THE ISOZYME PATTERN OF CYCLIC AMP-DEPENDENT PROTEIN KINASE AND THE DISTRIBUTION OF A CERVICOVAGINAL ANTIGEN IN EXPERIMENTAL CARCINOMA OF THE CERVIX UTERI OF MICE

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Tissue-sections from 12 methylcholanthrene-induced carcinomas of the cervix uteri of mice were tested for the presence of an antigen normally confined to the cervicovaginal epithelium. The antigen was detected in 10 of the 12 tumours investigated with indirect immunofluorescence, and in all 7 tumours studied with the more sensitive method of mixed hemagglutination. The concentration of the antigen was generally higher in the well-differentiated areas of the tumours, but it was also found associated with solitary tumour cells, apparently invading the stroma. The presence of CVA in the tumours suggests an origin of the tumour cells from the cervicovaginal epithelium. The cyclic AMP dependent protein kinase (EC 2.7.1.37) in the tumour cytosols was studied by chromatography on agarose and DEAE-cellulose. The enzyme showed the same properties as that from normal vaginal epithelium. The tumour cells thus contain an apparently normal complement of this enzyme, which is believed to be responsible for most of the intracellular actions of cyclic AMP.

Key words: Cervixcarcinoma; methylcholanthrene; mouse; immunological marker; protein kinase; cyclic AMP; chromosomes.

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The effect of prolactin on the incidence of 3-methylcholanthreneinduced carcinomas of the uterine cervix in mice (18) and on some growth parameters of such cells in vitro (17) have been reported from this laboratory. Current studies of the effect of dibutyryl cyclic AMP on such cells prompted us to investigate whether the cyclic AMP dependent protein kinase(s) in the tumours differed from the enzyme found in normal vaginal epithelium from the same strain of mice (10). Cyclic AMP (25, 26), as well as its butyrate analogue (21, 22), are believed to influence the cell by activation of the intracellular protein kinase. Prolactin has been shown to affect the intracellular concentration of protein kinases in mammary gland epithelium (29). It was therefore decided to study tumours from both neonatally untreated animals and neonatally estrogenized animals, as the latter have an elevated level of circulating endogenous prolactin (31).

An antigenic substance (cervicovaginal antigen = CVA) accumulates in the vaginal tract in response to prolactin (23), as well as in explants of neonatal uterine cervix exposed to dibutyryl cyclic AMP (27). Since the concentration of CVA is governed by factors (cyclic AMP, prolactin) which
may also affect the protein kinase, it was considered of interest also to study the distribution and concentration of CVA in the tumours. In the normal mouse, CVA is confined exclusively to the cervicovaginal epithelium. Its possible occurrence in the carcinomas might thus give clues to the histogenesis of the carcinomas.

MATERIALS AND METHODS

Treatment of Experimental Animals
The mice belonged to a closed, randomly-bred NMRI strain. They were fed a standard pellet diet and given water ad libitum. Some of the animals were given subcutaneous injections of 5 μg estradiol-17β (Sigma Chemical Co) in 25 μl olive oil on each of the first five days of life; others were given olive oil only. At the age of 6–9 weeks the animals were laparotomized, and a cotton thread impregnated with a mixture of 3-methylcholanthrene (Sigma Chemical Co) and beeswax (1:3 w/w) was inserted into the uterine cervix (15). The mice that developed palpable tumours were used for experiments (about 2½–3½ months after the threads had been inserted).

Routine Histological Examination
Several specimens were taken from each tumour used in the study, fixed in Bouin's solution, embedded in paraffin, sectioned at 6 μm, and stained in haematoxylin and eosin for histological study.

Preparation of Tissue Sections, Procedure for Immunofluorescence and Mixed Hemagglutination on Tissue Sections.
Three specimens were rapidly removed from different parts of the tumour while the animal was in ether anesthesia, quickly frozen in a jet of CO₂, and sectioned transversely in a Dite's cryostat kept at about −20°C. The method of indirect immunofluorescence was as described by Forsberg & Kvistnland (16). Briefly, the sections were air-dried at 56°C, immersed in acetone, dried again, and incubated with antiserum against CVA for 30 min at 37°C. The sections (after rinsing in phosphate buffered saline pH 7.4) were then incubated for 15 min at 22°C with FITC-conjugated antirabbit IgG globulin serum from goat (Behringwerke AG). The fluorescence was exited by transmitted ultraviolet light (from a high pressure mercury lamp). Preparation of the anti-serum against (partially purified) CVA was as described earlier (13). The indirect mixed hemagglutination technique for the demonstration of antigen in tissue sections was based on the method of Tender et al. (34). The procedure was modified (13) to allow a relative quantitation of the CVA-concentrations in the sections. The highest dilution of the anti CVA serum giving a density of the indicator corpuscles corresponding to a + (+) reaction on a scale from 0 to + + +, is defined as the titer of CVA. This is a measure of the CVA-concentration. Two tumours from estrogenized animals and three from non-estrogenized animals were only studied by immunofluorescence.

