Immunohistochemical expression of tumour-associated glycoprotein and polymorphic epithelial mucin in the human endometrium during the menstrual cycle

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An epitope defined by a second generation murine monoclonal antibody (LU-BCRU-G7) produced against a breast tumour-associated fucosylated glycoprotein of \(M_1\) 230 000 was found to exhibit phase specific reactivity in human endometrium during the menstrual cycle. Distribution and cycle related expression of the LU-BCRU-G7 determinant was different from the staining patterns observed with antibodies that react with the polymorphic epithelial mucins, HMFG 1 and NCRC 11, as determined by immunohistochemistry. Initial expression of the LU-BCRU-G7 determinant was associated with the peri-basal region of glandular epithelial cells with maximal staining during the mid-secretory phase. Diffuse cytoplasmic staining was also observed from day 10 to day 26, with a marked increase in the early secretory phase. Reactivity of NCRC 11 also showed maximal expression in the mid-secretory phase with no reactivity detected in early and mid-proliferative phases. The HMFG 1 defined epitope exhibited the opposite pattern of expression with maximal reactivity in the proliferative phase and little or no reactivity in the secretory phase. These findings suggest that expression of the LU-BCRU-G7, HMFG 1 and NCRC 11 defined determinants in the human glandular epithelium of the endometrium is differentially hormonally regulated, and may be of value as markers for endometrial function.

Introduction

The inability to determine the functional state of human endometrium and its readiness for implantation remains a stumbling block for improving pregnancy rates in in vitro fertilization (IVF). Since morphological assessment of the endometrium cannot predict its functional state as defined by specific protein production (Bell, 1990), the endometrial response to implantation must be determined using biochemical markers of function. The involvement of glycosylated components in many cell–cell and cell–substrate interactions (Kojima and Hakomori, 1989) suggests that glycoconjugates expressed by the epithelium of the endometrium represent likely candidates for mediating specific events surrounding implantation. They could be of value as markers in this role providing their expression follows a controlled pattern during the luteal phase (Bell and Drife, 1989). Evidence from both animal and human studies on glycoconjugates expressed by glandular epithelium of the endometrium supports the view that glycosylation changes are associated with implantation (Anderson et al., 1986; Aplin, 1991). Studies of the glandular epithelium of the breast, which in common with the endometrial epithelium exhibits hormone dependency, have shown similar alterations in the glycosylation state with respect to differentiation as determined by lectins (Walker, 1983), and antibodies, i.e. NCRC-11 (Price et al., 1985) and HMFG 1 (Arklie et al., 1981).

In the study reported here we have assessed and compared the staining patterns of three monoclonal antibodies raised against glycoconjugate antigens expressed by breast epithelium: the anti-human milk-fat globule membrane antibody HMFG 1 (Taylor-Papadimitriou et al., 1981) and the anti-human breast cancer-associated antigen NCRC 11 (Ellis et al., 1984), both of which are reactive against the high molecular weight polymorphic epithelial mucins (PEMs) and the other murine monoclonal antibody was raised against a specific glycoprotein isolated from human breast carcinomas (Rye and Walker, 1989). It was proposed to determine their reactivity in glandular epithelium of the endometrium during the menstrual cycle, to ascertain whether, as in the breast, they exhibit hormone-dependent expression.

Materials and Methods

Tissues

Endometrial tissues were obtained from dilatation and curettage (D and C) operations, e.g. investigation for dysmenor-
rhoea, association with laporascopic sterilization and infertility investigation. Patients selected had no apparent endocrinological problems and no local organic pathologies. All tissues were obtained from 20–30 year old patients, who had a history of regular cycles. Tissues were immersed in 4% formaldehyde in saline and routinely processed for paraffin wax embedding.

Serial 5 μm paraffin wax sections were used for routine haematoxylin and eosin staining and for routine immunohistochemistry. Endometrial specimens were dated from the last menstrual period and were used in the study only if there was corroborration by independent histological dating which followed standard histological criteria (Hendrickson and Kempson, 1980). A total of 36 specimens were collected; there were 3, 7 and 9 from the early, mid- and late proliferative phases, respectively, and 6, 4 and 9 samples from the early, mid- and late secretory phases, respectively.

Antibodies

The murine monoclonal antibodies used in this study were HMFG 1 (Unipath, Bedford), NCRC 11 (gift from M. R. Price and I. Ellis, Nottingham), and LU-BCRU-G7, a second generation murine monoclonal antibody derived from a polyclonal antiserum P5252 (Rye and Walker, in press) that was raised against a fucosylated glycoprotein identified in medium from primary cultures of human breast carcinomas (Rye and Walker, 1989).

