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A histological study of the Shionogi adenocarcinoma 115 grown in male and female mice

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Summary. We have previously demonstrated that growth rate and morphology differ between androgen-responsive Shionogi mouse mammary tumours maintained in male and female mice. Furthermore, we can modulate the growth rate of these tumours in male mice by exposing the mice to psychosocial stressors. In the present study, we were interested in determining if tumours in male mice with a comparable growth rate to that in females, also had a morphology similar to that in females. SC115 tumours were examined using histochemical and immunohistochemical techniques. Tumours in male mice were easily distinguishable from tumours in female mice regardless of growth rate. Tumours maintained in female mice contained osteoid-like regions which stained positive for sialic acid and sulphate moieties. No such regions were observed in any of the tumours from male mice. In addition, although all tumours contained MSA (muscle specific actin) -positive and S100 protein-positive cells, these regions were more extensive in the tumours of female mice. This study suggests that tumour growth rate and morphology are independently regulated by the host environment.

Key words: Shionogi SC115 tumour, S-100 protein, Muscle specific actin, Carbohydrate histochemistry

Introduction

Breast cancers are composed of a heterogeneous population of cells including hormone-responsive and hormone-independent cells. This heterogeneity results in metabolic and functional variability within a tumour and subsequent variation in response to treatments such as chemotherapy and hormone therapy (Emerman and Semiatowskli, 1984). A clearer understanding of this heterogeneity may facilitate the development of improved treatment regimens for breast cancer patients.

The mouse mammary tumour Shionogi adenocarcinoma 115 (SC115) is an example of a tumour that is heterogeneous in its response to hormones (Emerman and Semiatkowski, 1984). This tumour arose spontaneously in a female mouse of the DD/S strain. Following 19 generations of transplantation, an androgen-responsive subline arose that grew more rapidly in males than in females (Bruchovsky and Rennie, 1978). We have previously shown that following the injection of SC115 tumour cells (2×10^6) into intact male mice raised under our standard laboratory conditions, (i.e. animals housed in sibling groups of 3), a palpable tumour arises in 8-10 days and grows to a mass of 2-3 grams in approximately 3 weeks (Emerman and Semiatkowski, 1984). In females and castrated males, tumour growth is much slower: a palpable tumour is observed after about a month and at 2 months, has only achieved a weight of 1 gram (Emerman and Semiatkowski, 1984).

Tumours growing in intact male and female mice (maintained in sibling groups of 3) have been shown to exhibit significant phenotypic differences (Emerman and Worth, 1984). Androgen-responsive tumours grown in intact male mice have a sheet-like growth pattern. The tumours are highly vascularized, but there are considerable areas of necrosis. Androgen-independent tumours grown in female and castrated male mice lose this cohesive growth pattern. The cells are dispersed into loose sheets and irregular cords growing in loose stroma.

We have developed a highly replicable model in which the growth rate of the SC115 tumour in intact males can be altered by psychosocial stressors (Weinberg and Emerman, 1989). These stressors consist of differential housing conditions and exposure to novel environments. Being reared individually and remaining individually housed or being reared in a social group and then singly housed following tumour cell injection markedly increased tumour growth compared to that of mice remaining in their standard sibling rearing groups. In contrast, being raised individually and then moved to a

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large social group following tumour cell injection markedly reduced tumour growth.

The tumour growth pattern observed in male mice moved from the individual to the group condition was of particular interest as it approximated the growth of the SC115 tumour maintained in female mice (Weinberg and Emerman, 1989). Considering the dramatic morphological differences between tumours grown in intact male and female mice under our standard housing conditions, the purpose of the present study was to investigate whether the slow-growing tumours seen in male mice moved from individual to group housing would display histological characteristics similar to those seen in the androgen-independent tumours grown in female mice.