Four tumours from neonatally estrogenized animals and 3 tumours from non-estrogenized animals were examined by both immunofluorescence and mixed hemagglutination. The CVA concentrations given refer to the groups of carcinoma cells with the highest titers of CVA in any particular tumour.

Assays for Protein Kinase Activity and Binding of Cyclic [³H]AMP
were as described earlier (9). The cyclic [8-³H]AMP (27 Ci/mmol) and [γ-³²P]ATP were from The Radiochemical Centre, Amersham. The substrate for the protein kinase was whole-calf thymus histone (Type II, Sigma Chem. Co). One unit of protein kinase activity is defined as the amount of activity transferring 1 pmol of phosphate into histone per min under the experimental conditions described above.

Preparation of Cytosol from Cervix Carcinomas
The tumour was quickly removed from the ether-anesthetized animal after biopsies had been taken for routine histological examination. It was then grasped in a large metal forceps cooled in liquid N₂, and plunged into the nitrogen. After 1–5 weeks in liquid N₂ the tumours were thawed in homogenization buffer. Homogenization and further preparation of cytosol was as described earlier (10).

Gel Chromatography, Ion Exchange Chromatography and Sucrose Density Gradient Centrifugation of Cytosol
The procedures for gel chromatography using Sepharose 4B (Pharmacia, Uppsala, Sweden) and ion-exchange chromatography using DEAE-cellulose (DE-52, Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) as well as sucrose density gradient centrifugation (5–20% w/v linear gradient spun for 20 hours at 40,000 rev/min) have been described previously (10).

Karyological Examination
Minced biopsies from carcinomas of the cervix were incubated for 2 hours at 37°C in tissue culture medium (Parker 199, Statens Bakteriologiska Laboratorium, Stockholm, Sweden) containing 0.05 μg/ml of colcemid. The tissue specimens were next placed in a hypotonic solution (75 mM KC1) for 10 min at 22°C, followed by methanol/acetic acid (3/1 v/v) for 2 hours and 60% (v/v) glacial acetic acid for another ½ hour. Droplets from the resulting cell suspension were placed on pre-warmed (35°C) slides, air-dried, and the preparation stained with Giemsa-solution as described by Buckland et al. (2). Three tumours, two of which were from neonatally estrogenized animals, were studied. Between 20 and 30 metaphases were counted for each tumour.

RESULTS

Characterization of the Protein Kinase Activity in Tumour Cytosol
The protein kinase activity was measured in desalted samples from two cytosols prepared from tumours of neonatally estrogenized mice and two
tumour cytosols from non-estrogenized animals. The reaction rate was linear with respect to time and concentration of cytosol protein under the experimental conditions used. The proportionality between reaction rate and concentration of cytosol protein ensured that variable tissue levels of the heatstable inhibitor protein did not affect the kinase activity (9). The kinase was stimulated about 4-fold in the presence of 2 µM cyclic AMP. The activity (in the presence of cyclic AMP) ranged from 210 to 280 units/mg cytosol protein. There was no indication that neonatal estrogenization influenced either the level or cyclic AMP dependence of cytosol protein kinase. The mean kinase activity per mg cytosol protein was about 20% lower than in pooled non-malignant vaginal epithelium (10), but higher than in vaginal stroma (Ueland & Doskeland unpublished observation).

Gel Chromatography and Ion-Exchange Chromatography of Cytosol
Samples from each of the four tumour cytosols referred to above, were subjected to agarose chromatography. The peak of cyclic AMP binding activity and the main peak of kinase activity eluted (Fig. 1) like the corresponding activities from normal vaginal epithelium (10). A shoulder of kinase activity was eluted after the main peak (Fig. 1).

