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Effects of tamoxifen and CV 205502 on the morphology and the evolution of the noncancerous mouse mammary gland

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Summary. Tamoxifen (TAM, 0.01 mg/animal, three times a week) and the experimental prolactin-lowering CV 205502 (CV, 1 μg/animal, daily) were administered prophylactically, alone or combined, to virgin C3H/Sy mice during the early period of promotion in this spontaneous mammary carcinogenesis system (end of 2nd-5th month of age), in order to study their influence on the morphology and evolution of the non-cancerous mammary gland during therapy and after treatment cessation. During TAM administration the epithelial cells of the growing part of the gland exhibited myoepithelial- and, late in the treatment period, apoptotic-like features instead of the secretory ones expected, accompanied by intense basement membrane alterations, thickening of the surrounding connective tissue and arrested adipocyte maturation. These effects reversed progressively after drug withdrawal. The epithelial alterations were more intense and longer lasting in the TAM+CV-group, while growth arrest of the glands was observed in both groups parallel to the degree and the duration of these morphological changes. In these groups, tumor incidence was diminished, as expected, but the tumors that developed late after treatment cessation were of low histological differentiation. The above morphological observations show that TAM inhibits noncancerous mammary gland growth during the reproductive period by altering stromal and epithelial differentiation, effects that reverse progressively after treatment discontinuation and are potentiated by a prolactin-lowering agent in this animal study.

Key words: Tamoxifen, CV 205502, Mammary gland, Histology, Ultrastructure, C3H mice

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Introduction

Regarding estrogens (Lippman and Dickson, 1991) or the estrogen-dependent action of other hormones (Topper et al., 1986; King, 1990) as the main promoters in mammary carcinogenesis, attempts to prevent tumor development have been focused on eliminating the action of these steroids on cancerous or potentially cancerous cells. Such an agent, the nonsteroidal antiestrogen tamoxifen (TAM), has been extensively studied in experimental models for its chemosuppressive and chemopreventive action (Jordan, 1976; Welsch et al., 1981; Gottardis and Jordan, 1987), and has been widely used in the adjuvant therapy of breast cancer to prevent tumor recurrence and the appearance of second primary breast cancers (Cuzik and Baum, 1985; Fisher et al., 1989); the same drug is currently being tested in human breast cancer prevention trials (Powles et al., 1990; Fisher, 1992), where it is administered to clinically healthy women with high risk of developing the disease, although information is lacking about its action on noncancerous tissues and about its influence on the after-treatment period (since a chemopreventive treatment in humans should have an established duration [Fentiman, 1990]).

The present study was undertaken in order to gain knowledge about the effects of this drug on the morphology and the evolution of the noncancerous mammary gland after prophylactic administration, and was performed in a model of spontaneous mammary carcinogenesis, the C3H mice, during the early period of promotion. In this model, which resembles the human disease perhaps only in familial breast cancer (in tumor incidence and timing, at least), the main promoters are established to be the ovarian steroids and the estrogen-dependent action of prolactin (Welsch and Nagasawa, 1977). The role of prolactin remains unclear in human breast cancer; nevertheless, even in rodent systems it appears to be mainly an early promoter, which could be the reason for the difficulty in elucidating its interference

with human carcinogenesis, where the timing of the early period of promotion in previously healthy women cannot be defined. In order to block more completely the main promoters action, TAM effects were evaluated in comparison with the action of CV 205502 (CV), a potent experimental prolactin-lowering substance.

Materials and methods

Animals

A total of 210 female virgin C3H/Sy inbred mice (from the Symeonideion Research Center, Thessaloniki, Greece), were used. The C3H/Sy-mice bear the MMTV and develop mammary tumors in approx. 75% of the cases until the 12th month of age, and in 96% until the 16th month. All animals, 2 months old at the beginning of the experiment, were housed separately in a temperature- (22-25 °C) and light-controlled (12 h/day) room and given the usual diet for laboratory mice, and water ad libitum.

Materials

Tamoxifen (TAM) citrate (Sigma Chem.) was dissolved in a vehicle of sesame oil and ethanol (9.5:0.5). CV 205502 (CV): the experimental 8-hydro-benzo-(g)-quinoline (Sandoz, Basel, Switzerland.) with the apomorphine-related structure and the highly selective dopaminomimetic (on the D2-receptors) action (Nordmann and Petcher, 1985) was dissolved in a vehicle of n.s., 96 ° ethanol and DMSO (9:0.5:0.5).

Methods

Treatments

The animals were divided into four groups: (A): 55 animals received no therapy and were used as controls; (B): 55 animals were treated with TAM alone; (C): 50 animals with CV alone; and (D): 50 animals with TAM+CV. Both drugs were administered i.p., TAM three times a week in a dose of 0.01 mg/animal (approx. 0.4 mg/kgr) and CV daily, in a dose of 1 µg/animal (approx. 0.04 mg/kgr). Drug administration began at the end of the 2nd month (9-10th week) and continued until the end of the 5th month of age. Treatment duration was established as the shortest time-period required to reveal TAM effects on the mammary gland morphology, while timing of therapy was adjusted to cover the early stage of promotion in this model of spontaneous mammary carcinogenesis.