Materials and methods

Animals

Male and female mice of the DD/S strain, 2 to 4 months of age, were used in this study. In accordance with our model, fourteen males were used to form 4 experimental housing groups. At the time of weaning, males were housed either individually or in sibling groups of 3, and on the day of tumour cell injection, were assigned to 1 of the 4 experimental groups:

1) mice raised individually housed remained individually housed (II, n = 3).

2) mice raised individually housed were placed in non-sibling group of 5 (IG, n = 5).

3) mice raised in a sibling group of 3 remained in their rearing groups (GG, n = 3).

4) mice raised in a sibling group of 3 were separated and housed individually (GI, n = 3).

Mice in all of these groups were also exposed to an acute stressor, consisting of exposure to 1 of 5 different novel environments for 15 min/day, 5 days/week as previously described (Weinberg and Emerman, 1989).

Two additional groups of animals were included for comparison: 1) Males and females raised in the standard sibling rearing groups were maintained in their groups following tumour cell injection but were not subjected to acute daily novelty stress. 2) Female mice raised and maintained in their sibling rearing group were used for histochemical examination of normal mammary gland.

Tumours

The androgen-responsive SC115 mammary carcinoma subline designated Class I (Bruchovsky and Rennie, 1978) was maintained by serial transplantation in intact male mice of the DD/S strain. Tumours weighing approximately 2 g were dissociated to single cells according to our standard protocol (Emerman and Semiatkowski, 1984) and 3 x 10^6 cells/mouse were injected into the interscapular region. Mice in all groups were palpated twice weekly; once tumours were palpable (6-8 days), caliper measurements were taken twice weekly. Tumour weights were calculated according to the formula (Simpson-Herren and Lloyd, 1970):

$$\frac{\text{length (cm)} \times [\text{width (cm)}]^2}{2} = \text{grams}$$

Three weeks following tumour cell injection, males were terminated by decapitation and tumours excised. Females were terminated when the tumours reached a weight of 1.5 g. At that time, normal mammary glands were removed from non tumour-bearing female mice for histochemical analysis.

Fixation and Staining

Tissue blocks approximately 0.125 cm^3 were cut from the center and periphery of the tumours and from normal mammary gland and placed immediately into 10% formalin (4% formaldehyde) containing 2% (w/v) calcium acetate. Blocks were routinely paraffin processed after one week of fixation. Serial sections cut at a thickness of 5 μm were stained with the following techniques:

I) Carbohydrate histochemistry

a) Selective periodate oxidation - Schiff (PA*/S). This permits the specific demonstration of sialic acids without side chain 0-acyl substituents or with an 0-acyl substituent at position C_7 (Volz et al., 1987).

b) Saponification selective periodate oxidation - Schiff (KOH/PA*/S). This permits the specific demonstration of all sialic acids (Volz et al., 1987).

c) Saponification selective periodate oxidation - Alcian blue pH 1.0-Schiff (KOH/PA*/AB1.O/S). With this stain, all sialic acids stain magenta, O-sulphate esters stain aquamarine blue; mixtures stain in various shades of purple (Reid et al., 1987).

d) KOH/AB2.5/PAS. This is the standard Alcian Blue pH 2.5 Periodic Acid Schiff of Mowry (Mowry, 1963) preceded by a saponification step to remove any O-acyl esters blocking vicinal diols. This stain serves as a control, demonstrating the presence of carboxyl groups and O-sulphate esters in aquamarine blue and all sugars containing vicinal diols magenta; mixtures stain in various shades of purple (Mowry, 1963).

e) Allochrome (Lillie, 1954). This provides a rapid visualization of the connective tissue present in the tumour.

f) Haematoxylin and Eosin, H & E (Culling, 1974). This is used for standard morphological analysis.

II) Immunohistochemistry

a) Representative sections from tumours of animals in each condition were stained with muscle specific actin (MSA) and visualized by the immunoperoxidase technique (Papotti et al., 1988) to determine if cells had characteristics of myoepithelial cells. Muscle tissue

served as a positive control.

b) Representative sections from tumours of animals in each condition were stained with S-100 protein antiserum and visualized by the immunoperoxidase technique (Dwarakanath et al., 1987). S-100 has been shown to be associated with myoepithelial cells. Peripheral nerves, which also stain with S-100, served as a positive control.

