SUMMARY

Five-month-old male goats were treated with 25 mg diethylstilbestrol dipropionate (DES-DP) by a single intramuscular injection, causing characteristic histological alterations in the peripheral glandular epithelium of the prostate, resulting in squamous metaplasia. Using a panel of monoclonal and polyclonal cytokeratin antibodies on frozen tissue sections of control prostates, we were able to immunohistochemically distinguish between the normal secretory cells, which are positive for cytokeratin 18 as detected with the antibody RGE 53, and the scattered basal cells, which could be specifically stained by the antibody RCK 103. Cytokeratins indicating squamous differentiation, i.e., nos 4 and 13, recognised by the antibodies 6B10 and 1C7, respectively, were found in sporadic cells throughout the normal goat prostate.

Profound changes in cytokeratin expression were observed in the metaplastic lesions as compared to control peripheral glandular tissue. In this respect three monoclonal antibodies are of special interest. RCK 103 is immunoreactive with resting and all stages of differentiating basal cells.

Antibodies 1C7 and 6B10 strongly stain the squamous cells in the metaplastic lesions, with 1C7 staining all the squamous cells in the lesions except the basal cell layer, and 6B10 being immunoreactive with the same suprabasal cells or the more differentiated cells in the upper strata.

As a result the number of cytokeratin 18-positive cells is drastically reduced upon metaplasia.

The results indicate that the goat system can be used as a suitable model system to further test the applicability of immunohistochemical methods in meat inspection and toxicological pathology.

INTRODUCTION

Earlier studies have shown that exposure to oestrogens causes hyperplastic and metaplastic lesions in prostatic glandular epithelium of several animal species (1, 3, 5, 6, 7, 8, 9, 10, 12, 22, 25, 27). In calf, the first changes observed after oestrogen treatment include a transformation of basal cells, as seen at the ultrastructural level (9). This phenomenon is followed by proliferation and subsequent differentiation into squamous cells (9). During this process of squamous differentiation the number of filaments in the basal cells increases. In epithelial cells this intracellular filamentous matrix partially consists of cytokeratins. Different combinations of cytokeratins have been found to occur in different stages of epithelial differentiation (11). For example, glanular epithelial cells contain, amongst others, cytokeratin 18 (15), whereas squamous differentiation is characterised by the presence of cytokeratins 4 and 13 (13).

Using cytokeratin polypeptide-specific monoclonal antibodies (MoAbs), different types of epithelia can be distinguished immunohistochemically. Earlier studies have shown that such MoAbs can discriminate between the luminal and basal cell compartments in the normal prostate of man (4), rat (20, 26) and calf (27).

The purpose of this study was to determine the expression of cytokeratins in diethylstilbestrol dipropionate (DES-DP)-induced hyperplastic and metaplastic lesions in the peripheral glandular epithelium of the goat prostate and to compare this to the normal epithelium. Furthermore, several monoclonal keratin antibodies were tested in order to select those that gave optimal and conclusive results.

MATERIALS AND METHODS

Animals, treatment and tissue sampling

Four 5-month-old male goats were used. They were housed individually and had ad libitum access to grass and water. Two animals were injected once intramuscularly in the neck with 2.5 ml of an anabolic steroid preparation containing 10 mg DES-DP/ml in oil. The other two animals were treated with the same volume of a placebo solution. The composition of the DES-DP oil solution and the placebo has been described previously (5).

All four animals were killed 21 days after injection, and the prostates dissected. The tissue was snap frozen in isopentane, quenched in liquid nitrogen and stored at -70°C until analysis.

Immunohistochemistry

Frozen sections of the prostate tissue, 6 µm thick, were air dried before fixation in acetone for 5 min at room temperature. After washing for 10 min in phosphate-buffered saline (PBS) containing 2.7 mM KCl, the tissue sections were incubated at room temperature for 30 min with the primary antibodies, diluted 1:10 for the MoAbs and 1:50 for the polyclonal keratin antisera. After repeated washing in PBS with 2.7 mM KCl, the sections were incubated with rabbit anti-mouse Ig conjugated to horseradish peroxidase (Dakopatts, Glostrup, Denmark) diluted 1:25, or with swine anti-rabbit Ig conjugated to peroxidase (Dakopatts, Glostrup, Denmark) diluted 1:40, both for 30 min at room temperature.

After another washing step, peroxidase activity was visualised...
using 3-amino-9-ethylcarbazole (AEC; Aldrich Chem. Co., USA) and 0.03% H₂O₂. After incubation for 10 min the sections were rinsed in tap water, counterstained with haematoxylin and mounted with polyvinyl-pyrolidone (PVP).

