Pergamon

0960-0760(94)E0060-Y

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 4–6, pp. 365–372, 1994

0960-0760/94 $7.00 + 0.00

Immunocytochemical Versus Biochemical Receptor Determination in Normal and Tumorous Tissues of the Female Reproductive Tract and the Breast

Alain G. Zeimet, Elisabeth Müller-Holzner, Christian Marth and Günter Daxenbichler*

Department of Obstetrics and Gynecology, Innsbruck University Hospital, Anichstrasse 35, 6020 Innsbruck, Austria

The development of highly specific and sensitive monoclonal antibodies directed against human estrogen (ER) and progesterone receptors (PR) provides a new approach in precise histochemical receptor location independent of hormone binding. Over the years receptor determination was the domain of the radioligand-binding assay, in which receptors are measured by tritiated ligand and unbound ligand is removed by the dextran-coated charcoal (DCC) procedure. Presented here are the results and experiences obtained by the classic DCC and the immunocytochemical method in the different normal and tumorous tissues of the female reproductive tract and the breast. The results of both methods were compared, and overall concordance of the results was found to vary considerably among the different types of tissue analyzed. Best agreement (86%) was found for PR determination in breast cancer, and the lowest rate of concordance for ER determination in fibrocystic disease of the breast. Special attention was directed toward the heterogeneity of receptor distribution in the specimens examined. In all tissues investigated, ER and PR were located in the nuclei of cells in both paraffin and frozen sections. Staining intensity varied among different cell types and from cell to cell for a single cell type, as well as in tumorous and normal tissues. In breast cancer, randomly scattered single cell receptor positivity was distinguished from focal/clonal positivity. Paraffin-embedded lymph node metastases showed significantly weaker staining as compared with their respective primary tumors. In the normal ovary, the corpus luteum and the stromal layer of the outer cortex were revealed as highly receptive elements for progestins, whereas ER was barely demonstrable in the normal ovary. Benign serous and mucinous ovarian tumors showed opposite ER and PR distribution among the stromal and epithelial components. Of special interest were the highly significant changes in ER and PR content in the stromal and glandular cells of the different layers of the normal endometrium throughout the menstrual cycle.

J. Steroid Biochem. Molec. Biol., Vol. 49, No. 4–6, pp. 365–372, 1994

INTRODUCTION

Estrogen and progesterone receptors (ER; PR) are members of a superfamily of nuclear regulatory proteins, which modulate the activity of target genes through interaction with their respective ligand [1–3]. Moreover, PR is an estrogen inducible protein in mammalian target tissues and thus represents a good tool for the evaluation of the functional integrity of the estrogenic action. Since the end of the 19th century, in breast cancer estrogens are known to stimulate tumor growth and promotion [4]. By reason of therapeutic implications of the steroid receptor status in breast and endometrial cancers [5–7], most of the research on ER and PR expression has focused on these malignancies. Little, however is known about the exact distribution of ER and PR in benign lesions and in the different components of normal breast and the reproductive tract.

In breast and endometrial cancer, ER and PR status has been evaluated over the years in homogenized tissue using binding assays such as the dextran-coated charcoal (DCC) method and, more recently, enzyme

Proceedings of the XVI Meeting of the International Study Group for Steriod Hormones, Vienna, Austria, 28 Nov.–1 Dec. 1993.
*Correspondence to G. Daxenbichler.

immunoassay. However, immunocytochemical (ICA) methods have been successfully applied to demonstrate the exact subcellular and tissue distribution of both ER and PR in frozen [8, 9] and paraffin-embedded [10, 11] sections. Cytosolic receptor determinations are quantitative methods, which can easily be subjected to external quality control (e.g. the EORTC receptor study group quality control program), whereas only first attempts at quality control and standardization were made by D. Barnes for the semiquantitative ICA methods (personal communication). Disadvantages of biochemical methods are the need for important equipment and relatively large amounts of representative tissue. ICA, however, can use the same, even small specimen as for histopathological examination. The latter argument will become more and more important in light of the fact that early detection screenings focus on tumors with small diameters.

In the present contribution we aim to give an overview of our experiences in receptor determination with the DCC-assay and ICA in different normal and tumorous tissues of the female genital tract and the breast.

MATERIALS AND METHODS

Biochemical receptor determination

ER and PR assessment by DCC technique has been described in detail elsewhere [12]. Briefly, tissue cytosol aliquots were incubated with tritium-labeled estradiol or R5020 in the presence or absence of an unlabeled competitor for the receptor. The difference in radiolabel between non-competed and competed tubes following removal of unbound steroid with DCC represented the specifically bound radioligand. Receptor concentration was related to the protein content of the cytosol.

Protein estimations of cytosol samples were performed using the protein-dye reagent method as described by Bradford [13]. Tumors were considered receptor positive if at least 20 fmol/mg protein were present.

