Comparative analysis of biotin intranuclear inclusions of gestational endometrium using the APAAP, ABC and the PAP immunodetection systems

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Abstract

Aims/background—Intranuclear inclusions have been observed in gestational endometrial glands. Although resembling Herpes simplex virus infected cells, these nuclei have been shown to contain endogenous biotin. Hence, immunohistochemical systems using the avidin-biotin complex will result in cross reaction and false positivity. This study investigates a series of 10 gestational endometria (formalin fixed and paraffin wax embedded) with intranuclear inclusions using three different immunodetection systems with primary anti-biotin.

Results—Both the alkaline phosphatase anti-alkaline phosphatase (APAAP) and peroxidase anti-peroxidase (PAP) systems confirmed that the intranuclear inclusions were endogenous biotin. The streptavidin biotin complex (StreptABC) system produced a positive reaction in both test sections and controls (omitting primary anti-biotin) in the presence of prior blocking with free avidin and biotin. In addition, aberrant immunoreactivity was observed in adjacent nuclei/cytoplasm of endometrial glands and decidualised stroma in the negative control of the PAP system. This was subsequently eliminated using microwave pretreatment prior to immunohistochemistry.

Conclusion—The use of either the APAAP or PAP immunodetection systems (the latter with microwave pretreatment) is recommended for any immunohistochemical or non-isotopic in situ hybridisation investigation undertaken on gestational endometria.

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Keywords: gestational endometrium; alkaline phosphate anti-alkaline phosphatase; avidin-biotin complex; peroxidase anti-peroxidase; in situ hybridisation.

Intranuclear inclusions have been occasionally observed in endometrial glands, particularly during gestation and puerperium. These optically clear nuclei with a ground-glass appearance resemble Herpes simplex virus (HSV) infected cells.^{1,2} However, these inclusions contain biotin, as shown immunohistochemically using monoclonal anti-biotin as the primary antibody and the peroxidase anti-peroxidase (PAP) method for detection.^{1,2}

Biotin is a co-enzyme that participates in gluconeogenesis and fatty acid biosynthesis. Endogenous biotin is present in fairly large amounts in kidney, liver, pancreas, mammary gland, adipose tissue, and skeletal muscle,^{3,4} and has been reported recently in pregnancy related endometria.^{1,2}

Previous researchers have recommended blocking endogenous biotin with 0.05% free avidin and 0.05% free biotin to abolish cross reaction with endogenous biotin when using the streptavidin biotin complex/horseradish peroxidase (StreptABC/HRP) method.² An alternate recommendation to abolish endogenous biotin in gestational endometria suggested the use of a detection system, such as the PAP method, which would avoid the avidin/biotin blocking procedure.²

This study evolved from a case that was routinely investigated for HSV (Biomed, USA) using immunohistochemistry. The sections were pre-incubated with free avidin (0.1%) and free biotin (0.01%) (Dako, Glostrup, Denmark) prior to immunodetection for HSV using the StreptABC/HRP method as recommended by the manufacturer (Dako Biotin blocking system). A negative control was included using a parallel section in which the primary antibody was omitted. Despite using the blocking procedure, positive intranuclear immunoreaction was noted in the negative control, prompting this study.

Methods

Ten cases of gestational endometrial curettages were retrieved from the archives of the Department of Anatomical Pathology. All tissue had been fixed in formalin and embedded in paraffin wax. Cases were included in the study if intranuclear ground-glass inclusions were present within endometrial glands. The HIV status of these patients was negative.

All biopsy specimens were examined immunohistochemically using monoclonal anti-biotin (Dako) as the primary antibody, followed by the ABC method, the PAP complex method and the alkaline phosphatase anti-alkaline phosphatase method (APAAP), for the detection of endogenous biotin intranuclear inclusions.