Results

H & E stained sections of the tumours grown in females and males maintained under our standard laboratory conditions conformed to previous morphological descriptions of these tumours (Fig. 1). The tumours from males had a cohesive epithelial-like growth pattern, high degree of vascularization and large areas of necrosis. The tumours from female mice contained cells dispersed into loose sheets and irregular strands growing in loose connective tissue. The morphology of tumours from the male mice exposed to the 4 experimental housing conditions were the same as that of the males in the standard conditions. Lillie's allochrome stain confirmed that the significant amounts of loose stromal connective tissue present throughout the female tumours were not present in any of the male tumours.

The tumours from female mice contained regions of extra-cellular material which appeared osteoid-like in H&E stained sections. As it has been shown that osteoid of cortical bone contains chondroitin sulphate and sialic acid-rich glycoproteins (Herring and Kent, 1963; Andrews et al., 1969), all male and female tumours were investigated histochemically for the presence of these moieties.

The development of osteoid-like regions in female mice could be seen to proceed in several defined steps. First a cluster of cells lost their attachments with neighbouring cells (Fig. 2A). The cell clusters then began to secrete an extracellular product, (Fig. 2B), spreading away from each other as they did so until they achieved an osteoid-like appearance (Fig. 2C). When these extracellular regions were investigated histochemically (Table 1), they stained moderately positive with the PA* S stain, indicating the presence of sialic acids. There was a moderate increase in the staining intensity of these extracellular regions when the KOH/PA*S technique was used indicating the presence of substituted sialic

