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Immunohistochemical analysis of the topographical relationship between the estrogen- and progesterone receptor in five human breast cancers

A simultaneous demonstration of both nuclear receptors in the same section by using a computer-assisted image processing

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Summary. A technique is described which allows precise assessment of the topographical relationship between the estrogen receptor (ER) and the progesterone receptor (PR) in the same histological section. It is based on the analysis of the results of immunohistochemical double staining by computer-assisted image processing. Five human ductal breast cancers were examined. The simultaneous demonstration of both receptors consists in the following principal steps:

The primary antisera against the ER (monoclonal rat antibody) and the PR (monoclonal mouse antibody) are incubated simultaneously, but only the anti-ER antibody is demonstrated in the first staining step by using a goat anti-rat antibody as the linking antibody and the PAP complex from the rat, both antisera from the ER-ICA kit. The result is stored as a digitized grey image ("1. object image"). Then the colored end product and the residual peroxidase activity of the PAP complex are removed. In the second staining sequence the anti-PR-antibody is demonstrated by using a rabbit anti-mouse antibody as the linking antibody and the PAP complex from the mouse. The result is exactly positioned and also stored as a digitized grey image ("2. object image"). Though antibodies raised in different species were used, cross-reactivity could not be avoided. Grey values generated by cross-reactivity between the different antibody systems are evaluated in negative controls and are eliminated in the object images. The remaining (specifically stained) structures of both object images are copied into a final image so that the topographical relationship of the ER and the PR becomes obvious. The results show that in the five carcinomas investigated three types of receptor-positive tumour cells can be distinguished: Cells which coexpress the ER and the PR (1), cells which express either the ER (2) or the PR (3). The number of tumour cells showing one of these expression patterns varies from tumour to tumour.

Key words: Breast cancer – Estrogen- and progesterone receptor – Immunohistochemical double staining technique – Computer-assisted image processing

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Introduction

It is the generally held opinion that determination of progesterone receptor in early and advanced human breast cancer is an important factor for the predictability of hormone responsiveness. Cumulative data in the literature show that patients with breast cancer that are positive for estrogen- (ER) and progesterone receptors (PR) respond to endocrine therapy in about 70% of cases while patients with tumours positive for ER and negative for PR do so in only 30% of cases (Sedlacek and Horwitz 1985).

The biological significance of PR has been studied in different human breast cancer cell lines (Horwitz and McGuire 1978a, b; Romić-Stogković and Gamulin 1980; Devluschouwer et al. 1986; Lykkesfeld and Briand 1986; Reiner and Katzenellenbogen 1986). Several reports in the literature suggested that synthesis of PR in human breast cancer is under the control of a mechanism involving the ER (Horwitz and McGuire 1978a, b; Romić-Stogković and Gamulin 1980; Lykkesfeld and Briand 1986; Thorpe 1987).

Consequently the presence of the PR in breast cancer might be regarded as an index that the tumour is still hormone-responsive. However, it was

reported that in some tumour cell lines the synthesis of PR is not regulated by estrogens (Devluschouwer et al. 1986; Lykkesfeld and Briand 1986; Reiner and Katzenellenbogen 1986).

These various findings in different tumour cell lines highlight the diversity of hormone responsiveness and favor the view that different functional relationships between ER and PR may exist in human breast cancer. Morphological data showing the topographical relationship between both receptors may make an important contribution to these findings.

In the present report, an immunohistochemical technique is described which allows assessment of the topographical relationship between the ER and the PR in the same histological slide of tumour samples using computer-assisted image processing. We investigated five human ductal breast cancers using this technique.

Materials and methods

Five human ductal breast cancers with different receptor values for the ER and the PR were examined. The immunobiochemical assay was carried out by Dr. med. Limbach (RIA-Laboratories, Heidelberg, FRG) with the dextran coated charcoal method, in accordance with the recommendations of EORTC Breast Co-operative Group (1980). Tumours with more than 10 fmol/mg protein for ER and more than 20 fmol/mg protein for PR were considered positive.

Tumour samples which had been kept frozen in liquid isopentane at −80° C were sectioned at 7 µm at −20° C, fixed immediately without drying in picric-paraformaldehyde (pH 7.4) and then rinsed in phosphate-buffered saline (PBS, 0.01 M, pH 7.4).