Experimental course

The animals from each group were sacrificed as follows: I) by the end of the 3rd, 4th, 5th, 6th, and 7th month of age (n=3-5 animals/group/date), in order to observe the influence of the treatments on the morphology of the mammary glands during, and shortly after drug administration; and II) by the end of the 10th, 12th and 14th month (n=5-12), to demonstrate tumor incidence in the different groups and to examine the morphology of the nontumorous mammary glands at these ages. Cancer-bearing animals were sacrificed one month after the tumors could originally be palpable and included in the subgroups mentioned in (II).

Specimen collection and preparation

Mammary fat pads were excised and stained with methylene blue, in order to obtain material from the mostly rich epithelial regions for electron miroscopy (EM) under stereoscopic guidance, which in each case was dissected from the 2nd and 3rd thoracic glands unilaterally. The remaining mammary tissue and the thoracic glands of the contralateral side were fixed together in an alcohol-formalin solution for light microscopy (LM). Specimens for LM were embedded in paraffin as usual and stained with HE, and in some cases with toluidine blue (to demonstrate mast cell condition). For EM, specimens were treated in 1% glutaraldehyde and osmium tetroxide, dehydrated with ethyl alcohol series, embedded in Epon, and finally stained with uranyl acetate and lead citrate. Only structures characterized as terminal end buds (TEB) or small ducts (SD) on semithin sections were further processed for EM. The transmission electron microscope used was a JEOL 100 CX.

Estimation of growth of the epithelial component of the mammary glands (by LM)

Serial sections from the mammary glands were examined for their content in SD and TEB. TEB are the main growing epithelial component of the gland, relatively undifferentiated, wherefrom the new ductules and hyperplastic alveolar nodules (HAN) evolute, while SD represent the next differentiated structures in the gland's physical history (Sekhri et al., 1967). Since strict morphological criteria for the distinction between TEB and the newly-formed ductules are lacking under light microscopy, both structures have been co-interpreted as TEB, while HAN were not included.

Morphological studies were performed in a blind fashion by four of us (V.K., G.K., L.E. and A.S.).

Statistics

Evaluation of the antitumor activity of TAM and CV was performed by using the chi-square test separately for each age period described in «experimental course» in this section, in order to avoid misleading results when estimating tumor incidence as a total phenomenon for each group.

Results

Treatment influence on the morphology of the mammary gland

Controls

The evolution of the TEB/SD ratio is shown in Fig. 1. There was an almost constrant (with a transient plateau during the 6th month) growth of the epithelial component of the gland until the 10th month, whereafter it regressed in the animals which developed no tumors.

There are several references about the development, histology and ultrastructure of the normal mammary gland of small rodents (Sekhri et al., 1967; Topper and Freeman, 1980; Dulbecco et al., 1982), as well as of the C3H mice in particular (Wellings et al., 1960; Pitelka et al., 1980a,b), and our findings were similar to these data (Figs. 2a, 3a, 3b). Cells in the growing part of the gland were cuboidal, of the secretory type, with much rough endoplasmic reticulum and densely packed free ribosomes (especially in the TEBs, where the nuclei comprised one or multiple nucleoli), plus typical secretory vesicles and surface microvilli (cells lining the SDs, where mouse mammary tumor virus-(MMTV-) particles were also observed, their number increasing with age); the secretory features became scarcer and more lipid bodies and intercellular microvilli were found in the cells lining the larger ducts (quiescent cells, after Yasui and Dethlefsen [1987]). Parts of myoepithelial

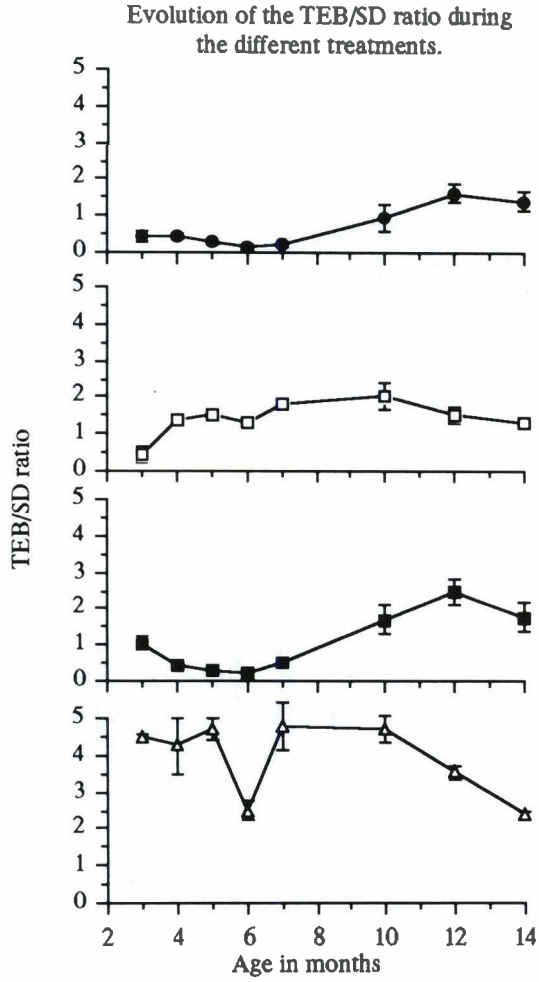

Fig. 1. Diagrammatic presentiation of the evolution of the TEB/SD ratio, which represents the growth rate of the epithelial component of the mammary gland, as observed during the different treatments. Although the particular points on the Y-axis are approximate (because of the semi-morphometric estimation), the trends are obvious.
Δ = controls, ■ = TAM, □ = CV, ● = TAM + CV.

