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High Rate of Expression of Multidrug Resistance-associated P-Glycoprotein in Human Endometrial Carcinoma and Normal Endometrial Tissue

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The overexpression of P-glycoprotein was studied in 10 normal endometrial controls (five from the proliferative and five from the secretory phase of the menstrual cycle) and in 23 endometrial carcinomas of different histological varieties, using the C219 and JSB-1 monoclonal antibodies. Three of the tumours had been previously treated with combination chemotherapy containing doxorubicin. All endometrial carcinomas, whether treated or untreated, as well as the normal endometrial controls from both the proliferative and the secretory phase of the menstrual cycle, overexpressed P-glycoprotein. This puts endometrial carcinoma into the same category as other tumours arising in organs which normally overexpress P-glycoprotein, all of which tend to be intrinsically resistant to chemotherapy.
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INTRODUCTION

P-GLYCOPROTEIN, a membrane-bound extrusion pump, is a mediator of multidrug resistance in experimental tumour cells, and seems also to play a role in the resistance to chemotherapy of human tumours [1–3]. Recently, the presence of P-glycoprotein in clinical tumour specimens has been correlated with their relapse rate after chemotherapeutic treatment [4]. More remarkably, the presence of P-glycoprotein has been inversely correlated with the survival of patients independently of the kind of treatment received by them [5]. It thus seems that P-glycoprotein may be of use for clinicians not only for discriminating which tumours will eventually respond or not to a certain kind of chemotherapy, but also as a powerful general prognostic factor. To define these applications of P-glycoprotein measurements in the future, however, detection and evaluation methods must still be optimised, to allow for the comparison of results between different investigators. One of the most widely employed assays for the detection of this and many other tumour markers in the clinical setting is immunohistochemistry, which is gaining acceptance as both a practical and reliable diagnostic method. One of its main advantages, for clinical purposes, over biochemical methods is the preservation of tissue architecture. This allows detection of P-glycoprotein in small cell subsets down to the single cell level, to determine its distribution in both tumoral and normal tissue and to account for the cellular heterogeneity present in every tumour. On the other hand, it is less sensitive than biochemical methods such as RNA and DNA measurements by means of cDNA probes, widely employed by other groups, which, however, are carried out on bulk tissue

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containing variable proportions of normal and neoplastic cells, both of which can express P-glycoprotein [6,7]. Another unsettled issue regarding immunohistochemistry is which of the various monoclonal antibodies developed against P-glycoprotein, several of which are by now commercially available, are best suited for future routine clinical use. They all recognise different epitopes of the P-glycoprotein molecule, require different fixation techniques and cross-react differently with normal tissues.

We have employed immunohistochemistry previously, using monoclonal antibodies to study P-glycoprotein overexpression in a variety of human tumours, including mammary, ovarian and cervical carcinomas [8–11]. In the present investigation, we have studied a series of endometrial adenocarcinomas, including the most common histological variants of this tumour. We have used two different monoclonal antibodies which are commercially available (C219 and JSB-1). Furthermore, we have also studied P-glycoprotein overexpression in normal endometrial tissue from both the proliferative and secretory phase of the menstrual cycle.

MATERIALS AND METHODS

Tumour and normal tissue specimens

The tumour specimens were obtained from patients of the University Department of Obstetrics and Gynaecology (Hospital de Cruces), Bilbao, Spain, either at initial surgery or by means of hysteroscopy. They were immediately snap-frozen in liquid nitrogen and stored at −70°C until they were used for the present investigation. 23 tumour samples and 10 normal endometrial biopsies (five from the proliferative and five from the secretory phase of the menstrual cycle, obtained by means of microcurettage) were studied. 20 tumours were from untreated patients, three from patients treated with five, four and three courses, respectively, of CAP polychemotherapy (cyclophosphamide 500 mg/m^2, doxorubicin 50 mg/m^2, cisplatin 50 mg/m^2). The clinical characteristics of the investigated patients, together with the pathological features of their tumours, are summarised in Table 1.

Monoclonal antibodies (Mab)

The C219 Mab (Centocor, Malvern, U.S.A.) was originally developed by Kartner *et al.* [12] using cell wall extracts from multidrug-resistant Chinese hamster ovary cells. It recognises two epitopes on the internal domain of the P-glycoprotein molecule. To make them accessible to the antibody, the membrane must be either air-dried or, better, subjected to acetone fixation. These epitopes do not resist well conventional fixation of tissues with formalin. When used for immunohistochemistry, the C219 antibody yields a crisp, mainly membrane-bound reaction, although some degree of cytoplasmic staining is always observable, especially at low degrees of expression of the antigen. Among the drawbacks of this antibody is its known ability to cross react with some normal tissues, such as connective tissue, striated muscle, normal mammary tissue and brain capillaries. This, however, can be easily identified by means of immunohistochemistry. We found the optimal concentration of this antibody to be 10 μg/ml when used with frozen tissue sections, like the ones of this investigation.