Immunocytochemistry

Frozen sections. Tissue samples were immediately frozen after surgical removal, and cryosections were stored at −80°C. The specimens were processed and stained with the ER-ICA and PR-ICA kit using the monoclonal antibodies H222 and K68, respectively (Abbott Labs, North Chicago, IL), without deviating from the recommended procedure.

Paraffin sections. Tissue samples were routinely fixed in 4% buffered formaldehyde (pH = 7.2) for 8–12 h; then specimens were paraffin-embedded at 56°C. Four-micron-thick paraffin sections were baked at 56°C for 1 h, deparaffinized in xylene (2 × 10 min) and immersed in two changes of 100% alcohol followed by three changes of 95% alcohol and one change of 50% alcohol for 1 min each. After incubation with a blocking reagent (Abbott Labs,) for 15 min, antibody KD68 of the Abbott Kit PR-ICA was used as primary antibody diluted 1:10 overnight. Thereafter, sections were washed with Triton X-100 (0.5%) containing phosphate buffered saline (PBS) for 2 × 5 min. For antigen staining the biotin–avidin system [14] was applied with biotinylated goat anti-rat immunoglobulin-G (IGG) (Chemicon, Temecula, CA) and peroxidase-conjugated extravidin (Biomarkor; Rehovot, Israel), both diluted at 1:1600. Finally, the sections were exposed to diaminobenzidine and counterstained with hematoxylin.

For negative control, parallel sections were processed in the same way as described except that an equivalent amount of non-immune rat IGG was used instead of the specific antibody. Slides from the Abbott kits with cells of known ER and PR positivity served as positive controls.

ICA scoring. ICA staining was evaluated by two independent observers. Differences >10% were resolved by consensus. The histoscore (HScore) was obtained by multiplying the intensity of staining (*i*: 0–3) by the proportion (in %) of staining cells. To better discriminate cyclic changes in endometrial receptor expression, the intensity scale was especially adapted (*i*: 1–5) for semiquantitative evaluation of normal endometria.

Statistical analysis

This was performed with the BMDP software package. Data were analyzed by non-parametric tests. Differences in median values were evaluated with the Mann-Whitney U-test. Correlations were estimated with the Spearman rank correlation coefficient. *P* values <0.05 were considered significant.

RESULTS

Normal breast and benign lesions

In normal breast tissue DCC determination showed ER concentrations ranging from 1 to 58 fmol/mg protein with a median value of 10 fmol/mg. Similar results were obtained for PR, with a range from 1 to 108 fmol/mg and a median value of 8 fmol/mg.

More informative, however, are the findings obtained by ICA in cryosections of the normal mammary gland. All sections analyzed contained a small but distinct population of ER and PR positive cells. Approximately 10% of total epithelial cell number showed nuclear staining for ER, in contrast to a significantly higher rate of about 24% nuclear staining for PR ($P < 0.05$). In postmenopausal women ICA revealed a tendency to higher ER than PR content (ER/PR ratios: premenopausal 0.22 vs postmenopausal 1.6). Receptor positive cells were distributed as scattered single cells, with highest frequency and intensity of staining in the lobules as compared to interlobular ducts.

Myoepithelial and stromal cells were found to be negative for ER and PR.

In fibrocystic disease graded I or II according to Prechtel [15], biochemically negative samples (62% for ER and 53% for PR) were contrasted by a 100% rate of positivity for both receptors when analyzed by ICA. No significant difference between normal and fibrocystic tissue was revealed by the DCC method, neither for ER (median value: 16 fmol/mg) nor for PR-content (median values: 21 fmol/mg). This was contrasted by ICA results, which showed a significantly higher staining intensity and frequency of positive cells for both receptors, predominantely with increasing grade of fibrocystic disease ($P < 0.03$). Unlike normal breast tissue, specific immunostaining was often noticed in cell groups and in some cases the majority of epithelial cells, lining lobules and ducts were found to be receptor positive.

Breast cancer

Biochemical ER values and semiquantified ER-ICA results showed overall agreement in 84% of 341 samples ($r = 0.64$; $P < 0.001$). Twenty-one cases (6%) were biochemically negative but were classed as receptor positive in ICA. In 33 cases (10%) positive DCC results were opposed by negative ICA outcome. The relationship between biochemical PR determination and PR-ICA resulted in an overall agreement of 86% ($r = 0.71$; $P < 0.001$). Five percent of the discordant cases were biochemically negative and ICA positive. The remaining 9% of cases were inversely positive in DCC-assay and negative in ICA.

In addition to a heterogeneity in intensity of nuclear staining, ICA investigations often revealed a marked distributional heterogeneity of receptor-containing cells in the same tumor. Among individual tumors, two main distribution patterns were found in more than 600 breast cancers examined for this purpose. A randomly scattered single cell positivity was distinguished from a focal staining pattern. In the latter, receptor positivity was either confined to small clusters of tumor cells, interspersing negative tissue at random, or occurred patchwork-like in larger areas. These findings may be one of the reasons for the discrepancy between ICA and DCC results.