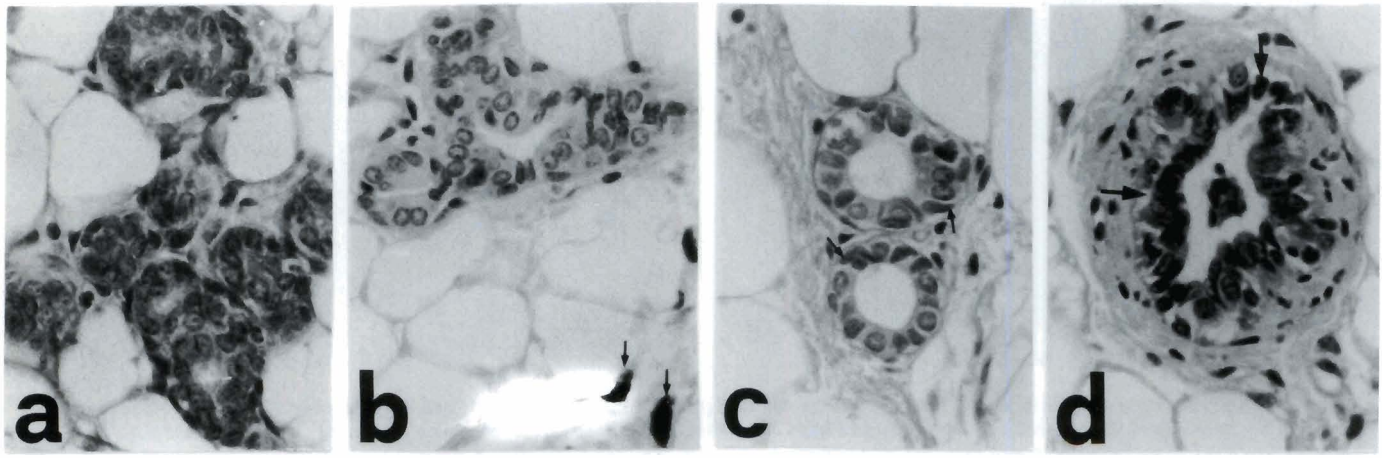

Fig. 2. Epithelial cell characteristics in the different groups (LM): **a)** Control, 3 months old, TEBs and newly-formed ductules: cells with little, delicate, often inconspicuous cytoplasm, without orientation. **b)** TAM, 3 months old, newly-formed ductules: abundant, dense eosinophilic cytoplasm. Arrows = mast cells, **c)** CV, 3 months old, small ductules with myoepithelial cells (arrows) and glycocalyceal borders on the luminal surface. **d)** TAM + CV, 4 months old: small duct with evidence of degeneration in the lumen. The clear rim recognized between epithelial cells and surrounding increased connective tissue (arrows) probably corresponds to the findings of Fig. 3f. a, b, c, d. H&E x 400