The second monoclonal antibody used by us, JSB-1 (Sanbio, Uden, The Netherlands), was developed by Scheper *et al.* [13] against complete multidrug-resistant Chinese hamster ovary cells. Nevertheless, it also recognises an internal epitope of P-

Table 1. Clinical data of the patients and P-glycoprotein expression of the tumours studied

Patient	Age	Histology	Stage	Grade	Treatment	C219	JSB-1
1	49		Ia	G1	–	++(c)	+ (c)
2	54		Ia	G1	–	++(c)	+ (c)
3	60		Ia	G1	–	++(c)	+ (a)
4	61		Ia	G1	–	++(c)	++(c)
5	55		Ib	G1–2	–	++(c)	++(c)
6	57		Ib	G1	–	++(c)	++(c)
7	62		Ib	G1	–	+ (a)	+ (a)
8	56	Adenocarcinoma	Ib	G1	–	+ (c)	++(b)
9	67		Ib	G1	–	+ (a)	++(c)
10	61		Ib	G1	–	+ (c)	+ (c)
11	76		IIa	G1	–	++(c)	++(c)
12	55		IIIb	G2	–	+ (c)	+ (b)
13	56		IIIb	G3	–	++(c)	++(c)
14	72		IIIb	G2	–	+ (c)	+ (b)
15	70		IVb	G3	–	++(c)	++(c)
16	53	Adenoacanthoma	Ia	G1	–	+ (c)	+ (c)
17	70	Adenosquamous	Ib	G2	–	+ (c)	+ (c)
18	57	Clear cell	Ib	G1	–	++(c)	++(c)
19	64	Serous papilloma	Ic	G1	–	++(c)	++(c)
20	56	Serous papilloma	IIIa	G1	–	+ (c)	++(c)
21	56		IIIb	G3	CAP × 1	++(c)	++(c)
22	77	Adenocarcinoma	IIa	G1	CAP × 4	++(c)	+ (b)
23	60		Ib	G1	CAP × 5	++(c)	+ (c)

+ Membrane-bound reaction, lower than positive controls; ++ membrane-bound reaction, equal to positive controls.

(a) Less than 10% of tumour cells positive; (b) 10–50% of tumour cells positive; and (c) 50–100% of tumour cells positive.

C : Cyclophosphamide; A : doxorubicin; P : cisplatin.

glycoprotein, and the cell membrane must be permeated in a way similar to the one described for C219. When used for immunohistochemistry, it shows a higher degree of cytoplasmic reaction, mainly localised at the Golgi apparatus, than C219. Its membrane-bound reaction, when present, is however as strong as the one described for C219. We used it at the concentration recommended by the manufacturer, 20 μg/ml, which we also found in a test series to represent the best compromise between specific signal strength and background staining.

Immunohistochemistry

The immunohistochemical technique employed has been described previously in detail [9,10]. Briefly, cryostat sections of the tumour and normal tissue samples, 6 μm thick, were made. They were allowed to dry overnight, then fixed in cold acetone at −20°C and stored at −20°C until they were processed. To perform the immunohistochemical procedure, the sections were rehydrated in phosphate-buffered saline, then incubated with normal sheep serum (1:10) for the blocking of unspecific binding to the second bridge antibody. Afterwards, the monoclonal antibodies were applied at the concentrations described above, and incubated overnight at 4°C. The second, sheep anti-mouse biotinylated antibody (1:50) and the streptavidin-biotinylated horseradish peroxidase complex (1:100) were applied in successive steps. The reaction was developed by means of 3-amino-9-ethylcarbazole, yielding a red reaction product. The preparations were finally counterstained with Mayer's haematoxylin and mounted with glycerol gelatine. Negative controls for each sample were carried in parallel by incubation with IgG fraction of normal preimmune mouse serum substituted for the first antibody, at the same concentration, the rest of the procedure being carried out as described. As positive controls we used doxorubicin-resistant sarcoma-180 cell smears and preparations from a solid sarcoma-180 nude-rat xenograft resistant to daunorubicin, expressing high levels of P-glycoprotein [14].