For demonstration of the ER the primary antibody, bridging antibody and the PAP complex of the commercial ER-ICA-kit from Abbott (Wiesbaden, FRG) were used. PR was demonstrated by using a commercial monoclonal mouse antibody against purified rabbit PR (mPRI, Transbio, SARL, Paris, France), a rabbit anti-mouse antibody (linking antibody, Dakopatts, Hamburg, FRG) and a PAP complex from the mouse (Dakopatts).

In addition, we used the following antisera for the demonstration of the epithelial tumour cells: A broadly reacting monoclonal mouse antibody, which is directed against most of the known cytokeratins (Cooper et al. 1984) as a primary antiserum (antibody AE1+AE3, Hybritech, San Diego, CA, USA), a rabbit anti-mouse antibody (Dakopatts) as the linking antibody and a PAP complex from the mouse (Dakopatts). The primary antibody against the PR was diluted in the primary antiserum provided by the ER-ICA-kit (the final concentration of the mPRI antiserum was 1:50) and was incubated for 60 min at 37° C. All other antisera, except those of the ER-ICA-kit, were diluted in PBS (0.01 M, pH 7.4) to a final concentration of 1:50 respectively 1:150 for the primary AE1+AE3 antiserum and were incubated at 37° C for 30 min, respectively 90 min, for the AE1+AE3 antiserum. After each incubation step the slides were rinsed in PBS.

The immunohistochemical demonstration of ER and PR was visualized by using a modified method based on the diaminobenzidin-nickel (DAB-nickel) technique described by Hancock (1984): Solution A: 25 mg in dimethylformamid dissolved DAB (Sigma, Deisenhofen, FRG) diluted in 20 ml PBS (0.01 M, pH 7.4). Solution B: 97.5 mg nickel II-sulfate-5-hydrate (Merck) diluted in 150 µl PBS. Just before use, 5 µl of H_2O_2 (30 p.c.) were added to the solutions A and B. The AE1/AE3 staining was developed by DAB only.

After each immunohistochemical staining the slides were dehydrated in graded alcohols and mounted in Entellan (Merck, Darmstadt, FRG).

The DAB-nickel complex was removed by coverslipping the slides in a mixture of phthalanhydride (Sigma) and glycerin (Merck): 5 mg phthalanhydride dissolved in 20 ml dimethylformamid and diluted in 80 ml glycerin. When the elution of the colored end product was complete, the slides were put in water and rinsed in PBS for 5 min.

Endogenous peroxidase was blocked by H_2O_2 (0.3 p.c.) prior to the application of the primary antisera. The residual enzyme activity of the PAP complex of the first staining step was inhibited by application of a solution of 2 ml H_2O_2 (30 p.c.) in 100 ml methanol for 8 min just before starting the second staining sequence.

The computer-assisted image analyzing of the results of the immunohistochemical stainings was carried out with the image analyzer IPS (Kontron, Munich, FRG). It consists of a microscope, a TV-camera, an image storage and a host computer. The TV-camera generates grey images, which are stored in a digitized form. A grey image is resolved into 512 × 512 pixels. Each pixel is codified by 255 grey values (0 = black, 255 = white). The digitized grey images are treated with several functions driven by the host computer.

The high sensitivity of the antigenic determinants of the ER and the PR required a special staining technique: In the first staining sequence, the primary antisera against the ER and the PR were incubated simultaneously (the mPRI serum was diluted in the primary antiserum provided by the ER-ICA-kit).

Next the secondary antibody and the PAP-complex of the ER-ICA-kit were applied. The reaction was demonstrated by using the DAB-nickel technique. The result of the first staining sequence was stored as a digitized grey image (1. object image, Fig. 1a).

After documentation, the slide was rehydrated in graded alcohols and the colored end product was removed. The residual peroxidase activity of the PAP complex was blocked. The second staining sequence consisted in incubation of the secondary antibody and the PAP complex against the anti-PR-antibody already incubated in the first staining step. The reaction was demonstrated by using the DAB-nickel technique. The result of the second staining sequence was exactly positioned and stored as a digitized grey image, in which structures occupied the same positions as identical structures in the 1. object image (2. object image, Fig. 1b).