Lymph node metastases

ICA investigations were made of 151 paraffin-embedded lymph node metastases and the corresponding primary breast carcinomas of 50 patients for their PR content. Reliability of PR-ICA on routinely fixed and paraffin-embedded tissue was shown by comparison with the immunostaining on frozen sections and values of DCC-PR, resulting in a concordance of 94% ($P < 0.0001$) and 80% ($P < 0.0001$), respectively.

Generally, PR content in metastases was lower than in primary tumors, ($P < 0.001$) and a marked heterogeneity in PR expression of metastases was noticed (Fig. 1). All metastases of PR negative primary tumors ($n = 14$) were negative. Metastases of carcinomas with more than 60% positive cells ($n = 9$) were PR positive, but 7 of these cases showed a markedly lower receptor content as compared to primary tumors. In malignancies with 10 to 60% PR positive tumor cells ($n = 27$), metastases again showed weaker immunostaining or were even negative for PR. Only in 5 cases was PR content found to be higher in metastases than in primary tumors.

Normal ovary

In biochemical assay ER was barely detectable in the examined cytosol fractions of normal ovarian tissue. Concentrations ranged from 1 to 26 fmol/mg, resulting in a median value of 7 fmol/mg. Cystolic PR content was found to be significantly higher as compared to ER values ($P < 0.001$). PR concentrations were distributed between 30 and 360 fmol/mg, and the obtained median value was 81 fmol/mg. In order to reveal a possible age dependency in estrogen and progesterone receptivity of the normal ovary or to establish an interference of endogenous steroids in receptor detection with labeled ligands, samples were subdivided into a premenopausal ($n = 16$) and a postmenopausal ($n = 19$) group. Neither ER nor PR was found to be significantly different in either group.

In agreement with biochemical data, ICA showed only weak staining for ER in 2 (6%) of 35 frozen sections. In these cases ER occurred in several clusters of stromal cells in the outer ovarian cortex. In contrast, no ER was detectable in the thecal or granulosa cells, or in the germinal epithelium covering the surface of the ovary (Table 1). Cells lining the occasionally encountered inclusion cysts, which are presumed to derive from the germinal epithelium were also found to be negative for ER.

A completely different situation was found in the distributional pattern of PR. In 33 (94%) of the 35 specimens a distinct immunoreactivity was detected in stroma cell layers adjoining the small acellular zone of the outer cortex of the normal ovary. In only 40% ($n = 14$) of the samples was PR poorly demonstrable in the stromal cells of the medulla. In most of these cases immunostaining was attributable to perivascular stromal cells. Unlike ER, PR was persistently demonstrable in the germinal epithelium and in epithelial cells lining inclusion cysts. On average 85% of the surface epithelial cells showed moderate to high expression of PR, whereas up to 100% of the epithelial cells of inclusion cysts contained PR. The corpus luteum was identified as a further constituent of high receptivity for progestins. While no specific immunostaining was apparent in follicular granulosa cells, between 20 and 60% of granulosa lutein cells stained positively for PR (Table 1).