RESULTS

23 endometrial carcinomas and 10 normal endometrial specimens were studied for the presence of P-glycoprotein by means of immunohistochemistry using two different monoclonal antibodies (Tables 1 and 2).

All analysed specimens, either from untreated (Fig. 1a) or treated (Fig. 1c) tumours, or from proliferative (Fig. 2a) or secretory normal endometrium (Fig. 2c) were positive for P-glycoprotein expression. The reaction was localised mainly at the apical border of the cells, towards the luminal end, when the glandular pattern of the tumour was well conserved (Fig. 1a). This is the typical pattern of P-glycoprotein expression in tissues with an excretory function, such as described by Deuchars and Ling [3]. When the glandular structure had been lost in solid, undifferentiated tumours (Fig. 1c), P-glycoprotein was localised pericellularly, at the membrane of the whole tumour cell. Two different patterns of staining intensity could be distinguished at the single cellular level: (1) mainly membrane-bound, with some degree of concomitant cytoplasmic staining, the membrane reaction being of lower intensity than the one shown by the positive controls (+); (2) heavily membrane-bound, still with some discernible degree of cytoplasmic staining and an intensity of the membrane reaction equal to that of the positive controls (++). The staining was especially strong and qualitatively different in 2 of the 3 cases previously treated with chemotherapy, which showed a strongly membrane-bound reaction and a coarseness of the deposit similar to the one described by us previously for P-glycoprotein overexpression in experimental, carcinogen-induced hepatomas [15]. An example of this is shown in Fig. 2(c), corresponding to patient no. 22.

Table 2. P-glycoprotein expression in normal endometrial tissue. All specimens showed overexpression in 50–100% of endometrial glandular cells

Specimen	C219	JSB-1
Proliferative		
1	+	++
2	++	+
3	++	++
4	++	++
5	++	++
Secretory		
6	+	+
7	+	++
8	++	+
9	++	++
10	++	++

+ Membrane-bound reaction, lower than positive controls; and ++ membrane-bound reaction, equal to positive controls.

The number of reactive tumour cells also varied between specimens, and the results were classified into three different categories according to it: less than 10% staining cells (a); between 10 and 50% staining cells (b); from 50 to 100% staining cells (c).

As can be seen from Table 1, the two antibodies used yielded the same results for staining intensity and percentage of reactive tumour cells in 13 out of 23 cases (56% agreement). However, if only either of the two categories is considered (i.e. only staining intensity or staining distribution), the results are coincident in 15 and 17 out of the 23 total cases (65 and 70% agreement, respectively). Thus, the results are overall comparable for both antibodies.

DISCUSSION

P-glycoprotein expression in endometrial tissue has been previously studied by Arceci *et al.* [16] in the mouse uterus. They found overexpression in the secretory epithelium of the pregnant uterus, but no expression in the normal, non-pregnant endometrium. Thiebaut *et al.* [17], using the MRK 16 antibody, were among the first to screen normal human organs for the expression of P-glycoprotein by means of immunohistochemistry. They found no expression in the uterus. However, it is not stated in their paper whether premenopausal (i.e. functional) endometrial glands were part of the specimen or specimens studied. The myometrium, indeed, which accounts for more than 95% of the uterine volume, does not express P-glycoprotein. Even endometrial stroma, which is a large part of the endometrial lining, does not express P-glycoprotein (e.g. Fig. 2a of this paper). The same may apply to the results reported by Hitchins *et al.*, who studied a variety of normal human tissues by means of immunoblotting using the C219 Mab. Immunoblotting is carried out on a homogenate of all the different tissues present in a biopsy. It is thus not surprising that they found no P-glycoprotein expression in their five "uterus" samples studied.

