4×10^6	3347	1942	0/2
	OF	4 weeks	3×10^3	2	18×10^6	3832	<150	0/2
	OF	4 weeks	3×10^3	1	40×10^6	1646	4827	1/1

[1]SC, subcutis; OF, ovarian fat; MESF, mesenteric fat.–[2]Primary tumors were excised at post-injection times shown, treated with collagenase and viable cells were counted. Mean cell number per tumor at the time of killing is shown.–[3]Receptors were determined by a ligand-binding assay, as described in the text, and results were expressed as the number of receptors per cell.–[4]Regional metastases to liver, spleen and diaphragm.–[5]These values represent the ER levels on SP1 cells in culture (with 7% FCS).

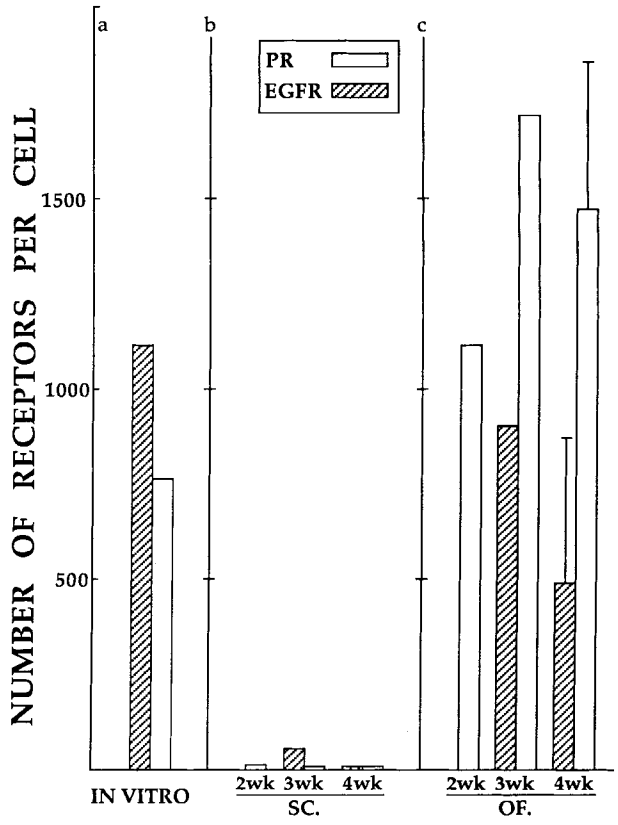

FIGURE 3 – Differential expression of receptors for progesterone and epidermal growth factor on SP1 tumors in the subcutis and in the ovarian fat pad. Tumors were excised at various times after injection of SP1 cells into the subcutis and ovarian fat pad. Tumor cells were dispersed with collagenase, and single cells were separated by Percoll gradient. PR and EGFR assays were carried out as described (Roos *et al.*, 1986; Horwitz *et al.*, 1981). An *in vitro* control consisted of SP1 cells grown on plastic with 7% FCS. Data at 2 weeks and 3 weeks are from pooled tumors; values at 4 weeks are from 3 individual tumors (standard error bars are shown).

mRNA observed did not correspond to changes in ER mRNA. These findings argue against a non-specific reduction of gene transcription in s.c. SP1 tumors.

DISCUSSION

The main conclusions from our study are that (*a*) adipose tissue can markedly influence the rate of growth and metastasis of the murine mammary carcinoma SP1, and (*b*) the mitogenic effect of adipose tissue on primary growth of SP1 cells is estrogen-dependent. The estrogen-dependence of SP1 growth was demonstrable only *in vivo,* consistent with a requirement for stromal cells. SP1 therefore appears to represent a hormone-dependent phase of tumor development, similar to early-stage human breast malignancies (Lippman and Dickson, 1990), and is a useful model for studying clinical aspects of tumor progression and metastasis.

Host-tissue interactions can markedly influence the metastatic phenotype of SP1 cells. In the s.c. site, the tumor grew as a benign lesion without metastasis. In the mammary, mesenteric and ovarian fat pads, SP1 tumors displayed faster rates of primary tumor growth and formed metastases. The pattern of metastatic spread clearly depended on the primary tumor site. Tumors from the mammary gland metastasized primarily to the lung; histological analysis revealed hematogenous spreading of tumor cells through capillary walls (data not shown). Tumors from the ovarian and mesenteric fat pads disseminated to regional visceral organs, including liver, spleen and diaphragm, implying local invasive spread from the primary tumor. The mechanisms of these 2 forms of metastases may be quite different. For hematogenous spread, cells must break down capillary basement membranes to pass from the primary tumor into the vasculature and into the target organ. For regional invasive spread, local production of growth factors by tumor cells or neighboring host mesenchyme (Chackal-Roy *et al.*, 1989) may be important. Mammary, mesenteric and ovarian tissue micro-environments may therefore promote different patterns of dissemination.