IMMUNODETECTION SYSTEMS

ABC method

Sections were dewaxed and rehydrated. Endogenous peroxidase was quenched using 3%

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Figure 1 ABC system. Intranuclear inclusions show immunopositivity, (A) with and (B) without (albeit lighter) primary anti-biotin, in gestational endometrial glands after blocking endogenous biotin with free avidin/free biotin.

hydrogen peroxide in methanol for 30 minutes. All sections were pre-incubated with 0.1% avidin for 10 minutes, rinsed in phosphate buffered saline (PBS) (pH 7.6) and re-incubated with 0.01% biotin for 10 minutes, then washed in PBS. The sections were then incubated with 5% normal horse serum for 20 minutes, monoclonal anti-biotin at a 1 in 20 dilution for 30 minutes, biotinylated secondary horse-anti-mouse IgG antibody for 30 minutes, avidin/biotin complex for 30 minutes, and finally 0.1% diaminobenzidine tetrahydrochloride (DAB)/0.03% hydrogen peroxide for five minutes. Mayer's haematoxylin was used as the counterstain. The sections were then dehydrated and mounted in Entellen.

APAAP method

Sections were dewaxed, rehydrated, and then incubated with normal rabbit serum (diluted 1 in 5) for 20 minutes, monoclonal anti-biotin (diluted 1 in 20) for 30 minutes, secondary rabbit-anti-mouse IgG for 30 minutes, APAAP kit (mouse) (Dako) for 30 minutes, and finally with substrate solution—alkaline phosphatase containing naphthol AS-MX phosphate/Fast Red TR as the chromogen. Mayer's haematoxylin was used as the counterstain and the

sections were mounted in an aqueous medium (Gycergel; Dako).

PAP method

Sections were dewaxed, rehydrated and quenched as for the ABC method, and then incubated with normal rabbit serum (diluted 1 in 5) for 20 minutes, monoclonal anti-biotin (diluted 1 in 20) for 30 minutes, rabbit-anti-mouse IgG secondary antibody for 30 minutes, PAP (rabbit) for 30 minutes, and finally with DAB as described for the ABC method. In addition, sections were rinsed in PBS/bovine serum albumin (BSA) for 2×5 minutes between every step.

Negative controls omitting the primary antibody were included, using normal serum as a substitute for the primary antibody in all three immunodetection systems.

Results

ABC METHOD

Biotin was detected in the inclusions in all 10 cases (fig 1) even though endogenous biotin was blocked with free avidin/biotin as recommended previously.² A positive immunoreaction was also seen in the negative control sections, coinciding with the intranuclear inclusions.

APAAP METHOD

This system was used as an alternative detection system, with monoclonal anti-biotin as the primary antibody to eliminate false positivity resulting from excess endogenous peroxidase or biotin. Endogenous biotin was detected within the intranuclear inclusions of the endometrial glands in all 10 cases (fig 2). The negative control sections were free of any non-specific staining in the endometrial glands and decidualised stroma.

PAP METHOD

Biotin was detected in all 10 cases within intranuclear inclusions in endometrial glands. Biotin was not detected within the endometrial glandular intranuclear inclusions in any of the negative control sections, in which anti-biotin was omitted.

However, two of the 10 cases showed aberrant positive staining in the negative controls. This positive reaction was present in the nuclei and cytoplasm of adjacent endometrial glandular epithelium without intranuclear inclusions (fig 3). Occasional decidual stromal cells also showed a similar aberrant immunoreaction within nuclei and cytoplasm. These two cases were then studied using a dual PAP/APAAP immunohistochemical detection system in which the primary antibody, anti-biotin, was omitted in the initial PAP procedure. After washing in PBS, sections were then immunostained using the APAAP method with monoclonal anti-biotin as the primary antibody. This double PAP/APAAP system did indeed show that the aberrant PAP positive cells were distinctly separate from the APAAP positive, endogenous biotin-containing intranuclear inclusions.

Figure 2 APAAP system. (A) Positive immunoreaction for biotin within intranuclear inclusions. (B) Negative control sections were free of any non-specific staining reaction.

Figure 3 PAP system. Aberrant positive immunoreaction in endometrial glands without intranuclear inclusions in negative control sections (large arrows). This was subsequently eliminated with microwave pretreatment. Note the adjacent intranuclear inclusions are negative (small arrows).