The peak fractions from the agarose column (Fr. 14–19) were pooled and chromatographed on DEAE-cellulose. Most of the protein kinase activity was eluted between 150 mM and 220 mM KCl, and was associated with cyclic AMP binding activity (Fig. 2). Another peak of cyclic AMP binding activity, devoid of kinase activity, was eluted at about 80 mM KCl. The material corresponding to this peak sedimented at about 4.5 S upon sucrose density gradient centrifugation (data not shown). The elution profiles shown in Fig. 1 and Fig. 2 are representative for all the four tumours studied, whether taken from estrogenized animals or not.

Histological Examination of the Tumours.
All the tumours investigated were confirmed to be frank carcinomas by routine histology. On the basis of the biopsies, nine tumours were classified as well-
differentiated squamous carcinomas, one as a poorly-differentiated carcinoma, and two were composed of squamous and adeno-carcinomatous elements.

The pieces of tumour used for biochemical and karyological investigations were taken from the main part of the tumour, and it was ensured that biopsies from the periphery of the pieces contained only tumour-tissue. For study of antigen, sections were taken both from the main part and the periphery (Fig. 3) of tumours.

Tests for Immunological Specificity of the Methods Used to Detect Antigen in Tissue Sections from Cervix Carcinoma

The sections appeared to be virtually free from indicator corpuscles or fluorescence when they had been incubated with pre-immune serum instead of immune serum. Only red corpuscles coated with both amboceptor and goat anti-rabbit antibody adhered to the sections. Furthermore, the fluorescence could be abolished by prior incubation of the immune serum with a partially purified preparation of CVA.

Occurrence and Localization of CVA in Carcinomas of the Cervix

The five first tumours were studied only by immunofluorescence. The seven following tumours were tested for CVA content by both immunofluorescence and mixed hemagglutination. Each of the two series contained one tumour in which immunofluorescence was barely detectable. The other ten tumours had areas of obvious immunofluorescence. Cervicovaginal antigen was detected in all the tumours studied by mixed hemagglutination. Eleven out of 12 tumours thus contained CVA, and it is possible that the tumour which was without

Fig. 3. Histologic appearance of a cervix carcinoma. Section from the periphery of a tumour, showing an early phase of tumour growth. Magnification: 50X.

Fig. 4. Association of indicator red blood corpuscles with the centres of nests of squamous epithelium directly below the surface (A) or in the periphery of the tumour (B). The anti CVA serum was diluted 1/50,000. Section fixed in formaline and stained with H-E. Magnification: A: 120 X, B: 160 X.
immunofluorescence contained CVA detectable by the more sensitive method of mixed haemagglutination.

The intensity of fluorescence and the density of indicator corpuscles varied within the same tumour. But the variation of CVA concentration between morphologically similar areas was less within one tumour than between different tumours.

In several tumours CVA was preferentially localized either in the centres of areas with stratified epithelium (Fig. 4 A, B) or associated with cyst-like structures (Fig. 5). The antigen was present in epithelial nests near the surface of the cervical canal (Fig. 4A), as well as in more deeply situated such structures (Fig. 4B). Cervicovaginal antigen could also be found in association with apparently isolated carcinoma cells. Fig. 6 shows the attachment of indicator corpuscles to an area of a section, where carcinoma cells are invading the connective tissue stroma.

Relative quantitation of the CVA concentration revealed no significant difference between tumours from neonatally estrogenized animals and control animals. The CVA titers ranged between 40,000 and 175,000 in tumours from estrogenized animals and between 100,000 and 150,000 in tumours from non-estrogenized animals. The highest concentrations of CVA were generally found in the lining of cysts like that shown in Fig. 5, but high

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Fig. 7. Spread metaphase from a tumour of a neonatally estrogenized animal. The number of chromosomes was 64. The arrow points to a metacentric chromosome. A similar chromosome was found in several other metaphases from the same tumour.
concentrations of CVA could also be found associated with apparently non-differentiated cells (Fig. 6).

Karyological Study
This was initiated because some evidence, summarized by Rowley (32), indicated that cancer cells had chromosomal changes characteristic of the etiological agent. If that theory were correct, one would expect to find changes characteristic of methylcholanthrene-induced tumours and possibly changes to be ascribed to viruses or other cocarcinogenic agents (30). The three tumours studied had chromosome numbers ranging from 51 to 90. We were unable to identify marker chromosomes for any of the tumours. In one tumour a metacentric chromosome was found in about 30% of the metaphases (Fig. 7), however.