In the present study, we have developed an adipose-tissue transplantation assay to study stromal interactions that promote tumor growth *in vivo.* A sub-threshold dose of SP1 cells grew when co-transplanted into the subcutis with mammary or ovarian fat-pad tissue. The same cell inoculum yielded virtually no tumors in control animals transplanted with SP1 cells by the same surgical procedure but without fat-pad tissue. Histologi-

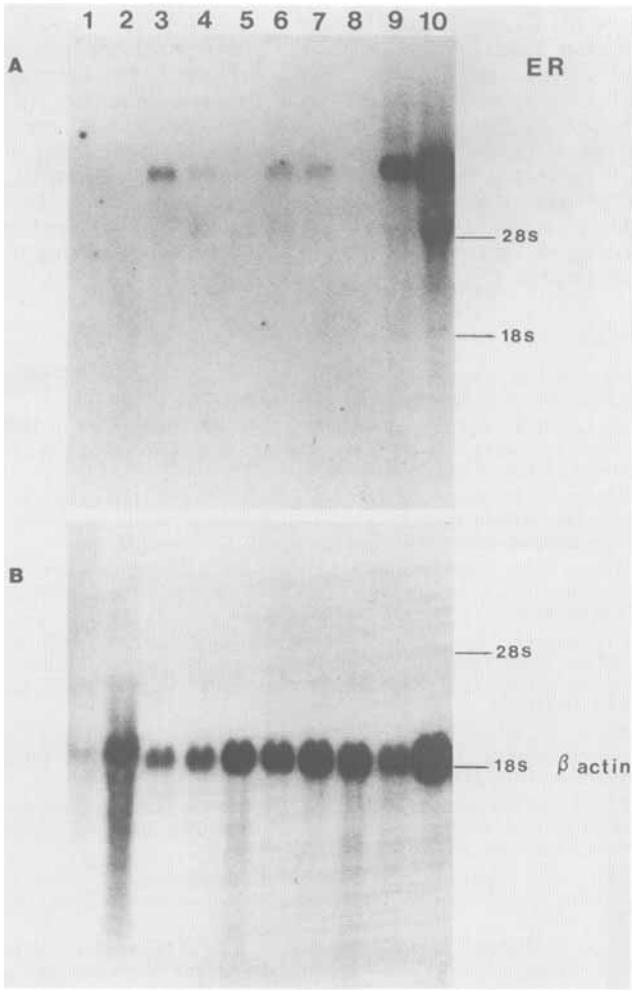

FIGURE 4 – Expression of estrogen-receptor mRNA in SP1 tumors *in vivo*. SP1 tumors from ovarian, mammary and subcutaneous sites were removed at 4 weeks. RNA was denatured and electrophoresed on a 1% agarose gel (20 μg/track). *(a)* Hybridization to the estrogen-receptor probe (Green and Chambon, 1990); *(b)* hybridization to a β-actin probe with the same blot (Ueyama *et al.*, 1984). Lanes 3 and 6, RNA from tumors in the ovarian fat pad; lanes 4 and 7, RNA from tumors in the mammary gland; lanes 5 and 8, RNA from tumors in the subcutis; lane 1, 2, and 9, RNA from kidney, spleen and uterus, respectively, of CBA female mice; lane 10, RNA from the human MCF-7 breast-cancer cell line after 48-hr culture with estrogen (50 ng per ml of medium). Tumor cells were purified from host tissue by Percoll-gradient centrifugation before extracting RNA (Elliott *et al.*, 1988).

cal analysis revealed close association of tumor cells and adipocytes; adipocytes were clearly distinguished by their large lipid vacuoles. These observations show that adipose tissue transplants can stimulate SP1 growth in the subcutis and that the transplantation procedure itself does not stimulate tumor growth. No metastases occurred from SP1 tumor cells co-transplanted with adipose tissue at the s.c. site; metastasis formation may therefore depend on additional stromal-cell components not present in the transplanted tissue. Experiments are in progress to examine the stromal-cell types (*e.g.*, adipocytes, fibroblasts and endothelial cells) that promote tumor growth and metastasis.