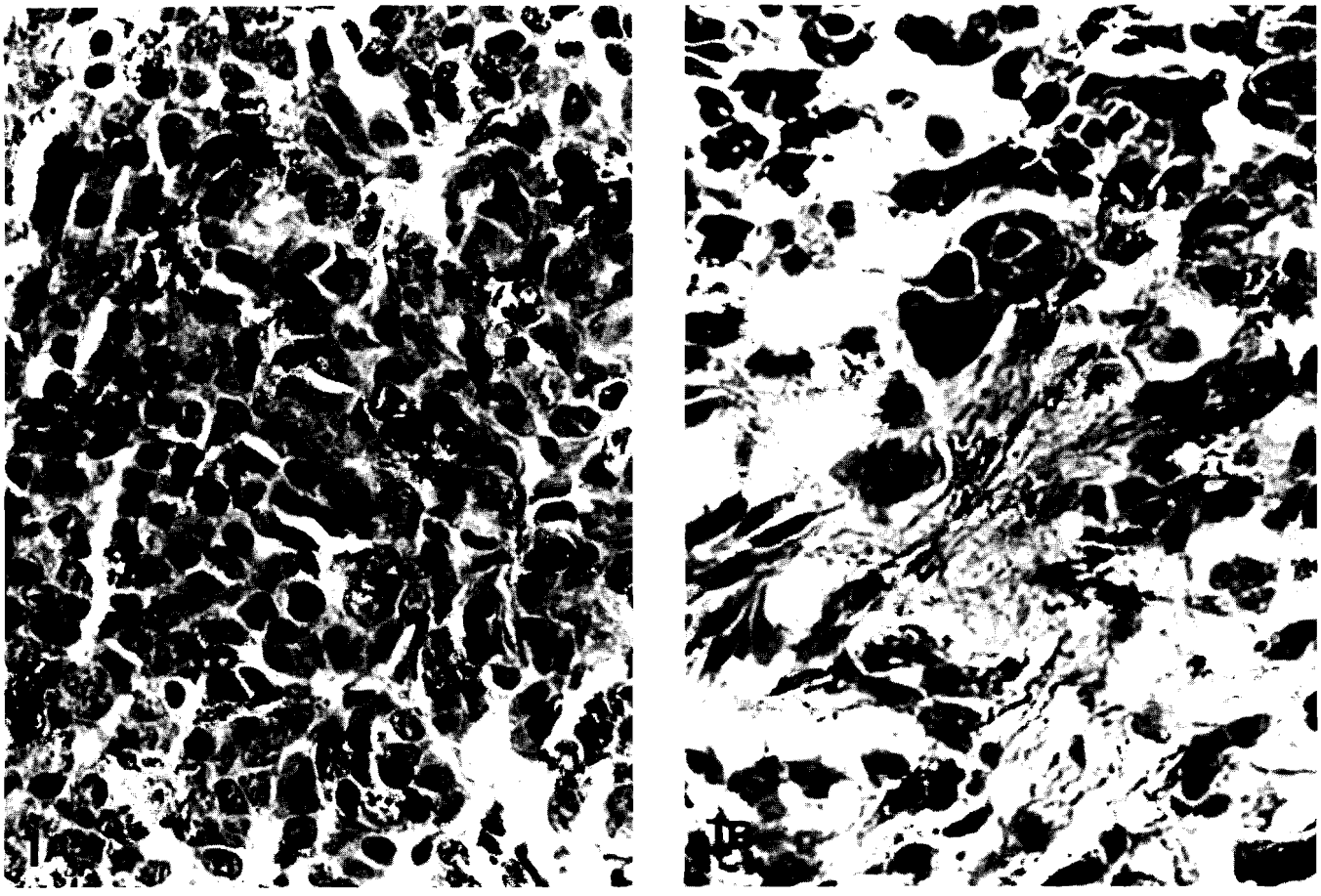

Fig. 1 The morphology of SC115 tumours grown in male **(A)** and female **(B)** mice raised under standard colony conditions conformed to previous descriptions. Tumours from males exhibited a cohesive epithelial-like growth pattern. Tumours from female mice contained cells dispersed into loose sheets and irregular strands growing in loose connective tissue. × 812.8

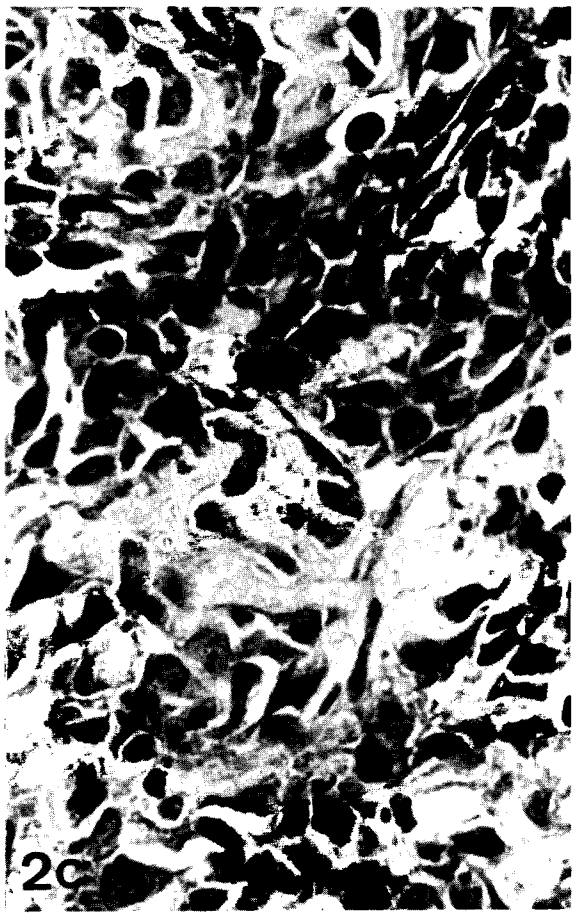

Fig. 2. The development of osteoid-like regions of female mice proceeded in several defined steps. **A.** First, a group of cells (arrowheads) lost their attachments with the surrounding cells. **B.** The cell clusters then began to secrete an extracellular product (arrowheads). **C.** Secretion continued until an osteoid-like appearance was achieved. × 812.8

acids. Further, when stained with the KOH/PA*/AB1.0/S technique these regions stained purple, indicating the presence of sulphated moieties as well as sialic acid moieties in the osteoid-like regions. No such osteoid-like regions were observed in any of the tumours grown in male mice with any of the stains used.