The two cases with the aberrant immunoreaction with the PAP detection system without primary anti-biotin were investigated further. Sections were pretreated in a microwave oven⁵

in 0.01 M citric acid buffer (pH 6.0) (2 × 5 minutes) at 700 watts, were allowed to cool in buffer for 25 minutes, and then rinsed in PBS. The PAP method was repeated, again without primary antibody. The non-specific immunoreaction seen in the cytoplasm/nuclei of endometrial glands (without intranuclear inclusions) and adjacent decidualised stroma was completely eliminated.

Discussion

Intranuclear inclusions in gestational/puerperal endometrial glands resemble HSV infection with peripheral margination of chromatin and central clearing of nuclei producing a ground-glass appearance.⁶ This morphological appearance may quite easily be mistaken for HSV infection. However, these inclusions have recently been shown to contain biotin.^{1,2} Hence, false positivity when using the StreptABC/HRP immunodetection system is a very real problem for the unwary.^{7,8} By blocking endogenous biotin with free avidin and free biotin,² it was anticipated that the false positivity associated with the StreptABC/HRP immunodetection system would be abolished. In our study, all 10 cases with intranuclear inclusions in gestational endometrial glands showed positive immunoreaction with the StreptABC/HRP system, with and without primary anti-biotin, even when endogenous biotin was quenched. This clearly shows that the StreptABC/HRP system is unreliable in the presence of endogenous biotin.

Our study also confirmed previous studies showing that the gestation-associated endometrial intranuclear inclusions are in fact endogenous biotin. This was accomplished using anti-biotin with two independent immunodetection systems (APAAP and PAP) that are related to neither avidin nor biotin. The use of the APAAP system has not been reported previously in this context. Surprisingly, however, aberrant staining in the nuclei and cytoplasm of adjacent endometrial glands and the decidual stromal cells was observed with the PAP system. This was also the case in the negative control sections without primary anti-biotin. These false positive cells seen with the PAP system could be clearly distinguished from the cells with biotin-containing intranuclear inclusions with the dual PAP/APAAP system. Further investigation using microwave pretreatment with citric acid buffer totally eliminated the aberrant false positivity in the PAP negative controls. Therefore, we recommend that studies on gestation/puerperal endometria using immunohistochemistry and in situ hybridisation should either use the APAAP or PAP immunodetection systems, the latter incorporating microwave pretreatment. Both systems avoid the problems related with endogenous biotin.

The aberrant false positivity associated with the negative control of the PAP system reaffirms the need for negative controls on all sections in every immunohistochemical run. Nevertheless, further studies with a variety of tissues, fixatives and pretreatment systems are warranted to

investigate the as yet uncharacterised aberrant false positivity associated with the PAP system.

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Supraregional Assay and Advisory Service

Notification of Tenders Accepted by the SAAS Management Executive Board

In response to tenders received, the following laboratories have been designated to offer the Supraregional Assay and Advisory Service in the areas of expertise as follows:

Uncommon Metabolic Thyroid Diseases

Department of Clinical Biochemistry

Director: Dr P R Raggatt

Addenbrooke's Hospital NHS Trust, Hills Road, Cambridge CB2 2QQ

Tel: 01223 217156; fax: 01223 216862

Metabolic Bone Disease

Department of Clinical Biochemistry

Director: Professor C P Price

St Barts & Royal London School of Medicine & Dentistry, Turner Street, London E1 2AD

Tel: 0171 377 7241; fax: 0171 377 1544

Department of Chemical Pathology

Director: Dr Trevor Gray

Northern General Hospital NHS Trust, Herries Road, Sheffield S5 7AU

Tel: 0114 243 4343; fax: 0114 256 0472

Department of Clinical Chemistry

Director: Dr William D Fraser

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Tel: 0151 706 4257; fax: 0151 706 5806

Department of Chemical Pathology

Director: Dr Angela Fairney

St Mary's Hospital, Praed Street, London W2 1NY

Tel: 0171 725 1097; fax: 0171 725 1097

The standard of responses to the invitations to tender were very high, and the Supraregional Assay and Advisory Service Board compliment all the laboratories who bid for these assays on the quality of their presentations.

The Board are currently soliciting tenders for the supply of Assay and Advisory Services in the area of Porphyrin Metabolism. Enquires about this area may be made from the Chairman, Professor Vincent Marks, at the following address:

Professor Vincent Marks

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