DISCUSSION
It has been suggested (36) that the defect in malignancy involves a failure of the cyclic AMP control mechanism. Whereas large amounts of data are available concerning the adenylate cyclase, the cyclic AMP phosphodiesterase and the intracellular concentration of cyclic AMP in malignancy, less is known about the role of protein kinase in neoplasia (33). The presence of functioning cyclic AMP dependent protein kinase in the cell is held to be a prerequisite for cyclic AMP action, the cyclic nucleotide acting by dissociating the inactive holoenzyme into a regulatory cyclic AMP binding subunit and an active catalytic subunit (25).

The protein kinase from normal liver and hepatomas behave differently when fractionated by isoelectric focusing (7). That method does not, however, separate the major isozyme forms of cyclic AMP dependent protein kinase (19). By DEAE-cellulose chromatography the two major protein kinase isozymes found in mammalian tissues (protein kinase I and II eluting from DEAE-cellulose at low and high ionic strength, respectively) can be separated (5, 10).

In the cervical carcinomas studied by us, protein kinase II was the dominating isozyme (Fig. 2). Another peak of cyclic AMP binding protein eluted from DEAE-cellulose was probably the regulatory subunit of protein kinase I. In a number of rat tissues (6), mouse liver (11), bovine adrenal cortex (12), and human renal cell carcinomas (Fossberg et al. unpublished work) the regulatory moiety of this isozyme is eluted from DEAE-cellulose in a position corresponding to the binding activity from cervix carcinoma. Furthermore, the sedimentation velocity (4.5 S) serves to differentiate the present binding protein from phospho-fructokinase (24) and a newly described binding protein not related to protein kinase (3, 35). Protein kinase I is generally more easily dissociated than protein kinase II (5), which may explain why the former enzyme is eluted as subunits and the latter in the holoenzyme form.

Fractionation of the protein kinases of vaginal epithelial cytosol on DEAE-cellulose (10) gave elution patterns nearly identical to those for tumour cytosol. Since the results obtained with the immunological marker CVA (to be discussed below) strongly suggested that the carcinoma cells were derived from the müllerian cervicovaginal epithelium, one can conclude that the protein kinase isozyme distribution was the same in the normal epithelium and its malignant derivative.

It has been demonstrated that the protein kinase I found in hepatocytes is lacking in hepatoma cell lines (20, 28). The results of the present investigation, as well as our recent finding that the proportion of protein kinase I relative to protein kinase II was not lower in human renal cell carcinomas than in renal cortex (Fossberg et al. unpublished work), indicate that disappearance of protein kinase I is not invariably associated with malignancy.

The binding protein corresponding to the regulatory moiety of protein kinase I might conceivably originate from the connective tissue elements of the tumours. The low concentration of protein kinase in cervicovaginal stroma (see results section) and the dominance of protein kinase II in that tissue (10) argue against such a possibility. Since only aneuploid metaphases were obtained from three tumours similar to those used for biochemical investigations, one would assume that the vast majority of the proliferating cells of such tumours were carcinoma cells. A contribution of macrophages of low proliferative activity and not discernible by routine histological investigation (14) cannot be excluded.

The present study was disgratifying with respect to marker chromosomes. Accumulating evidence, recently summarized by De Paolo & Popescu (8), indicates that the chromosomal changes in malignant cells need not be specific for the etiological agent.

The presence of a highly specific immunological marker for the müllerian cervicovaginal epithelium (CVA) in the cervix carcinomas is strong evidence that these tumours are derived from that epithelium. A stromal origin of the epithelial cells which later develop into carcinoma cells has been suggested by Coppleston & Reid (4). It should be noted that CVA is not a carcinoembryonic antigen as it is not detectable until 2 days before birth, and its intracellular concentration rises during the differen-
The highest concentrations of CVA were found in the morphologically more differentiated areas of the tumour, but high concentrations of the antigen could also be found in association with apparently isolated carcinoma cells infiltrating the connective tissue in the periphery of the tumour. The latter observation shows that an ordered alignment of the cells is not required for the production of CVA.

It also suggests that the generally rapid proliferation of the peripherally infiltrating carcinoma cells (1) and the production of a differentiated cell product like CVA are not mutually exclusive.

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REFERENCES