The polyclonal antiserum P5252 was coupled to sepharose 6B (Pharmacia-LKB, Milton Keynes) in borate buffer (pH 11). Culture medium from the human breast cell line HS578T was applied to the column in Tris buffer (pH 8.6) and bound glycoprotein was eluted with acetic buffer (pH 3.6). This preparation was identified as a broad band of Mr 230 000 on SDS-PAGE and was used as the immunogen for raising the monoclonal antibody LU-BCRU-G7.

This second generation antibody was raised using an in vitro immunization method. For each immunization, a spleen cell suspension was prepared from a 3-week-old BALB/c mouse and it was resuspended in 30 ml of IVIM; DMEM (Dulbecco’s Modified Eagles Medium) supplemented with 2 mmol L-glutamine 1⁻¹, 1 mmol sodium pyruvate 1⁻¹, 100 i.u. penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹, 10% thymoma cell medium (Sigma, Poole), 1% MEM non-essential amino acids (Sigma Chemicals) and 15% fetal calf serum containing 5 × 10⁻⁶ mol 2-mercaptoethanol 1⁻¹. The P5252 affinity isolate was added to the cell suspension at a concentration of 1 μg ml⁻¹, and the spleen cell suspension was seeded in a 75 cm² flask at 10⁶ cells ml⁻¹ and incubated for 5 days undisturbed at 37°C in an atmosphere of 5% CO₂, 95% air. After incubation, the flask contents were agitated to dislodge the loosely adherent blast cells and the resulting cell suspension was centrifuged at 500g for 5 min. Adherent cells were scraped off, washed in serum-free DMEM, and seeded in the fusion plates. After washing the non-adherent blast cell pellet once in serum-free DMEM, the cells were fused with 5–8 × 10⁵ cells of the myeloma line SP2-0-Ag14 in the presence of 50% polyethylene glycol (1500 kDa). After diluting with 40 ml SDMEM; DMEM containing 10% FCS, 2 mmol L-glutamine 1⁻¹ and 1% MEM non-essential amino acids, aliquots of the cell suspension were dispensed into 96 16 mm wells, and incubated at 37°C in an atmosphere of 5% CO₂, 95% air. After 24 h, 0.5 ml of SDMEM containing the additional supplements at double strength, hypoxanthine–aminopterin–thymidine (HAT) medium, 20% thymoma cell medium (Sigma) and 20% hybridoma supplement (Sigma) were added to each well. Half the medium was changed every 3 days using SDMEM with single strength supplements. The supernatant from each well was assayed after day 12, using a cell based ELISA method using the human breast cancer cell line HS578T, which is known to express the 230 000 kDa glycoprotein. Positive wells were identified, cloned by serial dilution and retested for antibody production. Antibody typing was determined by a murine isotyping kit (Amersham International, Aylesbury).

Of those monoclonal antibodies generated against the high molecular weight glycoprotein (Mr, 230 000), only one exhibited variable staining in the endometrium, and was determined to be of the IgM class, with kappa light chain. This antibody was given the notation LU-BCRU-G7.

Immunohistological techniques

Before applying the primary antibody, all sections were blocked with non-immune rabbit serum. Endogenous peroxidase activity in tissue sections was inhibited by incubation with 0.3% hydrogen peroxide in ultra pure water for 30 min. The mouse monoclonals LU-BCRU-G7, HMFG 1 and NCRC 11 were used at 1/5, 1/20 and 1/10 000 dilution respectively, before using an indirect peroxidase anti-peroxidase (PAP) detection system (Sternerberger, 1979).

Positive staining was graded according to an arbitrary four-point scale taking into account the intensity of glandular staining and the proportion of positively stained cells and glands. A score of 4 indicates intense staining of more than 95% of glands compared with a score of 1 which indicates occasional or very weak glandular epithelial staining.

Results

The three monoclonal antibodies tested, HMFG 1, NCRC 11 and LU-BCRU-G7, showed a variable pattern of staining in glandular endometrium during the menstrual cycle, using the indirect PAP detection method. A schematic representation of the degree of staining throughout the cycle for all three antibodies is shown (Fig. 1).

Early proliferative phase (days 5–7)

During the early phase of the menstrual cycle the glandular epithelium showed only minimal diffuse cytoplasmic or no immunoreactivity at all with the LU-BCRU-G7 antibody (Fig. 2a). No reactivity was detected with NCRC 11 at this phase of the cycle. Strong reactivity was observed with the monoclonal antibody HMFG 1 which was localized at the gland luminal border. No cytoplasmic or stromal staining was observed.