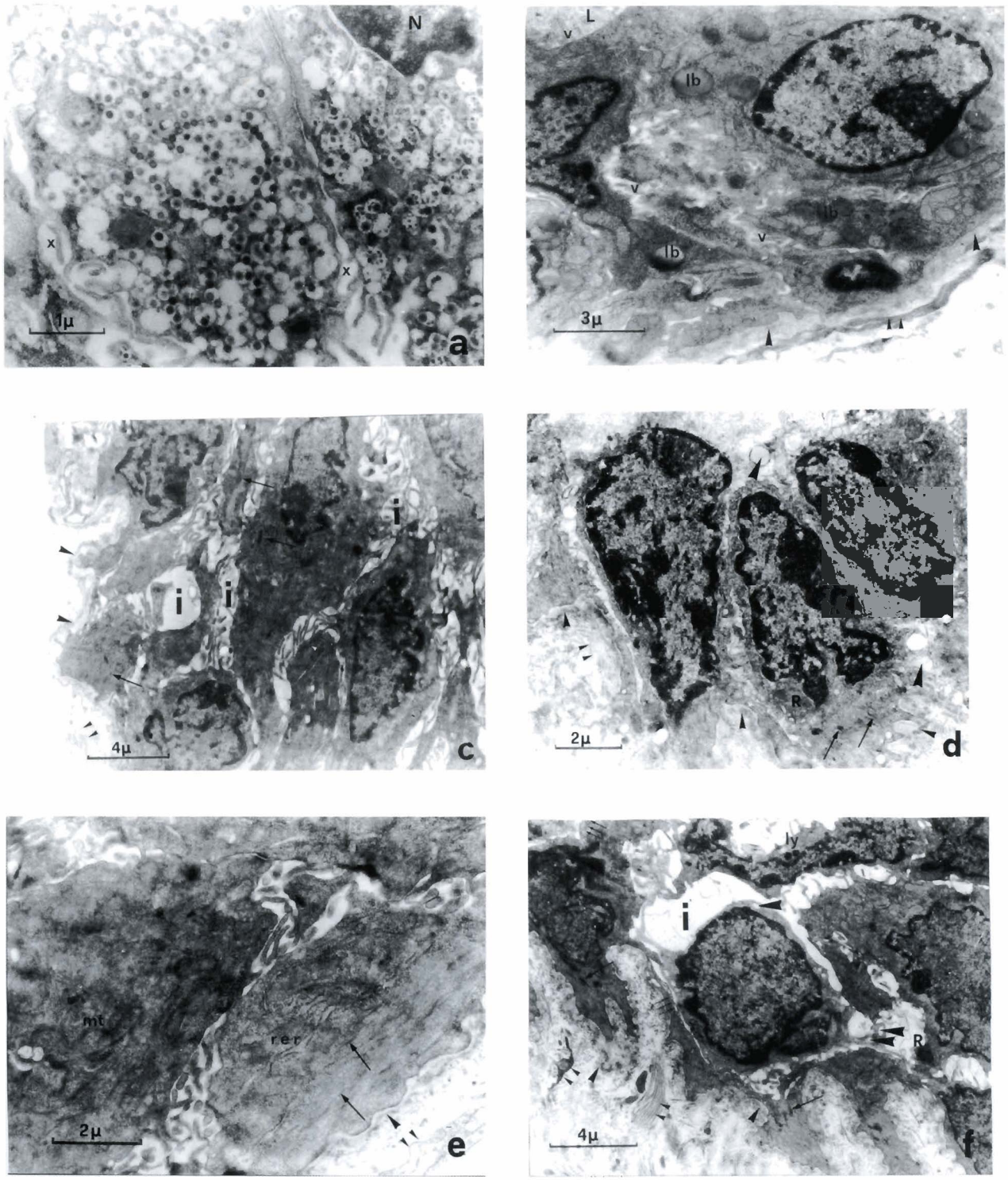

Fig. 3. Ultrastructural characteristics of the epithelial cells in the different groups. **a)** Control, 5 months old: hypersecretory cells seen in a newly-formed ductule, already differentiated as HAN-cells with abundant MMTV-particles. N= nucleus, x = intercellular spaces. **b)** Control, 4 months old: part of a small ductule lined by secretory epithelial cells. lb= lipid bodies, v= microvilli, L= lumen. **c)** TAM, 4 months old: cells in the periphery of a small duct without secretory features. **d)** TAM, 6 months old: degenerating myoepithelial-like cells (shrunken cytoplasm, clamped chromatin, **e)** CV, 4 months old: myoepithelial hyperplasia. rer= dilated cisternae of rough endoplasmic reticulum, mt= microtubules. **f)** TAM + CV, 7 months old: cells detached from their neighbouring ones, preceding cell-death. m= convoluted mitochondrion, triple arrows= pinocytotic vesicles, observed in the preserved myoepithelial cells. Symbols used in 3a-f: arrows= tonofilaments, large arrowheads= degeneration vacuoles, medium arrowheads= basement membrane, double arrowheads= collagen fibres.

cells could rarely be observed, the basement membrane (BM) was thin and continuous with fibroblastic endings attaching to it, collagen fibres were scarce around the growing epithelial component (but connective tissue was dense around the large, «older» ducts), and the whole was embedded in white mature adipose tissue (Fig. 4a).

Tamoxifen

Epithelial component. A considerable growth arrest of the mammary glands was observed in this group during the ages of 4-6 months (until 1 month after treatment cessation), a phenomenon which was progressively reversed during the next months, although the values of the TEB/SD ratio never reached that observed in controls (Fig. 1). Under LM, in contrast to that observed in controls, the epithelial cells in TEBs and SDs (Fig. 2b) were in most cases columnar in shape, with a dense eosinophilic cytoplasm and nuclei with a blunted or pycnotic appearance. Mitoses were not observed during the 4th-6th month. Typical HANs were absent until the end of the 7th month (they were found in 1 of 5 cases at this age), while more of them appeared in the next.

Under EM, by the end of the 1st month after TAM administration, the epithelial cells in the TEBs and SDs showed a myoepithelial-like differentiation; i.e. an increase in cytoplasmic filaments (Fig. 3c), especially tonofilaments (Fig. 3d). In the distended intercellular spaces there were interlacing elongated cytoplasmic projections (altered microvilli perhaps, with a high content in filaments). The myoepithelial differentiation of the cells became more prominent during the following months of therapy, when also an increase in lysosomes, autophagosomes and residual bodies was observed (animals 4-6 months old) (Fig. 3d). At the same ages, cells with shrunken cytoplasm and pycnotic nuclei, which could be characterized as apoptotic cells, were apparent among the ones mentioned with the degenerative changes. No virus particles could be found during therapy or 1 month after cessation of treatment. These altered characteristics of the epithelial cells in the TEBs and SDs, which were present for an additional month after treatment cessation, seemed to reverse progressively during the 7th month, and the majority of the epithelial cells by the end of the 10th month exhibited the secretory features described in controls.

Stromal component. The periTEB and periductal connective tissue was considerably more abundant during TAM therapy compared with controls, while the fibroblastic endings (in the cases where they were present) were always located at a distance from the epithelial BM. The latter was thickened, often doubled, and diversely dense, and continuously connected with the abundant collagen fibres. These phenomena were already partially reversed by the end of the 6th month (sooner than the rest of the epithelial cell alterations) and were not observed thereafter. The mammary gland adipose tissue was constituted by small, multilocular, often granular, eosinophilic cells, separated in lobules by connective tissue strands, ultrastructurally resembling adipocytes blocked at an early stage of differentiation. At the same age (4-6 months [Fig. 4b]), a great number of mast cells appeared to «infiltrate» the mammary gland adipose and periductal connective tissue, which, as could be proven with toluidine blue and with EM, showed no degranulation. Until the end of the 6th month the number and size of the mature mast cells increased and the adipose tissue appeared more shrunken. These phenomena also reversed after ceasing therapy, so that by the end of the 7th month the mammary tissue stroma did not differ from that described in controls.

