Liberation of a Mouse Uterus-specific Protein in the Serum during Uterine Carcinogenesis

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SUMMARY

An alkaline phosphatase that exhibited immunological specificity towards the mouse uterus was liberated in the serum during uterine carcinogenesis induced by dimethylbenzanthracene. A correlation was observed between the presence of neoplastic lesions and the appearance of this alkaline phosphatase in the serum. This specific protein, which was immunohistochemically localized in the endometrial epithelium of normal mice, was found in the tumor cells exhibiting keratinization. Diagnostic value of these findings has been implicated.

INTRODUCTION

Investigations of Abelev et al. (1–3) and Tatarinov(16) have shown that an α-fetoprotein is liberated in the serum during hepatic carcinogenesis. A carcinoembryogenic antigen has also been detected in the serum of patients suffering from intestinal cancer (10). These findings strongly suggest that cancer of a given type can be localized by the detection of a specific protein in the serum.

In earlier studies in our laboratory, an AP2 was isolated from mouse uterus (7). Tissue specificity of this AP was established by immunological studies, and it was localized in the endometrial epithelium. The main purpose of this study was to determine whether this uterus-specific AP was liberated in the serum during carcinogenesis of endometrial epithelium induced by DMBA (12).

MATERIALS AND METHODS

The studies were carried out with Swiss mice propagated by random mating at the Biomedical Centre, Laval University, Quebec, Quebec, Canada.

Induction of Uterine Tumor by DMBA. Although induction of uterine tumor can be accomplished by painting the tissue with carcinogen (13, 20), inserting a pellet containing carcinogen (9), or introducing a carcinogen-impregnated thread (13, 15), the latter method has produced the highest yield of tumors with the shortest latent period (12, 13).

Moreover, this method permits the prolonged contact of DMBA with endometrial epithelium, which subsequently induces endometrial carcinomas. Hence, the same method was used in the present studies. A triple overhand knot was made 3 cm from the end of mounted silk thread and was briefly dipped into melted DMBA. When this cooled, a small pearl of DMBA was formed around the knot. The pearl was placed directly into the uterus by introduction through the vaginal orifice. A small incision was made in the skin at the emergence of the needle, and the knot was buried in the s.c. tissue. The pearl was left in place throughout the life. Latent period for the development of the tumor was approximately 30 weeks.

One month after the beginning of experimental induction of the tumor, a blood sample of each mouse was taken through intraorbital sinus, and the serum was subjected to double diffusion test in agar gel (14), as well as to immunodiffusion test on cellulose acetate membrane (18), for detection of the presence of uterine AP. The blood sample was obtained every week thereafter for study of the correlation between the liberation of AP in the serum and the degree of neoplasm in the uterus.

A control group was maintained to verify whether a nonspecific liberation of uterine AP into the serum could be due to DMBA treatment or to a specific type of tumor in the uterus. This group of mice received DMBA, 1 mg/mouse, by the tail vein, and subsequently developed fibrosarcomas in the thigh. The serum of these mice was also subjected to the immunological test for detection of the presence of uterine AP.

Preparation of the Immune Serum. The following method has been used for purification of mouse uterus AP: 100 mg of a protein extract from the uterus, soluble in 0.9% NaCl solution, prepared by centrifugation at 74,000 X g (Beckman Model L) for 45 min, were applied to an LKB-8100-20 electrofocusing column. A pH gradient was created by passing the current through carrier ampholyte solution (Ampholine, LKB-8143). This ampholyte covered the pH range from 5 to 8, and was used at a concentration of 1% in 6 M urea. A density gradient was prepared from sucrose solution (50% concentration) to avoid remixing of the separated proteins. The electrofocusing column contained NaOH (0.2 M in 55% sucrose) at the cathode, and phosphoric acid (1.5%) at the anode, while the protein sample was layered in the middle portion. A voltage of 500 Volts was applied to the column, and the experiment was conducted for 48 hr. Convection currents were minimized by constant circulation of water at 4° around the column. All the electrofocusing fractions are studied in terms of the pH of their protein content (11) and...
the AP activity level, as determined by the method of Babson (6).

On the other hand, the qualitative protein content of the fraction is determined by immunoelectrophoresis and double diffusion. Furthermore, AP activity is detected in immune precipitates (17). Fraction 6 of the 1st electrofocusing elution, which shows the highest AP activity, is obtained at pH 6.8. For better purification, this fraction has been submitted to a 2nd electrofocusing with the aid of Ampholine LKB-8153, covering a pH range from 5 to 7. Fig. 1 illustrates the electrophoretic purity of the uterine AP content of the 2nd electrofocusing elution of Fraction 6, eluted at pH 6.85.