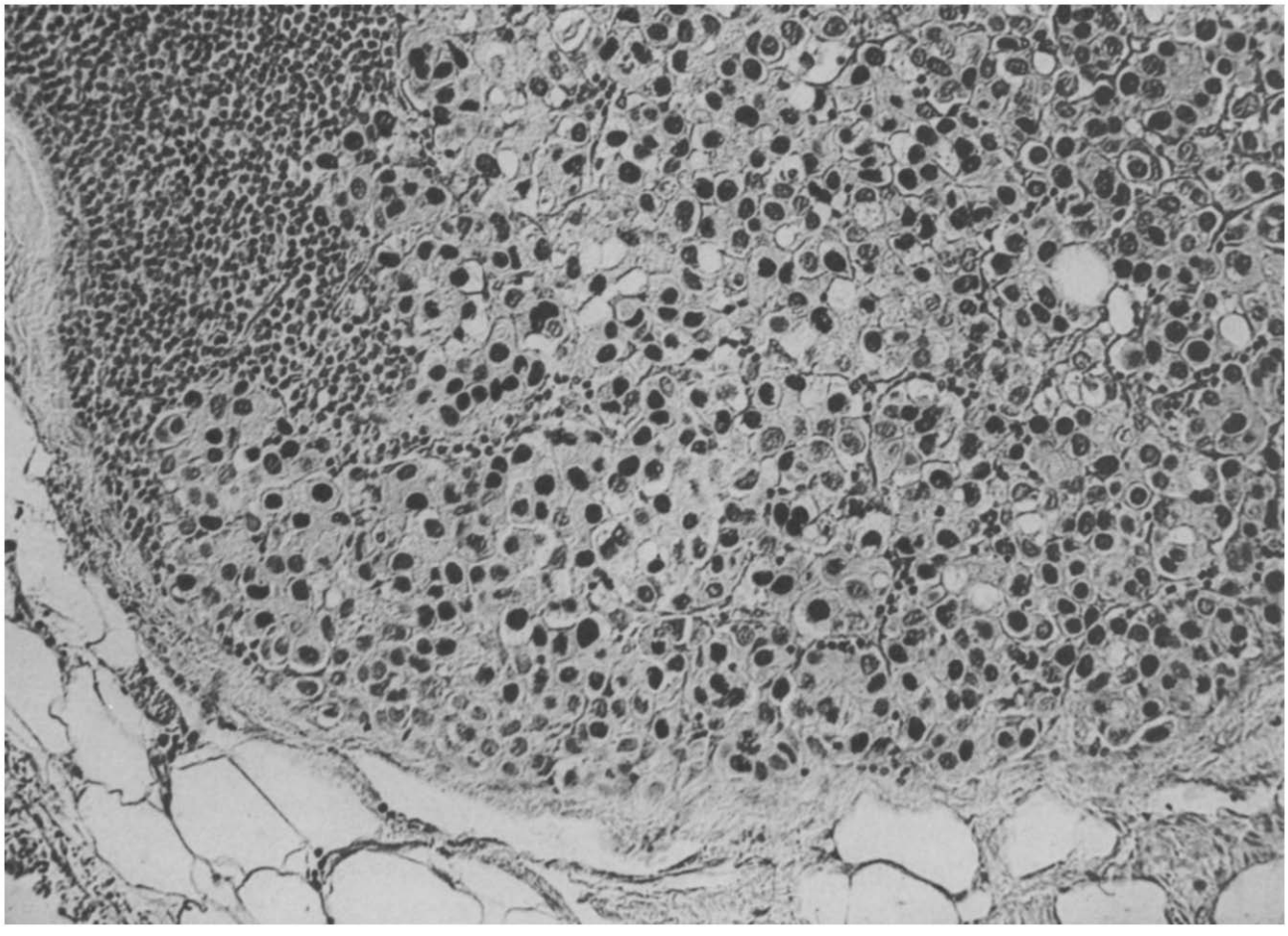

Fig. 1. Demonstration of PR in a paraffin section of a lymph node metastasis.

Common benign epithelial ovarian tumors

Biochemical receptor determination resulted in a significantly higher PR than ER content in the benign ovarian tumors ($P < 0.01$). While differences in ER content between serous and mucinous cystadenomas were not significant (median value: 12 and 7 fmol/mg, respectively), significantly ($P < 0.01$) higher PR expression was found in serous (median value: 75 fmol/mg; range 55–123) as compared to mucinous tumors (median value: 21 fmol/mg; range 6–30).

Table 1. *Summary of ICA results in normal and tumorous ovarian tissues*

	ER	PR
Normal ovary		
Stroma cortex	(+)	+ + +
Stroma medulla	–	(+)
Granulosa cells	–	–
Lutein cells	–	+ +
Germinal epithelium	–	+ + +
Benign serous tumors		
Epithelium	+ +	+ + +
Stroma	(+)	(+)
Benign mucinous tumors		
Epithelium	–	+
Stroma	+/(+ +)	+ + +
Ovarian cancers		
Serous	+ +	+ +
Mucinous	(+)	+
Endometrioid	+ +	+ + +
Clear cell	–	(+)
Undifferentiated	–	–

In ICA, 7 of the 10 examined serous cystadenomas and adenofibromas showed moderate staining for ER in the single-layered or pseudostratified epithelial lining of the cyst. In general, no specific staining for ER was exhibited in the stroma of these benign neoplasms. Mucinous cystadenomas were found to be receptive for estrogens in 4 of 15 cases (26.6%). These tumors showed very weak immunostaining in randomly scattered stromal cells of the cyst wall. In none of the examined sections, however was ER present in the mucin-filled epithelial cells lining larger cysts, small acini or daughter cysts. In PR-ICA a high degree of expression was revealed in the flattened and cuboidal epithelium lining serous cysts. In 8 of 10 samples high intensity PR immunostaining was observed in about 70% of cells. The stroma of the cyst walls, as well as that of the papillary structures, showed either weak immunostaining or was negative for PR. Contrarily, 73% (11/15) of the mucinous tumors were shown to