CV

As can be seen in Fig. 1, mammary gland growth progressed in this group -after a significant arrest at the end of the 1st month- despite treatment continuation. Epithelial cell morphology observed during treatment was that of controls, except the hyperplasia of myoepithelial cells (seen in almost all SDs examined [Fig. 3e]), accompanied by a slightly thickened BM, a phenomenon that reversed completely during the next months (after withdrawal of treatment).

The connective tissue around the epithelial component showed a mild hyperplasia (considerably slighter than in the TAM-group), mast cells were

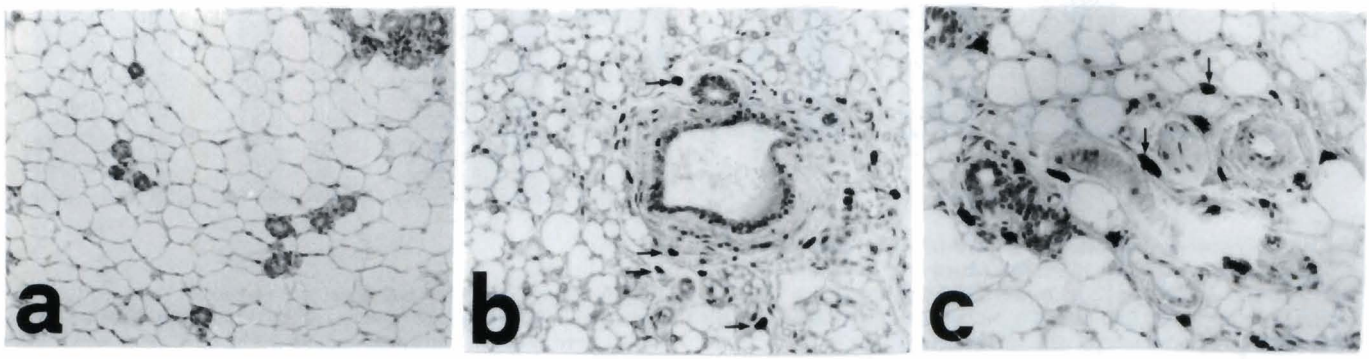

Fig. 4. Stromal changes during TAM- and TAM + CV-treatment: **a)** Control, 3 months old. **b)** TAM, 6 months old and **c)** TAM + CV, 4 months old: multilocular adipocytes, increased connective tissue, prominent mast cell infiltration (arrows). a) H&E, x 100; b) H&E, x 160; c) toluidine blue, x 160.

observed especially during the first two months of treatment, but in a degranulating condition, and most adipocytes comprised lipolytic characteristics.

CV + TAM

In this group, mammary gland growth inhibition lasted throughout the treatment period and in the next, until the 7th month, as shown in Fig. 1. The cells lining the SDs were mainly myoepithelial or of myoepithelial differentiation, devoid of secretory vesicles, and the intercellular spaces were distended, as described in the TAM-group. Evidence of degeneration was detectable early in this group (by the end of the 1st month of treatment [Fig. 2d]), with the corresponding ultrastructural features in these myoepithelial-like cells (lysosomes and phagosomes), which often displayed the characteristics of apoptotic cells, as in the TAM-group (Fig. 3f). These phenomena continued to be apparent in the SDs during the next months (until the 10th month). In contrast to all other groups, the cells in the TEBs, and SDs examined with EM and LM at the age of 7-12 months rarely reached the secretory characters of that observed in controls, so that also fewer MMTV-particles were observed in this group in comparison with all others. During the treatment period the morphology of the BM and periductal connective tissue, the number and condition of mast cells, as well as the adipocyte differentiation state (Fig. 4c) showed the characteristics described in the TAM-group (although to a lesser degree). HANs (consisting of tubular structures around a centrally-located proliferating duct, in most cases without evident secretory features) were observed in this group initially after the age of 7 months.

Treatment influence on mammary tumor incidence (Statistical data are shown in Fig. 5)

In the treatment-groups multiple tumors were not observed in the same animal, in contrast to controls. TAM obviously inhibited tumor development in the C3H/Sy mice, when administered alone or in combination with CV. In contrast to controls and the CV-group, where the tumor incidence progressively rose with age (until the 14th month), in the groups where TAM was administered the lowest tumor incidence was observed at the age of 12 months. CV alone was not effective in tumor inhibition, despite the lower incidence in comparison to controls observed at the age of 8-10 months. In this group tumors were detectable earlier than in all others, including controls (one type B, highly metastatic thoracic gland tumor at the age of 7 months).

Tumor histology (Data shown in Fig. 6)

Controls

The tumors examined in the control group (n = 24) were classified after Dunn (1959) and Sass and Dunn (1979) as type A (high differentiation, n= 4), type B (low differentiation, n= 18) and mixed or undifferentiated (n= 2). The type A/type B ratio observed during this experiment in the C3H/Sy mice is in contrast to that proposed by Dunn, and lies more closely to the results of Ames et al. (1984).