For the double-staining immunoperoxidase technique the procedure for the first stop is the same as described above, using as the primary antibody a mouse MoAb and AEC as a chromogen. The sections were, however, not counterstained with haematoxylin. After the precipitation reaction with AEC, the sections were treated in 0.1 M glycine-HCl buffer at pH 3 (3 times 15 min). After repeated washing in PBS/KCl, the incubation steps as described above for the first antiserum were repeated, using another mouse monoclonal antibody as the primary antibody. Instead of AEC, 4-chloro-1-napthol (Koch-Light Laboratories Ltd., UK) was used as the second chromogen.

Antibodies

The following antibodies were used in this study:
1. An affinity purified polyclonal rabbit antiserum directed against human skin keratins (pKer). This antiserum reacts with several epithelial tissues, but in general not with non-epithelial tissues (17, 21).
2. A mouse monoclonal antibody RCK 102, directed against keratins 5 and 8 (19, 23). This antibody stains virtually all epithelial tissues, but in general no non-epithelial tissues. When tested on cultured epithelial cells this antibody shows a typical intermediate filament staining pattern virtually identical to that seen with the polyclonal antiserum pKer.
3. The mouse monoclonal antibody RGE 53 directed against cytokeratin 18, which specifically recognises columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues, and mesothelial cells. No significant reaction is normally found in squamous epithelial or non-epithelial tissues (15). In bladder epithelium this antibody only stains the superficial cells (16).
4. The mouse monoclonal antibody RCK 103, which stains several types of glandular and squamous epithelium as well as transitional epithelium. Most important is its reaction with basal cell compartments (4, 20, 26) and myoepithelial cells. Since such cells have been described as containing keratins 5 and 14, one of these proteins is among the candidates for antigens to RCK 103 (18). Besides a reaction in epithelial cells, neural tissues are also stained by this antibody.
5. The mouse monoclonal antibody RKSE 60, directed against human skin keratin 10 (17), and specific for keratinising stratified squamous cells. No reaction is found with this antibody in columnar epithelial cells, non-keratinising squamous cells or non-epithelial cells (17).
6. The mouse monoclonal antibody IC7, directed against cytokeratin 13 (13). This antibody stains non-cornifying squamous epithelium with the exception of basal cell layers of some stratified epithelia. In the human prostate gland a few sporadic cells are immunoreactive with this antibody.
7. The mouse monoclonal antibody 6B10, directed against cytokeratin 4 (13), which also stains non-cornifying squamous epithelium. In contrast to the antibody IC7, not only the basal cell layer but also one or several suprabasal cell layers of these squamous epithelia are negative. In human prostate tissue it stains sporadic cells or clusters of cells.

The antibodies were obtained from Euro-Diagnostics B.V., Apeldoorn, The Netherlands.

RESULTS

The results of our immunohistochemical studies on controls and DES-DP-treated goats are summarised in Table 1 and illustrated in Figs. 1-12.

In haematoxylin-eosin stained frozen sections of the prostate tissue of the DES-DP-treated goats, many areas of squamous metaplasia were visible in peripheral glandular prostate epithelium (Fig. 1). Such areas did not occur in the prostate of untreated goats. The cytology of the metaplastic cells was distinctly hyposinophilic in comparison with normal epithelium. Although basal cells in control goats were immunoreactive with the MoAb RCK 103 (Fig. 2), the number of basal cells positive for this antibody was much less than has been described in the rat and calf prostate. Strikingly, RCK 103 stains virtually all the cells in the DES-DP-induced lesions in the goat glandular prostate (Figs. 3 and 6), which is in accordance with findings in the prostate tissue of the call (27). Polyclonal antibody pKer (in a dilution of 1:50) exhibited a similar staining pattern, although it also weakly stained the luminal secretory cells (Fig. 4). In higher concentrations this antibody distinctly stains these luminal cells.
The MoAb RGE 53 to cytokeratin 18 reacted strongly with the secretory cells in the prostate of the control (Fig. 5) as well as the treated goats, but there was no expression of cytokeratin 18 in the lesions (Fig. 6). The pre-existing secretory cells in close proximity to the lesions did, however, retain their cytokeratin 18 expression.

In accordance with findings in the human glandular prostate tissue (13), the monoclonal antibodies IC7 and 6B10 reacted with scattered cells and minor cell populations in the prostate glandular epithelium of the control goats (Fig. 7 and 9). Both

### Table 1. Cytokeratin expression in normal and metastatic goat prostate epithelium.

<table>
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<th>Control</th>
<th>DES-DP-treated</th>
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<td></td>
<td>Basal cells</td>
<td>Luminal secr. cells</td>
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<tr>
<td>RGE 53</td>
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<td>RCK 103</td>
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<td>IC7</td>
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<td>6B10</td>
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<td>RCK 102</td>
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<td>RKSE 60</td>
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4 all cells positive; 4/- all cells weakly stained; - all cells negative.
1 Scattered cells may be positive in the normal prostate epithelium.
2 Staining in all the cells in the metastatic lesions except the basal cell layer.
3 Staining as for IC7, but occasionally only staining of the cells in the upper strata of the lesions was seen.
Fig. 1. Hematoxylin-eosin staining of DES-DP-induced metaplastic lesions in goat prostate (obj. 40x).