After documentation, the slide was once more rehydrated in graded alcohols. Next an immunohistochemical demonstration of the cytokeratins and haematoxylin counterstaining were carried out. The resulting preparation was exactly positioned again and stored in a digitized form (3. object image). In the first staining sequence cross-reactivity occurred between the second and/or third antiserum (PAP complex) of the ER-ICA-kit and the primary antiserum against the PR. In the second staining step cross-reactivity could be demonstrated between the second and/or third antiserum (PAP complex) for the demonstration of the PR and the antisera of the ER-ICA-kit, incubated in the first staining step. Negative controls were carried out in two adjacent sections to the sample (reference slides). In the first reference slide, only the first staining step was carried out, but the primary anti-ER-antiserum was replaced by an identical volume of buffer. The result of this staining was stored as a digitized grey image (1. reference image, Fig. 1c). A second

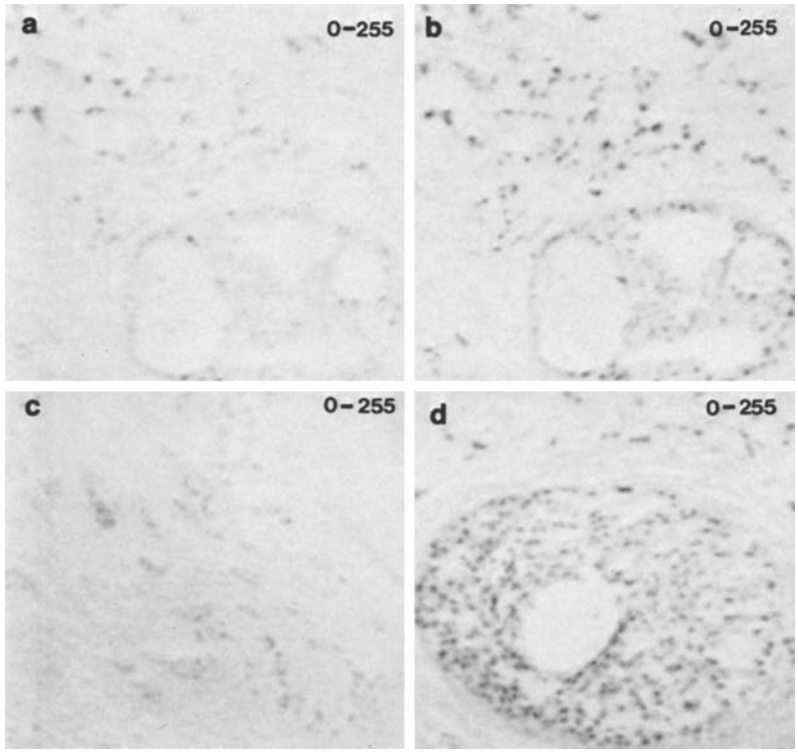

Fig. 1. Digitized grey images of the results of two sequential staining steps. Notice that the whole scala of grey values (0–255) is represented in all grey images.
(**a**) 1. object image
(**b**) 2. object image
(**c**) 1. reference image
(**d**) 2. reference image

reference slide was treated like the sample in the first and second staining sequence, except that the primary anti-PR-antibody was replaced by an identical volume of buffer. The result of the second staining sequence was stored as a digitized grey image (2. reference image, Fig. 1d).

To identify the specifically stained structures in both the 1. and the 2. object image, the grey values which were generated by cross-reactivity were evaluated in the reference images and then eliminated in the corresponding object images as follows:

Starting at the grey value 255 (=white) the grey threshold was progressively lowered until every structure in the reference images had disappeared (Fig. 2c, d). The grey threshold at which no more structures were visible in the 1. reference image, was applied to discriminate the 1. object image (Fig. 2a). The grey threshold to discriminate the 2. object image was evaluated from the 2. reference image in the same way (Fig. 2b).

After this manipulation, all structures remaining in both object images have higher staining intensities than the strongest staining intensity generated by cross-reactivity. Next the evaluated structures of both object images were transduced into binary images (1. and 2. binary image), which were overlayed with arbitrarily chosen colors. These were copied into the 3. object image. Coexpression in identical structures was demonstrated by copying the contour of the 1. binary image and the 2. binary image in the 3. object image (final image, Fig. 3a, b).

The figures are photographs directly taken from the TV-monitor having thus a relatively poor resolving power for the cytoplasmatic and nuclear structures.