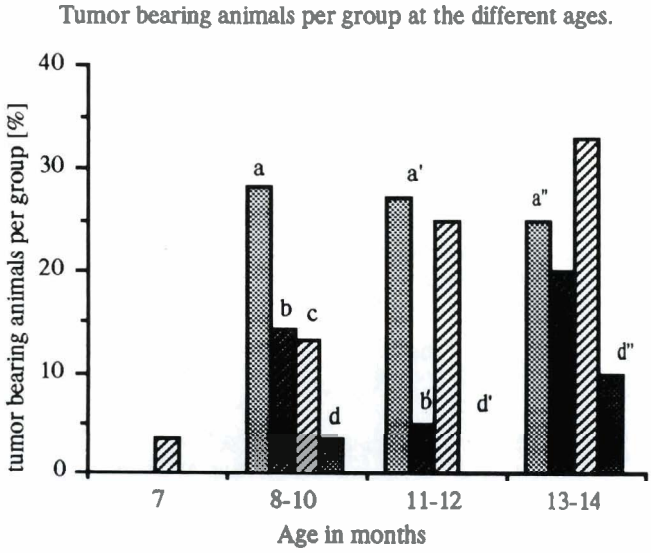

Fig. 5. Tumor-preventive efficiency of the different treatments compared to controls: a) at the age of 8-10 months: xsq (a-b)= 13.7 ($p < 0.01$), xsq (a-c)= 16.8 ($p < 0.01$) and xsq (a-d)= 188.8 ($p < 0.001$). a') 11-12 months: xsq (a'-b')= 99.2 ($p < 0.001$). No tumors are observed in the TAM + CV-group at this age. a") 13-14 months: xsq (a"-d")= 22.5 ($p < 0.01$). Tumor incidence is still very low in the combination group, while their percentage in the CV-group supersedes that of controls.
■ = %Con (a), ▨ = %TAM (b), ▨ = %CV (c), ▩ = %CV+TAM (d).

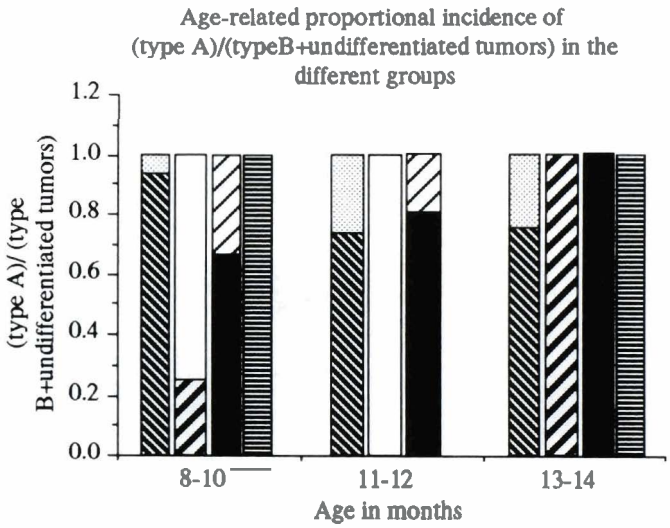

Fig. 6. Proportional presentation of the histological types of tumors observed in the experimental groups at the different ages. in the TAM-group, type A tumors (proportionally more than in all other grups) are detected until the 10th month.
□ = Con A, ▧ = Con B, □ = TAM A, ▨ = TAM B, ▨ = CV A, ▩ = CV B, ▤ = TAM+CV B.

TAM

Tumors developed after TAM treatment (n= 8) were in general better differentiated in comparison to all other groups (number of type A tumors = 3). In one case mixed features of a florid fibroadenoma (in the tumor periphery) and large type B areas located centrally were observed (Fig. 7). Yet, the type A tumors were detected until the 12th month, while all tumors that developed in the next months (n= 3) were of type B with a high degree of squamous neoplastic component and variable amounts of stroma (Fig. 8a).

CV

No differences in tumor histology were observed in this group compared to controls (number of tumors developed = 11).

TAM + CV

In this group only 2 tumors were detected until the 14th month. Both tumors were of poor differentiation (they could not be classified as type A or B), where the squamous component was invariably present (in fact the tumor detected at 14 months of age can be considered as a squamous carcinoma [Fig. 8b], with foci of keratinization, many necrotic areas, increased stromal component and a few places suggesting a glandular origin).

Discussion

The effect of TAM, alone or in combination with CV, on the normal C3H/Sy mammary gland histology can be briefly described as growth arresting on the ground of a modified differentiational state (for the stromal, as well as for the epithelial component). Evaluating a drug's action on a multi-tissue organ *in vivo*, as is the mammary gland, always comprises difficulties and uncertainities, since the effect observed is the result of multiple factors (inter)acting on the same cells. The morphological observations in this case show that TAM originally alters the differentiation of the mammary epithelial cells rendering them to myoepithelial-like cells instead of the secretory type expected. This action of TAM (reported also *in vitro* [Sapino et al., 1987; Chouvet et al., 1988]) could perhaps be interpreted as estrogenic, since estrogens are known to induce an increase in cytoplasm, especially stress filaments in cancer cell lines (Sapino et al., 1987). On the other hand, the disappearance of the secretory characteristics of the epithelial cells can be definitely interpreted as an antiestrogenic action, since estrogens seem to be necessary for the development of these characters in mammary cells (Vic et al., 1982). The above considerations as a whole come to the definitely established and well-defined, at the receptor level (Green, 1990), mixed agonistic/antagonistic effects of TAM, which are observed in the C3H/Sy mammary glands already by the end of the first month of drug administration. Despite the bad role in cancer promotion, which estrogens are charged with (including the estrogen-dependent progesterone action), it must be considered that these steroids also act on the more differentiated structures of the mammary gland; i.e. the larger, «aged» ducts (Topper and Freeman, 1980), though the molecular basis whereby they exert their differentiating action is not clear. Concerning TAM action on the epithelial component (cyto)morphology, the characterization of causing «aging of the ducts» would perhaps be the most proper one.