This purified fraction (e2 6) has been used for immunization; 10 mg of this purified AP fraction was dissolved in a mixture of 0.5 ml 0.9% NaCl solution and 0.5 ml Freund’s complete adjuvant. The suspension was injected intradermally into the footpads of a rabbit. A month after sensitization, 3 booster injections were given as follows. We dissolved 20 mg of the protein in 3 ml of 0.9% NaCl solution. The animals were given injections of 0.5 ml of this solution s.c. on the 1st day, 1 ml i.v. on the 2nd day, and 1.5 ml i.v. on the 3rd day. The animals were bled 1 week after the booster dose, thus giving rise to a monospecific antibody (Fig. 2). Electrofocusing fractions, rich in AP activity, have been prepared from the liver, intestine, placenta, and kidney for induction of rabbit antibodies against the AP of those organs for confirmation of organ specificity of the uterine AP, previously studied with the aid of total extracts from the same tissues (7).

**RESULTS**

**Double Diffusion in Agar Gel**

Immunological specificity of the uterine AP was studied by the method of double diffusion in agar gel (14). Specificity of the immune reaction was confirmed by maintenance of the following absorption controls. Immune serum against purified uterus AP was divided into the aliquots of 1 ml and absorbed 3 times (10 mg each) with the serum, as well as with the lyophilized extracts from different tissues, such as liver, intestine, placenta, kidney, and uterus (Fig. 3). The absorption control tests have been repeated by use of the fractions rich in AP activity that were obtained by electrofocusing from the liver, intestine, placenta, and kidney: 3 consecutive absorptions, with the use of 5 mg protein each, have been performed.

Antiserum against purified uterine AP reacted uniquely with the uterus and failed to react with other tissues as visualized by double diffusion test (7). These observations were further confirmed by the absorption control tests in which the antiserum retained its capacity to react with uterine AP after extensive absorption with other organs (Fig. 3). This characteristic was confirmed by absorption with purified fractions rich in AP activity from the same tissue. These antiseras, produced against different organs such as kidney, liver, intestine, placenta, and lung, failed to react with purified uterine AP (Fig. 4).

**Immunodiffusion on Cellulose Acetate Membranes**

We have used acetate membranes for immunodiffusion (18) for detection of microquantities of uterine AP in the serum of mice bearing uterine tumors. However, this method cannot yet be used for quantitative immunodiffusion. Work is in progress to obtain the degree of purity of the uterus protein required for these quantitative studies.

The membranes were soaked in the γ-globulin fraction from the immune serum directed against uterine AP. The method described by Avrameas (5) was used for the isolation of γ-globulin. Immunoadsorbent was prepared from the uterine extract by use of glutaraldehyde. Immune serum was kept in contact with the immunoadsorbent for 1.5 hr. Immunoadsorbent was washed several times with phosphate-buffered saline, pH 7.2. Elution of the γ-globulin was performed in batches, with glycine-HCl buffer, pH 2.8.

Undiluted sera of DMBA-treated mice were applied on the surface of the membrane. The reaction is positive when a radial immunoprecipitation appears after a 48-hr incubation period. Each mouse in which the serum is positive is immediately sacrificed so that we may establish a correlation between the positive reaction and the presence of a uterine tumor revealed by histopathology (Fig. 5).

The serum samples were taken from DMBA-treated mice through intraorbital sinus and applied to the membrane without prior dilution. On the basis of the preliminary observations, the 1st batch of serum samples was obtained 1 month after the beginning of DMBA treatment and approximately once a week thereafter.

**Immunohistochemical Localization**

The uterine AP was localized in the tumor by enzyme-labeled antibody (4). In brief, the method consisted of incubation of the tissue sections with γ-globulin fraction from antuterine AP produced in rabbit. The sensitized tissue was further washed and treated with goat anti-rabbit γ-globulin which was previously coupled with glucose oxidase. The sections were again washed several times. Localization of the antigen-antibody complex was shown by enzymatic activity of the glucose oxidase (7). The tissue AP activity has been verified by histochemistry for establishment of a relationship between the immunohistochemical localization of AP and histochemical AP activity in the tumor.

**Liberation of the Uterus-specific AP into the Serum of Tumor-bearing Mice.** When the serum of mice bearing well-developed uterine tumor was subjected to double diffusion test in agar gel, presence of uterine AP was observed, as shown in Fig. 6 in which we can see a reaction of identity between AP (Well 7), the tumor (Well 6), and sera from 9 mice bearing DMBA-induced tumors (Wells 3 to 5). Wells 1 and 2 contain normal mice sera concentrated by ultrafiltration. Sera of all the mice that showed uterine tumor also showed positive reaction (Fig. 5). For investigation of whether the liberation of uterine AP into the serum was specific for uterine carcinogenesis, a control was maintained, as described in the "Materials and Methods." These mice developed tumors in the thigh after 8 to 10 months. Histopathologically, the tumors were diagnosed as fibrosarcomas. Sera from these mice did not
show a positive reaction in the immunodiffusion test. These results establish the specificity of the uterine AP towards uterine carcinogenesis.