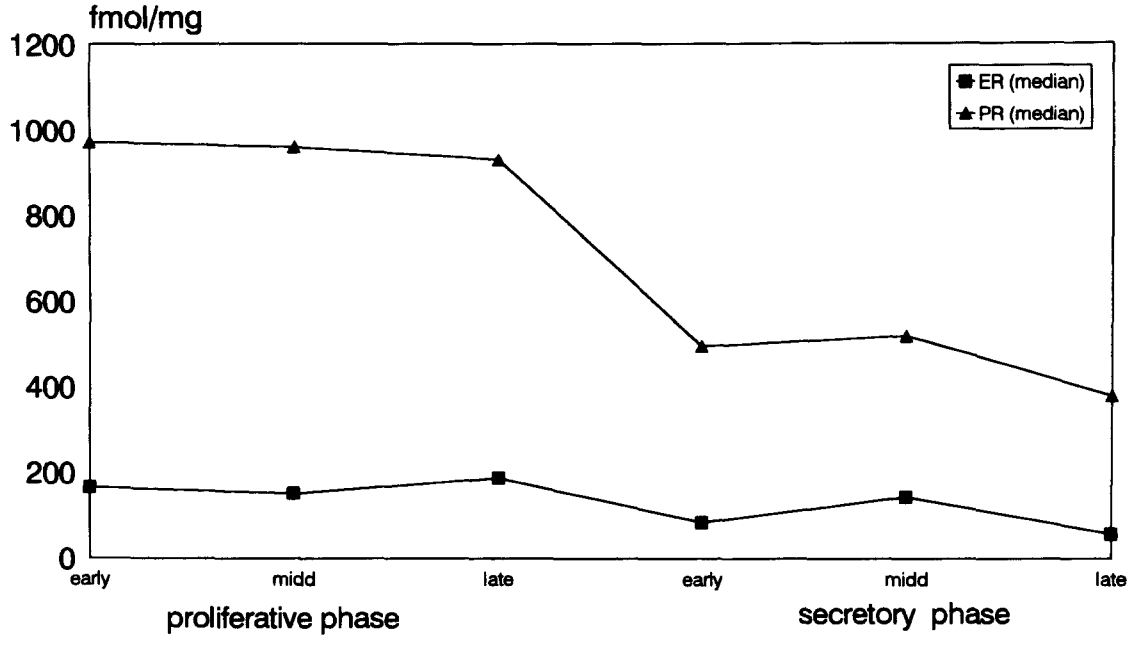

Fig. 2. Biochemically determined ER and PR content of normal endometria of the different cycle phases, as shown by the respective median values.

contain detectable PR in the stromal component. Only 3 of these 11 tumors showed faint nuclear staining in the mucinous epithelium, and in only 1 single case the mucinous cells lining the cysts were seen to be highly positive for PR. The remaining mucinous cystadenomas were completely deprived of PR (Table 1).

Epithelial ovarian cancer

ER positivity was biochemically demonstrated in 57% ($n = 34$) and PR positivity in 53% ($n = 32$) of the 60 investigated cytosol fractions. Only 33% ($n = 20$) of the specimens, however, were found to be positive for both steroid receptors. Concentrations determined in malignant ovarian tissue resulted in an equal median value of 28 fmol/mg for ER and PR. Mucinous and clear cell carcinomas tended to have especially poor receptor expression. Compared to all other histological types, endometrioid cancers showed a significantly higher ER and PR content ($P < 0.05$).

In ovarian cancer ER- and PR-ICA staining was confined to tumor areas with a high degree of differentiation. In endometrioid and serous cystadenocarcinomas the presence of ER- or PR-containing tumor cells was strongly associated with well differentiated glandular and papillary structures. Mucinous and undifferentiated carcinomas generally, exhibited neither ER nor PR. Immunocytochemical results obtained in the different histological types of ovarian cancer are summarized in Table 1.

Normal endometrium

Changes in median values throughout the menstrual cycle as determined by DCC-ER and -PR in 53 cytosol fractions are summarized in Fig. 2. In most cases DCC-PR values (median: 720 fmol/mg) were higher than DCC-ER levels (median: 164 fmol/mg) ($P < 0.001$). Endometria of the early and late secretory phase contained significantly lower ER concentrations as compared to the other cycle phases ($P < 0.05$). PR was found to be higher in the proliferative phase than in the secretory phase ($P < 0.02$).

In ICA, ER was detected in the vast majority of epithelial and stromal cells from endometria of the early, middle, and late proliferative phase. Throughout the secretory phase a reduction in immunostaining was noticed in both layers, the basalis and functionalis, resulting in a weak and scattered ER positivity in stromal and glandular cells of the functionalis. In contrast, strong staining was observed in epithelial cells of some glands in the basalis. In agreement with biochemical results, the immunostaining obtained in the functionalis and basalis by PR-ICA was substantially stronger than that obtained by ER-ICA. During the proliferative phase stromal and glandular PR increased in the functionalis. In the postovulatory period a continuous decrease in staining intensity was noticed in all the components of the normal endometrium. In the late secretory phase glandular epithelium of the functionalis was absolutely PR negative. In contrast, some basalis glands showed moderate immunostaining for PR. Moreover, the stromal component of the basalis and functionalis remained

positive for PR. ICA results from the different components of the endometrium are depicted in Figs 3 (a and b).