It has been suggested that breast cancer develops from undifferentiated cells which have the ability to become either epithelial cells or myoepithelial cells, depending upon their endocrine environment (Hayashi et al., 1984). It is possible that the different hormonal milieu of the male and female mice induced different phenotypes in the breast tumour cells. If so, it was of interest to determine if the slow growing tumours of the IG male mice had a phenotype similar to that of female mice. To investigate this possibility, tumours were stained with the myoepithelial cell specific markers S-100 and MSA to display myoepithelial cells present in the tumours.

All tumours stained for MSA to some degree. Tumours from both males and females contained small clusters of strongly positive cells within the regions of viable cells. In addition, in tumours taken from female mice, a layer of MSA positive cells lined the osteoid-like regions (Fig. 3A).

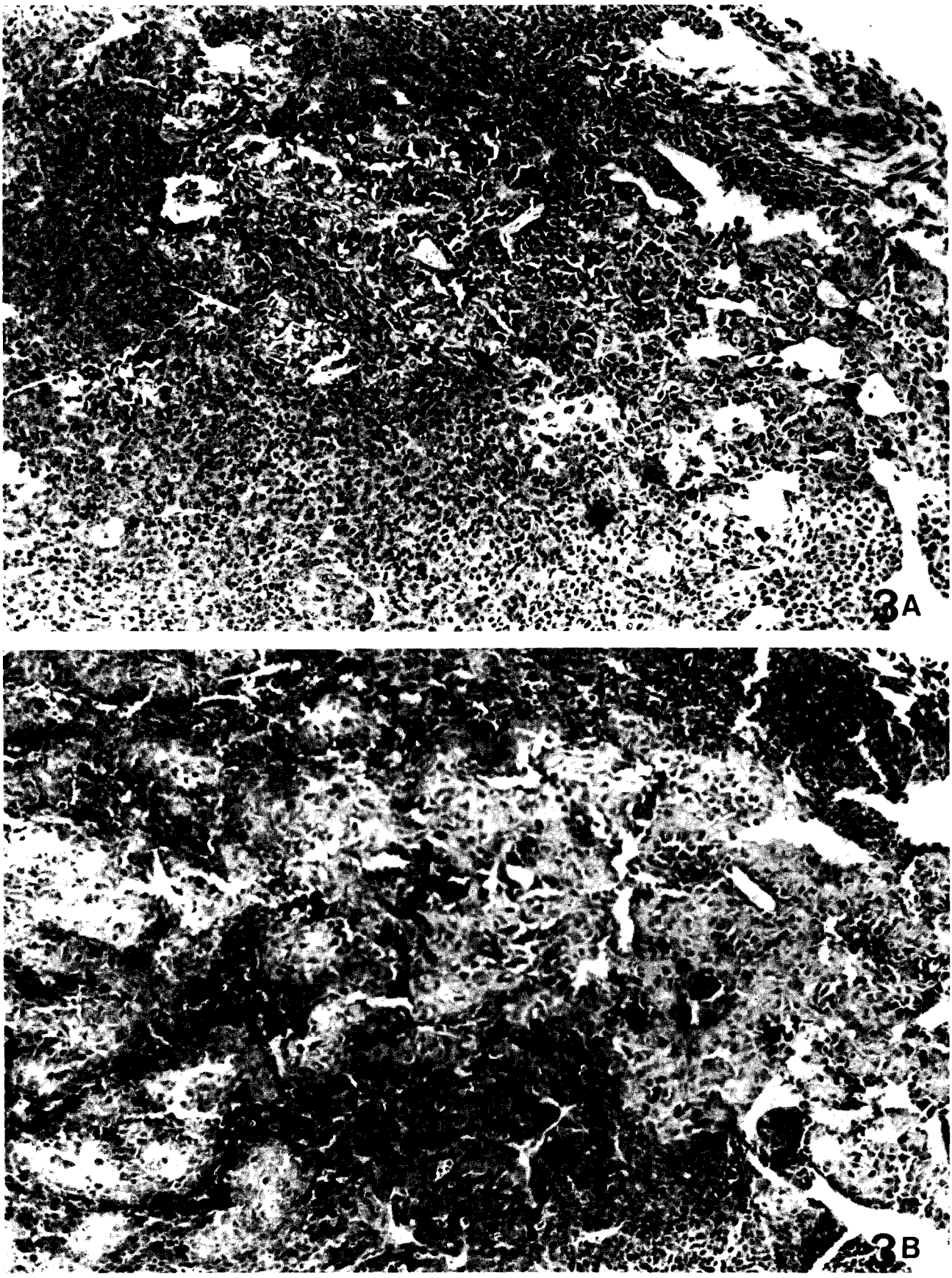

Fig. 3. Comparison of MSA **(A)** and S-100 **(B)** staining of serial sections from osteoid-like regions of a tumour grown in a female mouse. The osteoid-like regions (arrows) are surrounded by MSA positive cells. In contrast, cells positive for S-100 (arrows) are found in the areas outside the MSA positive regions. × 203.2

The S-100 staining was less extensive than the MSA staining in tumours from male mice. In contrast, the tumours grown in female mice had large regions of viable cells which stained intensely S-100 positive. S-100 stained the cells in areas surrounding the osteoid-like regions, but did not stain the MSA positive cells immediately adjacent to the osteoid-like regions (Fig. 3B). Further, in both males and females there appeared to be little overlap in the areas that were stained by these two techniques.