Our observation, that primary growth of SP1 and SP1-3M cells in adipose-tissue sites is abrogated by ovariectomy and is restored in 17-β-estradiol-supplemented animals, indicates that adipose-tissue-mediated mitogenesis is dependent on estrogen. The mechanism of hormone action on SP1 cells is not known. We have been unable to stimulate SP1 growth directly *in vitro* with 17-β-estradiol or the stable estrogen analogue, diethylstilbestrol, or with EGF (data not shown). Adipose stromal tissue may therefore be required for delivery of the estrogen signal to SP1 cells, or for release of growth substances from stromal tissue in response to hormone. Another possibility is that estrogen acts indirectly via prolactin (Ojeda *et al.*, 1980).

Several investigators have proposed that in normal mammary epithelial-cell development, delivery of the estrogen signal requires stromal cells. Estrogen may stimulate the local production of EGF/TGF-α (Snedeker *et al.*, 1991), or of EGFR (Stancel *et al.*, 1990); these changes may lead to the activation of the EGF/TGF-α pathway by an autocrine or paracrine mechanism. The estrogen-dependent growth of SP1 cells may also involve the EGF/TGF-α pathway. Stromal cells could stimulate production of EGF/TGF-α by SP1 cells; alternatively, SP1 cells may be stimulated by estrogen to release cytokines that stimulate surrounding stromal cells to secrete growth factors, such as TGF-α, that then act on the tumor cells to stimulate growth.

Interestingly, the epididymis fat pad and the kidney subcapsule can strongly support growth of SP1 cells. Both tissues display constitutively high levels of EGF precursor molecules (Pascall *et al.*, 1989), which could stimulate tumor growth independent of estrogen. It is also possible that certain adipose tissues in male mice can synthesize estradiol from androstenedione via aromatase enzymes (Perel *et al.*, 1980). Thus many mechanisms may contribute to the adipose-tissue-mediated enhancement of SP1 tumor growth.

The increase in ER, PR and EGFR levels on SP1 cells during growth in adipose-tissue sites is similar to the response of certain normal and malignant epithelial cells (Stancel *et al.*, 1990), and may be important in the stromal-cell interactions that promote growth of SP1 cells. A 6.5-Kb ER mRNA species corresponding to a mature ER molecule was present in significant abundance in intramammary and ovarian fat-pad tumors, but was only weakly present in tumors in the subcutis. Although variations in β-actin mRNA (Fig. 4) or glyceroaldehyde transferase mRNA (data not shown) occurred in the same RNA samples, these fluctuations did not correlate with ER mRNA levels. These results imply that induction of ER expression on SP1 cells during growth in adipose-tissue sites is regulated at least in part at the mRNA level. The possibility that altered hormone and growth-factor receptors can affect the growth potential of SP1 cells is currently being investigated.

Our finding that growth of SP1 cells was inhibited by progesterone is similar to the observation that early stage PR^+ human breast malignancies usually respond to the anti-proliferative effects of progesterone (Lippman and Dickson, 1990). Interestingly, growth of the metastatic SP1-3M cell line was not inhibited by progesterone. SP1-3M cells therefore may be similar to certain late-stage human breast malignancies which respond poorly to progesterone therapy. The mechanisms of action of progesterone on breast tumors and of reduced sensitivity to progesterone of certain late-stage tumors are not known. Murphy *et al.* (1991) have shown that endogenous TGF-α and EGFR mRNAs were consistently elevated in progesterone-resistant T-47D cells, as compared with the progesterone-sensitive parent cell line; whereas both cell types expressed ER and PR. These findings suggest that progesterone resistance is a multifactorial process, possibly involving autocrine growth stimulation.

In summary, we have shown that adipose tissue can augment the growth of SP1 cells and that this effect is modulated by steroid sex hormones. Our findings are highly relevant to the understanding of the role of tissue micro-environment in

promoting tumor growth and metastasis. In addition to being an important component of glandular tissue, adipose tissue is also present in bone marrow, one of the first sites for breast- and prostate-cancer recurrence. Adipose tissue may therefore provide the appropriate stimuli for growth of malignant tissue in certain target organs.

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