Endometrial cancer

In the biochemical assay 65% of the 45 investigated specimens showed ER concentrations >20 fmol/mg (median value: 57 fmol/mg). These concentrations were significantly lower than those measured in normal endometria of the proliferative phase ($P < 0.05$), but were not different from those obtained in endometria of the secretory phase. At a median value of 137 fmol/mg, DCC-PR values tended to be higher than cytosolic ER content, but this difference did not reach statistical significance. It is worth mentioning that PR content determined in the normal endometria was significantly higher than that measured in endometrial cancer ($P < 0.01$).

Only 45% of the investigated cryosections were classed ER-ICA positive. Of these samples, 11 were considered weakly positive. In contrast, PR-ICA resulted in a positive rate of 80%, whereas 14 cases were evaluated as only weakly PR positive. High

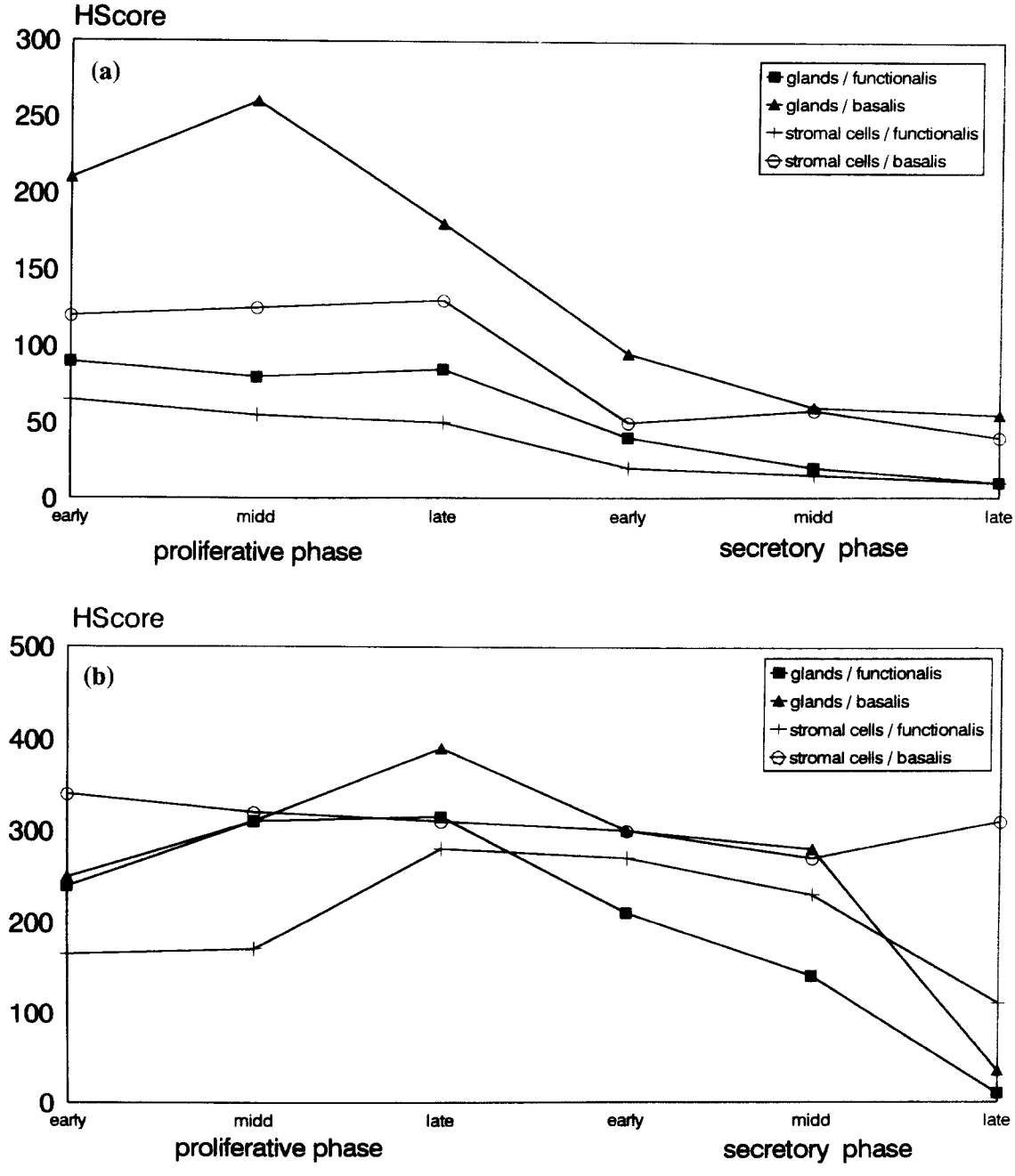

Fig. 3. Median values of semiquantitative ICA-ER (a) and -PR (b) evaluation for the various cycle phases and constituents of the normal endometrium.