Table 1. Histochemical staining of the osteoid-like pools in the female mice.

Stain	Female Osteoid Pools
PA*S	++
KOH/PA*/S	++
KOH/PA*/AB1.0/S	P,M,A
KOH/PA*/AB2.5/S	P,M,A
PAS	+++
AB1.0	+
AB2.5	++
Lilles Allochrome	P,B

+, weakly positive; ++, moderately positive; +++, strongly positive. P, purple; M, magenta; A, aquamarine blue. B, blue.

Discussion

We and others have shown that the SC115 mammary carcinoma consists of a heterogeneous population of androgen-responsive and androgen-independent cells (Bruchovsky and Rennie, 1978; Emerman and Semiatkowski, 1984). The predominant subpopulation of the SC115 tumour grown in intact male mice is androgen-responsive, whereas an androgen-independent subpopulation is selected for when SC115 cells are grown in female or castrated male mice. These two subpopulations of cells have different growth rates and morphologies (Emerman and Semiatkowski, 1984; Emerman and Worth, 1984). Based on our previous data (Weinberg and Emerman, 1989) indicating that tumours grown in male mice moved from individual to group housing conditions (IG) and tumours grown in female mice have similar slow growth rates, we hypothesized that in the present study, tumours grown in IG males might also share other characteristics of the predominantly androgen-independent subpopulation of cells.

This study confirmed our previous work (Emerman and Worth, 1984) showing morphological differences between tumours grown in female and intact male mice housed under our standard conditions. Interestingly, however, tumours grown in IG males had a morphology similar to tumours of the male controls.

The presence of osteoid-like extra-cellular material was observed in tumours grown in female mice but not in male mice. It is notable that the slow growing tumours in IG males were again similar to other male tumours rather than to the female tumours.