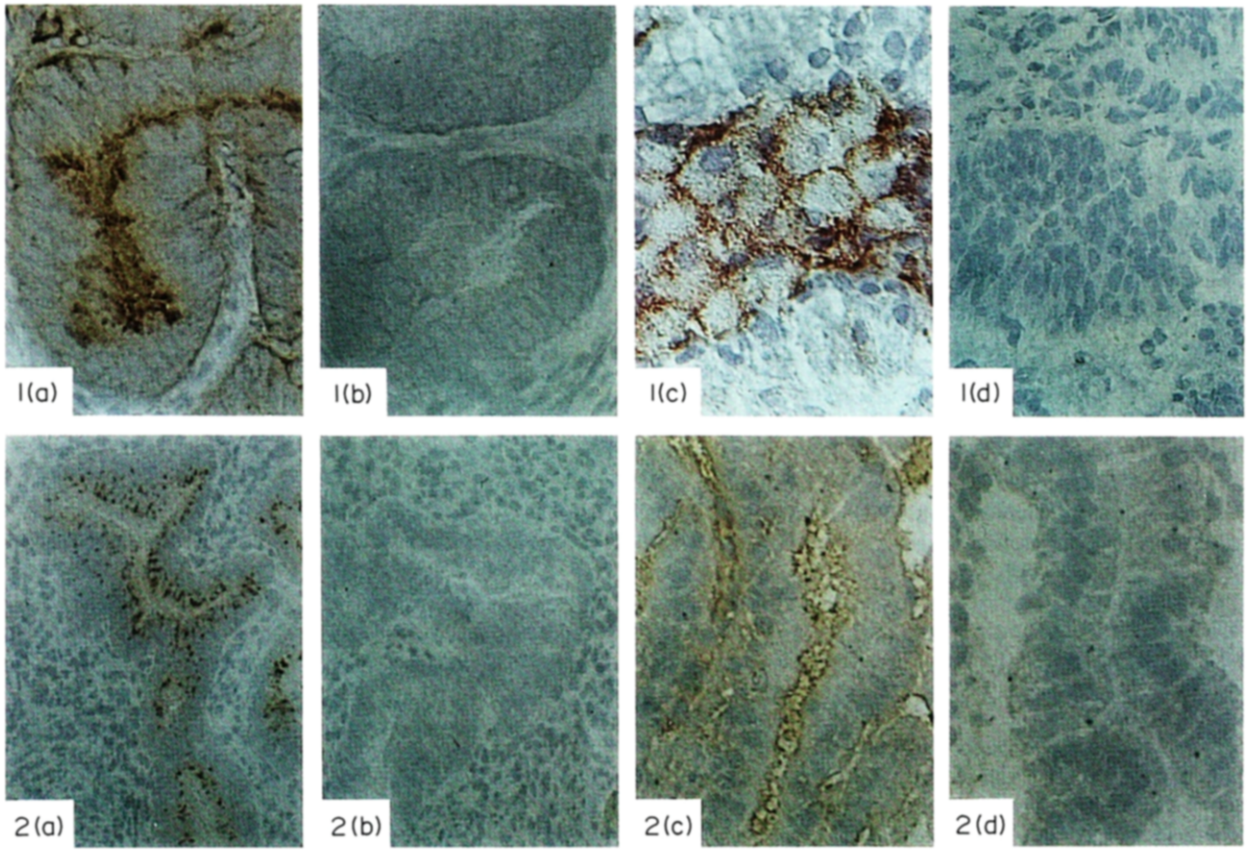

Fig. 1. Immunohistochemical detection of P-glycoprotein in endometrial carcinoma and normal endometrial tissue. Streptavidin-biotin-peroxidase method. C219 monoclonal antibody. Comparable results were obtained with the JSB-1 Mab. 1(a) Untreated, well-differentiated endometrial carcinoma. 1(b) Negative control of 1(a). 1(c) Endometrial carcinoma treated with four courses of CAP. 1(d) Negative control of 1(c). 2(a) Normal proliferative endometrium. 2(b) Negative control of 2(a). 2(c) Normal secretory endometrium. 2(d) Negative control of 2(c).

The proportion of endometrial cells expressing P-glycoprotein vs. myometrial cells in a biopsy comprising the whole uterine wall is insignificant, as has been said, and this necessarily dilutes the concentration of the antigen. This may explain why Hitchins *et al.* also failed to detect P-glycoprotein expression among 12 kidney samples in that same study. However, proximal renal tubular cells are known to express P-glycoprotein in a constant fashion, while the rest of the organ does not [3].