Table 2. Advantages and disadvantages of the biochemical method

Principal advantages of biochemical determination:
(a) high reproductivity confirmed by regular external quality control.
(b) long-time acquisition of experience with the method and the data collected
Principal disadvantages of biochemical determination:
(a) limited control over quality and representativity of specimens
(b) no assessment of tissular heterogeneity or distribution pattern of receptors
(c) relatively high amounts of tissue required

expression of both receptors was predominately observed in well or moderately differentiated tumor areas. However, no ER and PR immunoreactivity was observed in the squamous elements of adenosquamous carcinomas and adenoacanthomas. In the majority of samples investigated, adjacent normal ER- and PR-containing endometrium and myometrium, were noticed. Overall concordance between DCC-assay and ICA in the discrimination of receptor positivity and negativity, was 75 and 80% for ER and PR, respectively. However, a clear tendency toward overestimation of the receptor content in cytosolic determination was revealed by direct comparison of the individual results.

DISCUSSION

Controversial opinions concerning the advantages and disadvantages of both methods for ER and PR detection hereby presented have been discussed in a number of publications [11, 16, 17]. The principal outcomes of this controversy are summarized in Tables 2 and 3. We feel that one of the most important points in this debate is that ICA is the most suitable method for examining tissular location and heterogeneity in receptor distribution, not only in the primary tumor but also in lymph node metastases and micrometastases when applying a method adapted for routinely fixed and paraffin-embedded sections [18]. Whether this heterogeneity in staining reflects polyclonal origin of the tumors or asynchrony of receptor expression due to physiological factors requires further investigation. However, in light of the scope of knowledge on autocrine and paracrine influences on tumor growth and progression [19, 20], intratumoral distribution of cells accessible for endocrine therapy appears to be of great significance in the choice of an individualized and appropriate therapeutic management. Gaskell *et al.* [21] reported that the proportion of cells staining gave a better prediction of hormonal responsiveness than did the intensity of staining or the receptor status determined biochemically. These results suggest that the percentage of receptor-containing cells is an important index of tumor heterogeneity and provides information supplementary to that obtained by biochemical assay of tissue extracts.

In endometrial cancer, PR expression seems to play a crucial role in prognosis of this disease [22]. In our opinion, ICA is the method of choice for this malignancy, because of the significant risk of sample contamination by receptor-containing normal endometrial and myometrial elements, which could lead to overestimation of the receptor content in homogenized tissue.

Furthermore, the use of ICA is not limited to evaluation of the hormonal dependency of malignant tumors. In complex normal tissues like the endometrium or the ovary this method makes it possible to study the exact receptor distribution among the different tissue elements and cell types, providing a better understanding of unresolved endocrinological questions.

The high concordance of results obtained with both methods (Table 4), together with one or the other decisive advantage of ICA as compared to DCC assay, has made ICA more than a second-line alternative in receptor determination. However, in order for ICA to overtake the classical DCC assay for diagnostic purposes, ICA must meet several further criteria. First of all, immunochemical procedures require standardization of each proceeding-step, beginning with unanimous use of a single, most suitable, commercially available antibody and a standard procedure, and ending with evaluation of the results according to an uniform scoring system. In addition, well defined standardized internal positive and negative controls are

Table 3. Advantages and disadvantages of the ICA method

Principal advantages of ICA:
(a) assessment of intratumoral distribution heterogeneity
(b) direct and simple control over representativity of examined specimens
(c) feasibility of the assay in very small amounts of tissue or cells of aspirates
(d) feasibility of the assay in paraffin-embedded tissue
Principal disadvantages of ICA:
(a) semiquantitative method that may depend on subjective factors of the observer
(b) limited standardization of assay procedures and internal controls
(c) absence of external quality controls

Table 4. Overall concordance between the results of DCC-assay and ICA for discrimination of receptor positivity and negativity

Tissue	ER (%)	PR (%)
Benign lesions (breast)	38	47
Breast cancer	84	86
Endometrial cancer	75	80
Benign ovarian tumors	85	77
Ovarian cancer	64	80

Biochemical cutoff value was set at 20 fmol/mg protein, and ICA threshold level was faint nuclear staining (+) in at least 10% of tumor cells.

needed together with regularly and rigorously performed external quality control. Furthermore, it is worth mentioning that the assay on cryosections presently remains the method of choice for ICA. Receptor determination using paraffin-embedded material should be limited to special applications, like determination of receptor status in small tumors as well as in lymph node metastases. Only after such a consensus is reached, will results be fully comparable, this being a prerequisite for an adequate external quality control and thus for general acceptance of this valuable method.