Mid-proliferative phase (days 8–10)

From days 8 to 10 there was diffuse cytoplasmic staining of glandular cells with LU-BCRU-G7 (Fig. 2b). Reactivity was noted in a few cells of some glands that showed staining localized at
Fig. 1. Graphical representation of antibody reactivity (a) HMFG 1, (b) NCRC 11 and (c) LU-BCRU-G7 in individual samples (o) of the endometrium during the menstrual cycle. The degree of change in intensity and numbers of cells staining was graded according to an arbitrary four-point scale. A score of 4 indicates intense staining of more than 95% of glands compared with a score of 1 which indicates occasional or very weak glandular epithelial staining. EP: early proliferative; MP: mid-proliferative; LP: late proliferative; ES: early secretory; MS: mid-secretory; LS: late secretory.

The peri-basal and glandular luminal membranes. Some generalized stromal staining was observed of minimal intensity and some cases showed areas of strong localized stromal reactions. Reactivity of NCRC 11 was the same as described in the previous phase (Fig. 3a). The pattern of reactivity with HMFG 1 was the same as that described for the previous phase with no apparent change in staining intensity (Fig. 4b).

Early secretory phase (days 15–18)

From days 17 to 19 stromal staining with LU-BCRU-G7 was detected only in one sample. Staining of the glandular epithelium was greater than that found in the late proliferative phase (Fig. 2d). The staining of the peri-basal region of glandular epithelial cells persisted, and more cells showed cytoplasmic reactivity. The NCRC 11 antibody showed luminal membrane reactivity in most glands, with staining of a greater intensity than in the previous phase (Fig. 3b and c). Reactivity with HMFG 1, although still apparent at this phase of the cycle, showed that staining intensity and numbers of cells staining were reduced (Fig. 4c). Some weak reactivity with glandular secretions was also noted.

Mid-secretory phase (days 19–23)

From days 20 to 26 there was a marked increase in intensity and number of cells staining with LU-BCRU-G7 in the glandular epithelium (Fig. 2e). Staining was localized at the peri-basal and luminal gland borders, with diffuse cytoplasmic reactivity. The glandular epithelial cell staining observed was expressed in over 50% of all cases studied. Stromal staining was either minimal or entirely absent. Reactivity with NCRC 11 was intense and localized at the glandular luminal membrane as detected in the previous phase. There was negligible staining of the cytoplasm of the glandular epithelium. There was also weak reactivity of NCRC 11 with glandular secretions. No reactivity was detected with HMFG 1.

Late secretory phase (days 24–28)

The staining distribution for LU-BCRU-G7 in glandular epithelial cells was similar to that described for the previous phase, but much weaker in intensity (Fig. 2f). Some moderate localized and weak general stromal staining was also observed. Reactivity of NCRC 11 was much weaker than in the mid-secretory phase. Staining with NCRC 11 of tissues dated at day 28 showed minimal staining of most glands with only a few showing the intense staining observed in previous phases (Fig. 3d). No reactivity was detected with HMFG 1 (Fig. 4d).

Discussion

In the study reported here we assessed the menstrual cycle phase specific reactivity of three breast tumour-associated monoclonal antibody defined epitopes. HMFG 1, NCRC 11 and a novel second generation murine monoclonal antibody LU-BCRU-G7. The antibodies are known to react with high molecular weight glycoconjugates: HMFG 1 and NCRC 11 with a group of high molecular weight mucins termed polymorphic epithelial mucin (PEM), and LU-BCRU-G7 with a fucosylated glycoprotein expressed in breast carcinomas (P. D. Rye and R. A. Walker, unpublished). The reactivity of these antibodies in epithelium of the glandular endometrium revealed three individual patterns. The epitopes of both NCRC 11 and
LU-BCRU-G7 showed maximal expression in the mid-secretory phase of the cycle and HMFG 1 showed an opposite pattern with no reactivity detected in the mid- and late secretory phases of the cycle.

The NCRC 11 and LU-BCRU-G7 antibodies show a decrease in reactivity during the late secretory phase, which is characterized by decidualization of the stroma and falling oestrogen and progesterone concentrations. The coincidence of rising progesterone concentrations and LU-BCRU-G7 reactivity suggests that this hormone may be responsible for the increased reactivity of LU-BCRU-G7 in glandular endometrium. Further studies of first trimester tissues would be required to confirm this link.