Fig. 11. Double immunoperoxidase staining pattern of DES-DP-induced metaplastic lesion, showing reactivity with IC7 (blue color of 4-chloro-1-naphtol) and GB10 (red-brown color of AECI) (obj. 40x).

Fig. 6 Double Immunoperoxidase staining pattern of DES-DP-induced metaplastic lesions, showing reactivity with RCK 103 (red-brown color of AECI) and RGE 53 (blue color of 4-chloro-1-naphtol) (obj. 40x).

Antibodies are immunoreactive with a much larger cell population in the DES-DP-induced lesions (Fig. 8 and 10). Here the MoAb IC7 stains all the cells in the lesions, except the basal cell layer (Fig. 8). The MoAb 6B10 showed a similar staining pattern (Fig. 10; arrow), but occasionally stains only the more differentiated cells in the upper strata (Fig. 10; arrowhead). This occasional difference in the staining patterns of IC7 and 6B10 is illustrated in a double immunoperoxidase staining reaction in Fig. 11.
Figs. 2-5. Immunoperoxidase staining patterns of normal prostate (Figs. 2 and 5) and prostate of a DES-DP-treated goat (Figs. 3 and 4), incubated with ACK 103 (Figs. 2 and 3), pKer (Fig. 4) and A1E 53 (Fig. 5) (all figures obj. 40x).

Figs. 7-10. Immunoperoxidase staining patterns of normal prostate (Figs. 7 and 9) and prostate of a DES-DP-treated goat (Figs. 8 and 10) incubated with 107 (Figs. 7 and 8) and 6B10 (Figs. 9 and 10). Fig. 10: Arrow indicates a metastatic lesion with all suprabasal cells staining for 6B10, while the arrowhead points to a lesion with a staining reaction only in the upper strata (all figures obj. 40x).
The broadly cross-reacting monoclonal antibody RCK 102 stains both basal cells and luminal secretory cells in the prostate tissue of the control and the oestrogen-treated goats. Moreover, it is immunoreactive with the proliferated and differentiated cells in the lesions (Fig. 12).

No reaction was found with the MoAb RKSE 60, indicating that cytokeratin 10 is not expressed in the hyperplastic and metaplastic lesions. As expected, goat epidermis was positive for RKSE 60, thus serving as a positive control.

DISCUSSION

This study demonstrates that profound changes in cytokeratin expression patterns occur upon metaplastic differentiation of prostatic epithelium induced by DES-DP in the goat. Cytokeratin 18, which is normally only found in the luminal cells of control animals, was not detected in the metaplastically altered epithelium but was found in the remaining secretory cells. In parallel, the metaplastic cells showed the expression of cytokeratins 4 and 13. The expression of cytokeratin 4 was sometimes restricted to the more differentiated cells in the upper strata.

Similar changes in cytokeratin expression patterns have been observed in squamous metaplasia of the human uterine cervix (14, 24) and in the human bronchial epithelium (2).

The immunoreactivity of the DES-DP-induced lesions with the MoAb RCK 103 is in accordance with earlier findings in the bovine prostate (27). The fact that MoAb RCK 103 reacts with scattered basal cells in the normal goat prostate provides a further indication of the relation of these cells and squamous metaplastic cells (6, 9). The negative reaction found with the MoAb RKSE 60, which is specific for keratinising squamous epithelial cells, indicates the absence of cornification in the metaplastic lesions. This finding is in agreement with results found by Kroes and Teppema (9) in DES-induced prostatic metaplasia in calves.

In the countries of the European Common Market the use of oestrogens in cattle is banned. Earlier studies showed that immunohistochemistry is a very sensitive method for detecting oestrogen-induced lesions in the glandular prostate tissue of calves (27) and bulls (5). This additional technique is often needed to obtain a conclusive diagnosis; this study underlines the potential value of cytokeratin immunohistochemistry as an inexpensive and sensitive method in visual inspection to detect the (illegal) administration of substances with oestrogenic potency.

The administration of combined oestrogen and androgen preparations does, however, diminish the histologically detectable effect on prostatic epithelium (8). Studies are in progress to detect whether or not changes in cytokeratin expression patterns, as seen in this study, can also be observed in these animals. In long-term experiments with calves treated with DES, a restoration of the glandular prostate tissue was also observed (9). In these cases it will be of interest to know how long cytokeratin 4 and 13 expression persists. Another application of cytokeratin immunohistochemistry lies in determining the oestrogenicity of substances in toxicological pathology.

In conclusion, it can be stated that cytokeratins characteristic of squamous differentiation as well as cytokeratins specific for different epithelial compartments of the prostate are valuable markers for oestrogen-induced changes in this organ.
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