Serum samples from the mice that received the DMBA treatment for up to 60 days showed a negative reaction when subjected to immunodiffusion test. After treatment for 75 days, the 1st positive reaction was obtained with the serum of 1 mouse. Histopathological examination of the uterus of this mouse showed the presence of a uterine carcinoma. The majority of the mice showed the presence of uterine AP in the serum after 107 days of treatment. Although concentration of uterine AP seemed to be low, these mice had developed neoplastic lesions, as revealed by histopathological examination. Apparently, there was no correlation between the size of the tumor and the liberation of uterine AP in the serum. However, all 9 mice that showed a positive reaction in immunodiffusion test also showed the presence of uterine tumor when examined histopathologically.

Cellular Localization of the Uterine AP in the Tumor. The majority of the DMBA-treated mice developed palpable tumors after a latent period of 4 to 5 months. Histopathologically, the tumors were diagnosed as endometrial carcinomas with abundant keratinization and pearl formation. The studies on cellular localization in these tumors led to the conclusion that the uterine AP was localized around keratinized cells and pearl formation (Figs. 7 and 8).

DISCUSSION

The present work has shown that a tissue-specific AP is liberated in the serum of mouse during uterine carcinogenesis induced by DMBA. Utilization of the integrated methods constitutes a valuable system for the diagnosis of cancer. Our results suggest that the approaches of Abelev (1–3) and Gold and Freedman (10) could be used for diagnosis of the cancer of different tissues. The uterine AP, that is immunologically different from the placenta AP, shows some differences from Regan isoenzyme of AP (8), which is immunologically similar to human placenta AP. On the other hand, the mechanisms by which these 2 phosphatases are liberated into the serum are also different. The presence of Regan AP in the serum of cancerous individuals is explained by a derepression of the genome of the cancer cell. We offer the following interpretation: this specific uterus AP is passively liberated in the serum by disruption of the integrity of secretory cells of uterine endometrium containing this protein (7) during DMBA-induced uterine carcinogenesis.

Our data show that there is a definite relation between the presence of uterus-specific AP in the endometrial epithelium and its appearance in the serum during carcinogenesis of the endometrium. This fact indicates that there is a correlation among a tissue-specific constituent, its association with the neoplastic lesion having same tissue origin, and its liberation in the serum during carcinogenesis. Presence of uterine AP even in microquantities can be considered an indication of the early neoplastic lesion, which cannot be always detected clinically. It appears that there is no correlation between the size of the tumor and the presence of the specific AP in the serum, despite its association with the uterine tumor.

Work is in progress to extend our observations for human tissues and to determine whether this approach could be used for the detection of uterine cancer.

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REFERENCES

Mouse Uterine AP and Uterine Carcinogenesis


Fig. 1. Degree of purification of mouse uterus AP as evaluated by electrophoresis on acetate membrane (see Fraction 6, Fig. 1). Alb, albumin; NMS, normal mouse serum; UAP, uterine AP; F, fraction no.

Fig. 2. Degree of purification of mouse uterus AP as evaluated, by the criterion of immunization. NMS, normal mouse serum; Uterus, total uterus soluble proteins. Fraction 6 induces in the rabbit a monospecific antibody.

Fig. 3. Organ specificity of the mouse uterine AP as envisaged by double immunodiffusion. Central well, purified AP obtained by isoelectric focusing of the mouse uterine extract. Peripheral wells, antiserum prepared against purified AP after it was absorbed 3 times (10 mg protein per ml antiserum each time) with the proteins, soluble in 0.9% NaCl solution, from: Well 1, serum; Well 2, uterus; Well 3, placenta; Well 4, intestine; Well 5, kidney; Well 6, liver, respectively.

Fig. 4. Central well, purified AP from mouse uterus. Peripheral wells, antiserum prepared against mouse: Well 1, uterus; Well 2, intestine; Well 3, placenta; Well 4, kidney; Well 5, liver; Well 6, serum, respectively.

Fig. 5. NMS, normal mouse serum. Immunodiffusion on acetate membranes for detection of uterus-specific AP in the serum of mice bearing DMBA-induced uterine carcinoma. Microscopic examination has shown the presence of uterine carcinoma in all of the 6 mice presenting a positive reaction. There is no correlation between the size of the tumor and the presence and intensity of the immunological reaction.

Fig. 6. Immunohistochemical identity between uterine AP and the protein liberated in the serum of tumor-bearing mice. Central well, antibody against mouse uterine AP. Peripheral wells, Well 7, AP; Well 6, tumor; Wells 5, 4, and 3, pooled serum samples from mice bearing endometrial carcinomas induced by DMBA; Wells 2 and 1, normal mouse serum.

Fig. 7. Section of endometrial carcinoma induced by DMBA in the uterus. The cells showed keratinization and pearl formation. HPS, × 125.

Fig. 8. Immunohistochemical localization of the uterine AP in DMBA-induced tumor by the aid of enzyme-labeled antibody. The uterine AP is localized around cells showing keratinization and pearl formation (arrow). × 250.
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