The variation of HMFG 1 staining during the cycle was not apparent when an alkaline phosphatase–anti-alkaline phosphatase (APAAP) detection system was used (S. C. Bell, unpublished). Cytoplasmic localization of staining for HMFG 1 in the endometrium has recently been noted (Graham and Aplin, 1991). Although this was not detected in our current study using the indirect PAP detection method, glandular epithelial cytoplasmic staining was detected with HMFG 1 antibody when using the APAAP technique (S. C. Bell and G. Waines, unpublished). These findings suggest that there is a quantitative variation throughout the cycle for this epitope which is undetectable when using more sensitive immunocytochemical detection techniques.
Fig. 3. Photomicrographs of endometrial tissues immunostained with monoclonal antibody NCRC 11, using the indirect PAP method and counterstained with haematoxylin. (a) Mid-proliferative phase. No staining in both early and mid-proliferative phase (scale bar = 50 µm). (b) Early secretory phase. Intense staining at the gland luminal border (scale bar = 20 µm). (c) Early secretory phase. Gland showing staining of secretions (scale bar = 20 µm). (d) Late secretory phase. Staining much weaker than in previous phase with only some reactivity persisting at the gland luminal borders (arrowed) (scale bar = 20 µm).

Fig. 4. Photomicrographs of endometrial tissues immunostained with monoclonal antibody HMFG 1, using the indirect PAP method and counterstained with haematoxylin. (a) Mid-proliferative phase. Intense gland luminal border staining (scale bar = 20 µm). (b) Late proliferative phase. Luminal border staining similar to previous phase (scale bar = 20 µm). (c) Early secretory phase. Reduced level of reactivity in the glandular epithelium. Staining where present was very weak (scale bar = 50 µm). (d) Late secretory phase. As with mid-secretory phase no staining was detected (scale bar = 20 µm).
The localization of LU-BCRU-G7 reactivity throughout the cycle suggests that the epitope resides on a secretory component. This is supported by the original isolation of the glycoprotein from medium conditioned during primary culture of breast carcinomas (Rye and Walker, 1989). However, staining of endometrial secretions was absent. Preliminary epitope mapping studies suggest that the epitope is a carbohydrate since binding of the LU-BCRU-G7 antibody can be blocked by normal human milk, and 0.1 mol D-glucosamine 1−1 (P. D. Rye, unpublished). Since the epitope appears to be of a glycosylated nature, it is possible that the LU-BCRU-G7 reactivity observed in this tissue is a result of crossreactivity between carbohydrate groups shared with the fucosylated glycoprotein identified in breast cancer and an unidentified endometrial protein. The loss or modification of these carbohydrate groups before secretion in the endometrium may account for the lack of staining in the luminal secretions. The pattern of reactivity of the LU-BCRU-G7 epitope is similar to that identified by the monoclonal antibody B72.3 (Johnson et al., 1986; Soisson et al., 1989), which also recognizes a high molecular weight glycoconjugate (TAG-72) identified in breast carcinomas. However, it is unlikely that this is the same as the LU-BCRU-G7 epitope since it is described as having characteristics that identify it as a mucin (Hanisch et al., 1989).

Staining of secretions was noted for NCRC 11 and to a lesser degree for HMFG 1; this was expected as these antibodies are known to react with the PEMs as detected in breast glandular epithelium. However, the opposite pattern of expression observed throughout the cycle with these antibodies was unexpected, and suggests that NCRC 11 recognizes a discrete subgroup of antigenic components within the PEM group. This may also apply to the pattern of expression observed with the monoclonal antibody B72.3 (Johnson et al., 1986; Soisson et al., 1989).

The altered binding patterns of antibodies and their epitopes in human endometrium during the menstrual cycle are not novel phenomena and indeed are not surprising considering the nature and function of this tissue. A number of groups have identified endometrial secretory proteins with secretory phase specific expression: prolactin (Maslar and Riddick, 1979), S1-5 (Strinden and Shapiro, 1983), EP9, 11 and α,2-PEG (Bell et al., 1986; Waites et al., 1988; Bell, 1990). However, proteins that have been studied exhibit increased synthesis and secretion in the late secretory phase, and are therefore unlikely to be involved in the initial phases of implantation. Other studies (Aplin, 1991; Seif and Aplin, 1990) have identified components of a glycoconjugate nature which show better correlation of expression with the early secretory phase. The reactivity patterns in human endometrium as seen with HMFG 1, NCRC 11 and LU-BCRU-G7 emphasize the potential importance of glycoconjugates as unique markers of endometrial function, particularly of secretory glandular epithelium. Further study of the reactivity patterns of these and other glycoconjugate targeted antibodies in endometrial biopsies from abnormal menstrual cycles and infertile patients could provide a valuable contribution to the study of fertility.

This work was supported in part by the Cancer Research Campaign. We thank S. Dearing and G. Waites for technical assistance.

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