In the present study, we found that all endometrial carcinomas and all normal endometrial glands expressed P-glycoprotein. This is consistent with the concept that P-glycoprotein is an efflux pump, forming part of a widespread cellular transport system. Tumours derived from organs naturally expressing P-glycoprotein, such as the colon, the kidney or the pancreas, are all known to be intrinsically resistant to therapy [1–3]. This also applies to endometrial carcinoma. Thipgen *et al.* [18] studied the sensitivity of endometrial carcinoma towards all currently used chemotherapeutic agents. Surprisingly enough, they found doxorubicin (a classic MDR compound) to be the most effective one. The response rate of endometrial carcinoma to doxorubicin, however, which is in the range of 20–30%, is extremely low if compared to typically chemosensitive tumours (e.g. germ-cell tumours), whose response rate is above 80%, so that the contradiction is only apparent. It may also be that the P-glycoprotein isoform naturally detected on endometrial carcinoma has not a preferential affinity for doxorubicin. This would be supported by the findings of Choi *et al.* [19], who found slightly different P-glycoproteins, with just one amino acid change (which they attributed to spontaneous mutation), to elicit overlapping but distinct multidrug resistance patterns. Along this same line, Devault and Gros [20], through transfection experiments, demonstrated that the mouse MDR1 and MDR3 genes encode for different P-glycoproteins, both of which produce the complete multidrug resistance phenotype, albeit with distinct drug specificities. So, they found that cells transfected with the MDR1 gene were more resistant against colchicine and doxorubicin, whereas those transfected with the MDR3 gene showed a preferential resistance against actinomycin-D. The antibodies employed by us recognise both MDR1 and MDR3 expression products. It is possible that some endometrial carcinomas express a higher proportion of the MDR3 isoform of P-glycoprotein. In the light of the just cited reports, this would explain a slightly different multidrug resistance pattern and the partial response to doxorubicin reported by Thipgen *et al.* [18]. Some batches of the antibodies employed by us (C219 and JSB-1), finally, have been shown by Finstad *et al.* [21] to cross-react with blood group A antigenic determinants, simulating a positive P-glycoprotein reaction in normal ovaries, fallopian tubes and ovarian carcinoma from blood group A carriers. All our specimens, and not only those of blood group A carriers, expressed P-glycoprotein. It is also not to be expected that both antibodies, from two different manufacturers, belonged to one of the false-reacting batches detected by Finstad *et al.* So far, we are confident that our results are consistent. Their finding, however,

is highly worrying and mandates for even more strict internal controls in the future.

The fact that P-glycoprotein was expressed by all the tumours of this study partly explains why these tumours are usually resistant to chemotherapy, although other mechanisms must certainly be involved. However, since all endometrial carcinomas seem to express P-glycoprotein, this cannot be used as a qualitative prognostic factor, as is the case with soft tissue sarcoma and mammary carcinoma. These tumours do not normally express P-glycoprotein, and if they do so, this is usually associated with an ominous prognosis. So, Chan *et al.* [5] found that the presence of P-glycoprotein in any amount negatively influenced the prognosis of children with soft tissue sarcoma. For mammary carcinoma, Verrelle *et al.* [4] also found a significant correlation between the recurrence rate and P-glycoprotein expression of locally advanced cases treated by means of chemotherapy. Furthermore, they attempted a semiquantitation of their immunohistochemical results. Using their scale, the highest level of P-glycoprotein expression defined by it, which largely corresponds to the "++(c)" level of our own scale, was associated with a significantly worse prognosis. However, Verrelle *et al.* used only one antibody in their study. From our own results using two different antibodies and an observed discordance of at least 30% between them, we would be very reluctant to quantitate the results of immunohistochemistry. Still referring to locally advanced mammary carcinoma, Ro *et al.* [22], finally, report a significantly higher response rate to induction chemotherapy in tumours not expressing P-glycoprotein before treatment when comparing them to their P-glycoprotein-negative counterparts. The conclusion seems to be that P-glycoprotein is only useful as a prognostic factor in those tumours that do not naturally express it. In those with high intrinsic levels of P-glycoprotein, like endometrial carcinoma, it would only predict a general resistance to chemotherapy, something we already knew from experience. The clinical implications are, however, more far-reaching than this. Multidrug resistance can be reversed experimentally by a variety of drugs, among which the best known are verapamil and trifluoperazine, which unfortunately are of limited use in practice due to severe collateral cardiac toxicity. Other less toxic compounds are currently under investigation [23]. One good candidate for multidrug resistance reversal, on purely theoretical grounds, would be tamoxifen. It is known that tamoxifen, an oestrogen receptor antagonist, which is active against several gynaecological tumours, among which also endometrial carcinoma [24], is also capable of completely reversing multidrug resistance *in vitro* [25]. Tamoxifen has also been extensively used together with multidrug resistant drugs such as doxorubicin and 4-epirubicin in the treatment of mammary carcinoma, and no alarming toxic effects have been reported from this association. Clinical trials combining multidrug resistant agents with such relatively innocuous multidrug resistance modulators would be thus warranted in the immediate future.

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