REFERENCES

1. Green S. and Chambon P.: A superfamily of potentially oncogenic hormone receptors. *Nature* **324** (1986) 615–617.
2. Beato M., Chaplepakis G., Schauer M. and Slater E. P.: DNA regulatory elements for steroid hormones. *J. Steroid Biochem.* **32** (1989) 737–748.
3. Baniahmad A. and Tsai M. J.: Mechanisms of transcriptional activation by steroid hormone receptors. *J. Cell. Biochem.* **51** (1993) 151–156.
4. Beatson G. T.: On treatment of inoperable cases of carcinoma of mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* **2** (1895) 104.
5. Kauppila A., Janne O., Kujansuu E. and Vihko R.: Treatment of advanced endometrial adenocarcinoma with combined cytotoxic therapy: predictive value of cytosol estrogen and progesterone receptor levels. *Cancer* **46** (1980) 2162–2167.
6. Reifenstein E. C.: The treatment of advanced endometrial cancer with hydroxyprogesterone caproate. *Gynec. Oncol.* **2** (1974) 377–414.
7. Cummings F. J., Gray R., Tormey D. C., Davis T. E., Volk H., Harris J., Falkson G. and Bennett J. M.: Adjuvant tamoxifen versus placebo in elderly women with node-positive breast cancer: Long-term follow-up and causes of death. *J. Clin. Oncol.* **11** (1993) 29–35.
8. King W. J. and Greene G. L.: Monoclonal antibodies localize estrogen receptor in the nuclei of target cells. *Nature* **307** (1984) 745–747.
9. Perrot-Applanat M., Logeat F., Groyer-Picard M. T. and Milgrom E.: Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology* **116** (1985) 1473–1484.
10. Müller-Holzner E., Zeimet A. G., Müller L. C., Daxenbichler G. and Dapunt O.: Monoclonal technique to aid decision on endocrine treatment in breast cancer. *Lancet* **i** (1989) 1147–1148.
11. Andersen J., Thorpe S. M., King W. J., Rose C., Christensen I., Rasmussen B. B. and Poulsen H. S.: The prognostic value of immunohistochemical estrogen receptor analysis in paraffin-embedded and frozen section versus that of steroid-binding assay. *Eur. J. Cancer* **26** (1990) 442–449.
12. Daxenbichler G., Forsthuber E. P., Marth C., Wiegele J., Margreiter R., Müller L., Hausmaninger H., Manfreda D. and Dapunt O.: Steroid hormone receptors and prognosis in breast cancer. *Breast Cancer Res. Treat.* **12** (1988) 267–273.
13. Bradford M.: A rapid and sensitive method of quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–256.
14. Hsu S. M., Raine L. and Fanger H. L.: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody PAP procedures. *J. Histochem. Cytochem.* **29** (1981) 577–580.
15. Prechtel K.: Mastopathie und altersabhängige Brustdrüsenveränderungen. *Fortschr. Med.* **89** (1971) 1312–1315.
16. McClelland R. A., Berger U., Miller L. S., Powles T. J., Jensen E. V. and Coombes R. C.: Immunocytochemical assay for estrogen receptor: Relationship to outcome of therapy in patients with advanced breast cancer. *Cancer Res.* **46** (1986) 4241s–4243s.
17. McCarty K. S. Jr, Szabo E., Flowers J. L., Cox E. B., Leight G. S., Miller L., Konrath J., Soper J. T., Budwit D. A., Creasman W. T., Seigler H. F. and McCarty K. S. Sr: Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res.* **46** (1986) 4244s–4248s.
18. Müller-Holzner E., Zeimet A. G., Daxenbichler G., Marth C., Müller L. C. and Dapunt O.: Progesterone receptors in routinely paraffin-embedded primary carcinomas and lymph node metastases. *Breast Cancer Res. Treat.* **25** (1993) 47–55.
19. Lippman M., Dickson R. B., Kasid A., Gelmann E. P., Davidson N., McManaway M., Huff K., Bonzert D., Bates S., Swain S. and Knabbe C.: Autocrine and paracrine growth regulation of human breast cancer. *J. Steroid Biochem.* **24** (1986) 147–154.
20. Westley B. R. and May F. E. B.: Estrogen regulates cathepsin D RNA levels in estrogen responsive human breast cancer. *Nucleic Acids Res.* **15** (1987) 3773–3786.
21. Gaskell D. J., Sangsterl K., Hawkins R. A. and Chetty U.: Relation between immunocytochemical estimation of oestrogen receptor in elderly patients with primary breast cancer and response to tamoxifen. *Lancet* **i** (1989) 1044–1046.
22. Creasman W. T.: Prognostic significance of hormone receptors in endometrial cancer. *Cancer* **71** (1993) 1467–1470.