The epithelial BM alterations and the increase in collagen fibres around the epithelial structures (especially the TEBs and SDs) may result from the observed myoepithelial differentiation (compared with and extrapolated from *in vitro* data [Ormerod and Rudland, 1984; Barraclough et al., 1990; Bronzert et al., 1990]), are possibly related with the increased TGF-ß activity attributed to TAM (Knabbe et al., 1987; Ethier and v. de Velde, 1990) and most probably play a part in

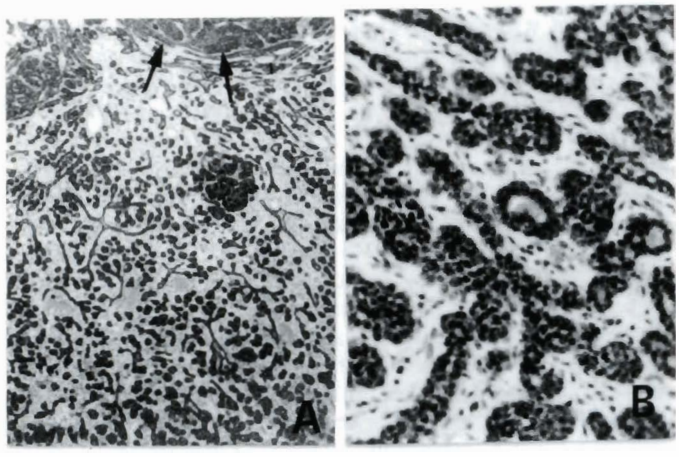

Fig. 7. Tumor with features of a florid fibroadenoma in the periphery and central type-B areas (arrows in A) in a 10-month-old C3H/Sy, TAM-group. A) H&E, x 25; B) H&E, x 160

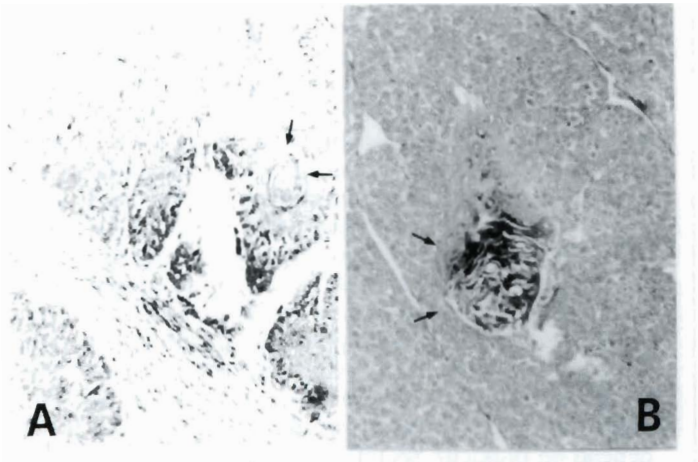

Fig. 8. Tumors with squamous neoplastic component in 14-month-old C3H/Sy observed after TAM-treatment: **A)** TAM-group: abundant stroma. x 100. **B)** TAM+CV-group. x 160. arrows= foci of keratinization.

the growth arrest of the epithelial tissue (Emerman et al., 1977; McGrath, 1983; Haslam, 1986; Silberstein and Daniel, 1987), observed during TAM administration. The alterations in stromal component morphology are more difficult to interpret with the existent data, but since the mammary gland epithelial component is able to grow only in white mature adipose tissue (Topper and Freeman, 1980), it seems that the paucity in adipocyte differentiation observed in the TAM groups provides an unsuitable blackground for epithelial growth. This effect of TAM (it can be resultantly attributed to TAM, since it was not observed in controls or in the CV group), appears later in the course of treatment and may be related to the action of the triphenylethylene derivative (i.e. calmodulin and PK-C antagonistic etc. [Lam, 1984; O'Brian et al., 1985]) achieved after reaching pharmacological concentrations in the different tissues. Moreover, adipose tissue alterations run parallel to the increased number of large, mature mast cells. Since mast cell degranulation depends on intracellular calcium mobilization (Chakravarty et al., 1990), the maturation state of these cells could be possibly a delayed effect of TAM, which in turn may be related to adipose tissue differentiation and epithelial growth (Folkman, 1985; García-Caballero et al., 1989). Also, the degenerative changes in the epithelial cells possibly represent a consequence of these pharmacological TAM actions, as has been described in breast cancer cell lines (Chouvet et al., 1988; Lazier and Bapat, 1988), while in addition, TAM has recently been found to induce cell apoptosis (Wilson et al., 1992).

The maintenance of the morphological alterations for at least one month after the end of treatment in the TAM-group is consistent with the once established prolonged action of TAM after withdrawal of drug administration (Pasqualini et al., 1988), while the reversal of the same alterations at organ level late after treatment cessation provides the basis for the tumor development observed during this study.