The osteoid-like regions of the female tumour contained sulphate and sialic acid moieties similar to the osteoid of cortical bone (Herring and Kent, 1963; Andrews et al., 1969). Although breast carcinomas are not reported to undergo differentiation to bone, calcification is a well documented occurrence in human breast tumours (Hatter et al., 1969; Sickles, 1980; Bouropoulou et al., 1984; Frappart et al., 1986). Such regions of calcification have been reported to contain sialic acid moieties (Bouropoulou et al., 1984). The production of extra-cellular material by the SC115 tumour grown in female mice may be similar to the production of regions of calcification reported in human breast cancers.

S-100 and MSA immunohistochemical staining patterns differed markedly from each other in both male and female tumours. Myoepithelial cells, as demonstrated by the MSA immunohistochemical stain, were found in small isolated clusters in all tumours investigated. These clusters were usually located in the center of sheets or cords of viable cells. Importantly, MSA staining was most prominent in female tumours and it was associated with the osteoid-like regions.

It has been suggested that the differentiation of breast tumour stem cells into epithelial or myoepithelial cells is controlled by local environmental conditions (Hayashi et al., 1984). It appears that the SC115 tumour is capable of myeopithelial cell differentiation and that this differentiation is promoted by the hormonal environment of the female. Furthermore, in tumours from female mice, myoepithelial cells appeared to be linked to the production of the osteoid-like extracellular material.

In contrast, the S-100 staining pattern in males was less extensive than that seen with the MSA technique. In tumours of females the S-100 staining was at least as extensive as seen with the MSA stain. In both males and females there was no overlap between the regions stained by the two techniques. The large regions of S-100 positive cells in tumours from female mice are of particular interest. It has been shown that the S-100 protein belongs to a family of structurally related proteins which share a high degree of sequence homology and exhibit extensive cross-reactivity immunologically (Kligman and Hilt, 1988). The S-100 family are calcium binding proteins which probably act as second messengers, similar to calmodulin (Kligman and Hilt, 1988), and may be involved in the promotion of cell division and the calcium induced depolymerization of microtubules. One S-100 protein, p9ka, has been isolated from rat myoepithelial cells (Barraclough et al., 1987). The lack of correlation between MSA and S-100 staining of tumours from female mice suggests that the S-100 protein being recognized is not the myoepithelial cell associated p9ka, but rather may be an S-100 protein involved in regulation of cell division or microtubule formation. This view is supported by studies of primary human breast cancer which find that both epithelial and myoepithelial cells stain positive with the S-100 technique (Dwarakanath et al., 1987; Stroup and Pinkus, 1988).

In this study, we have demonstrated that although tumours grown in male mice moved from the individual to group housing are similar to tumours grown in female mice with regard to their growth rate, they clearly do not resemble tumours grown in female mice in their histology. Rather, slow growing tumours of IG males are histologically similar to the fast growing tumours of control males. These results suggest that the growth of tumours in IG mice may be affected by selective pressures operating on the cells to produce a population of cells that is different from those grown in male and female mice in standard conditions but shares properties of both. There are several ways this could occur. Firstly, it is possible that there has been selection for a cell type that shares characteristics of androgen-responsive cells, but has the slow growth rate characteristic of androgen-independent cells. Secondly, the tumours grown in IG mice may be composed of androgen-responsive cells whose growth rate has been modulated by unknown factors present in their environment. Finally, the SC115 tumour may be composed of a single cell population whose phenotype is modulated by the environment. In this case, we see a gradation of changes from the androgen-responsive to the androgen-independent state with the cells in the tumours of the IG mice being in transition between the two states. Thus, in tumours of IG mice, it is possible that only some of the altered environmental factors responsible for the production of androgen-independent tumours are present. An understanding of the mechanism regulating the heterogeneity seen in the SC115 tumour may be important in light of findings that the androgen-responsive and androgen-independent SC115 subpopulations have different sensitivities to chemotherapy (Emerman and Semiatkowski, 1984).

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