The reversal of CV-effects on the C3H/Sy mammary gland morphology during therapy was unexpected, since related references are missing in the literature. Approaching the phenomenon, there is no evidence that the drug's action on lowering prolactin levels is reversed after a period of time (Vance et al., 1989; Khalfallah et al., 1990), but there are some data which show that the mere lowering of prolactin level in rodents does not necessarily mean the elimination of the hormone's effects in the periphery (Nagy and Berczi, 1991). The myoepithelial differentiation observed during the first 2 months of treatment, as well as the partial growth arrest of the mammary gland may result in addition to the diminished offer of a main promoter, i.e. prolactin, to the mammary epithelium, from the altered estrogen/progesterone ratio supposed to follow the lowered prolactin levels (concerning that progesterone levels depend on prolactin secretion in rodents [Cooke, 1989]). The lipolytic changes during the months of treatment could be related to the findings of Elisova (1990) or to the observed extensive mast cell degranulation (after Franzen and Norrby [1980]) and may in addition be responsible for the persistence of further epithelial growth during treatment.

Concerning the antitumor activity of the drugs, which is parallel to their antigrowth properties, TAM, when used alone or in combination with CV, can successfully prevent mammary carcinogenesis in the C3H study model. TAM alone is known to do so in this system (Jordan et al., 1990, 1991), as well as in various experimental mammary carcinogenesis systems, as already mentioned. TAM, combined with a prolactin-inhibiting agent, is, to our knowledge, for the first time tested in a system of spontaneous mammary carcinogenesis; the low incidence of tumors in the TAM+CV group may make it interesting to further define the role of this combination in mammary cancer prevention models. The antitumor activity of the «antiestrogen» on the C3H mice seems to be potentiated by CV. TAM has been found to block prolactin action in the mammary gland, especially when acting in a low prolactin environment (Biswas and Vonderhaar, 1991), which could be the reason for the even greater growth arresting and antitumor efficacy of the drug when combined with a prolactin lowering substance (in this case CV). This obvious synergism between the two drugs lies somehow in contrast with the findings of Welsch et al. (1982), where a rat-DMBA model was used and TAM was combined with bromocryptine. CV alone proved to be less efficient in overall prevention of mammary carcinogenesis under the conditions used, whereby it must be stressed again that the therapy duration was short for the prolactin lowering substance, in comparison to earlier related studies (Welsch and Nagasawa, 1977). Nevertheless, tumors in the CV group were detected earlier than in all other experimental groups, a fact that should be enough to exclude CV alone as a candidate in similar cancer prevention attempts.

TAM prevented the appearance of tumors in the groups where it was administered as expected; a finding consistent with recent works in the same model (Jordan et al., 1991). Interestingly, tumor incidence in these groups showed a biphasic pattern, with the nadir at the age of 11-12 months, in contrast to controls and the CV-group, where tumor number increased with age. This can be partly approached on the basis of the slow cytokinetics of the C3H tumors, which need about 5-6 months to be clinically detectable (Braunschweiger and Schiffer, 1978). Tumors detected during the 8-10th month can, in this way, be considered to have developed before TAM full antigrowth action was established (during the 4th month of age), while those detected during the 13-14th month, after withdrawal of the drug's effects (during the 7-8th month, as evidenced from the alterations in mammary gland morphology). This latter finding can be compared with data from experimental (Jordan, 1988), as well as clinical studies (adjuvant trials [Rutqvist et al., 1991]) concerning tumor development

after TAM discontinuation, and is in this case probably due to the retention of the tumorigenic capacity of the mammary cells, which is expressed when tumor promoters act on these cells after treatment cessation (Jordan et al., 1991). Additional evidence in this area is the appearance of MMTV particles in the TAM groups initially 2 or 3 months after cessation of therapy (for TAM and TAM+CV group respectively). Nevertheless, the fact that the lowest incidence of tumors was detected by the age of 11-12 months can be attributed, in the cytokinetical way of thinking, to the complete antigrowth effect of TAM on the mammary glands observed during the 5-7th month.

The biphasic pattern of tumor incidence observed in this study in the TAM-groups seemed to be related to the histological grading of the tumors. Although the overall tumor number was small, it was striking that the well differentiated type A tumors were all observed during the 8-12 months, while the undifferentiated ones after the 13th month. Starting with the latter, it can be speculated that these high grade tumors with the squamous and stromal component may be the result of the promoter action on an altered epithelium (i.e. myoepithelial-like, instead of secretory), as has been described for the squamous carcinomas of the breast on the ground of myoepithelial differentiation (Raju, 1990; Wargotz and Norris, 1990). On the other hand, TAM can be considered to have positively affected the differentiation of the tumors which developed during the treatment period. It must be stressed that the C3H tumor grading is not closely related to human breast cancer; however, the above comments perhaps provide an approach to the phenomenon that contralateral cancers or recurrences developing in adjuvantly TAM-treated patients are more often estrogen receptor negative (without implications in the overall prognosis [Rutqvist et al., 1991]). Post-TAM-tumor biology and morphology is indeed an issue of great interest and there is a great lack of information, so far.

This study has shown that TAM, when acting alone or in combination with a prolactin-lowering substance on the C3H noncancerous mammary gland during the early period of promotion, exerts a growth-arresting effect, which is expressed on the ground of an altered histological differentiation, but both facts reverse after drug discontinuation. Possible implications of this altered differentiation in post-TAM-tumor biology should be investigated, with regard to drug application to healthy women for breast cancer prevention.

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