Results

In the two color representations of the results of two sequential immunohistochemical stainings in one final image a coexpression of the ER and the PR is indicated, when the content of the blue contour (ER) is red (PR) (Fig. 3b).

In cells which express the PR independently from the ER the nucleus is marked in red not being surrounded by a blue contour (Fig. 3b).

The blue contour without red content signifies that the ER is expressed without the PR (Fig. 3b).

In the five carcinomas investigated, three types of tumour cells with different receptor expression patterns can be distinguished:

(1) Tumour cells which coexpress both the ER and the PR.
(2) Tumour cells that express the ER only.
(3) Tumour cells that express the PR only.

In each of the five carcinomas investigated, all these three expression patterns could be found. Nevertheless, the number of tumour cells showing one of these receptor expression patterns varies from tumour to tumour.

One carcinoma showed all three expression patterns in about the same number of tumour cells (Fig. 3a, b).

In three carcinomas, the first expression pattern predominates (Fig. 4).

One tumour was found in which the second expression pattern predominates (Fig. 5).

Discussion

The immunohistochemical demonstration of two different antigens in one histological section can only be regarded as specific on condition that: no

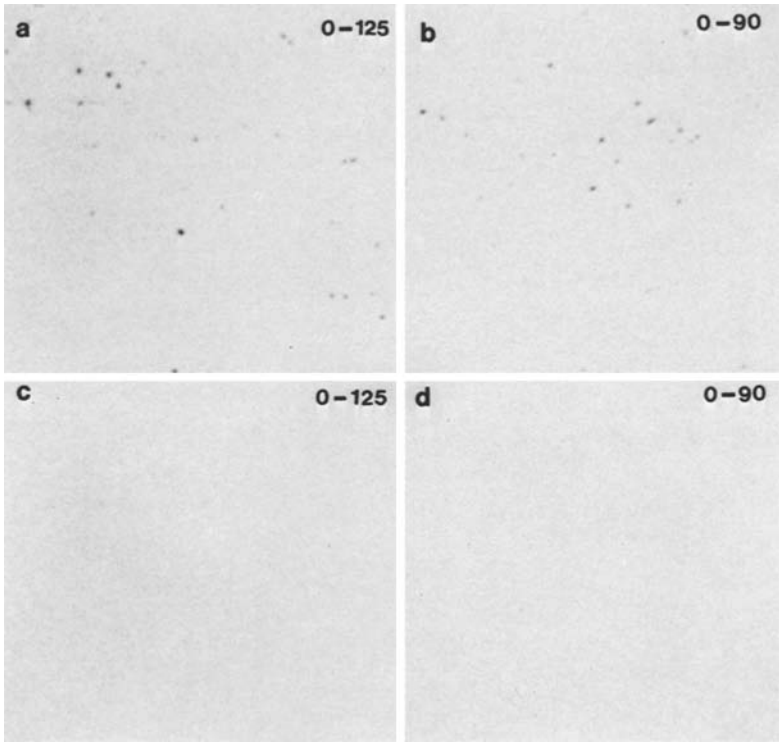

Fig. 2. Evaluation of the grey thresholds of the specifically stained structures in the first and second object image. The 1. reference image becomes entirely white at the grey value 125 (**c**), the 2. reference image at 90 (**d**). Notice that at the grey value 125, respectively 90, there are still structures visible in the 1. (**a**) and the 2. (**b**) object image (specifically stained structures)

cross-reactivity occurs between the antibodies used to detect each antigen specifically, and that the dye-stuffs generated to visualize the different immunoreactions can be clearly distinguished.

The interpretation of the results of conventional immunohistochemical double staining techniques using different enzyme detection systems (Mason et al. 1983) may be difficult, because coexpression of antigens in identical cell compartment is deduced by the appearance of a mixture of the dye-stuffs generated by the different chromagens used. The method described here allows a precise detection of coexpression which is characterized by an easily recognizable pattern (differently colored contour) of well distinguishable colors.

Referring to the first and, probably, the most important condition, conventional simultaneous double-staining techniques can only be used when no cross-reactivity between the antibody system used for the demonstration of both antigens takes place. Fig. 1 c clearly demonstrates that non-specific staining occurs in the simultaneous immunohistochemical detection of the ER and the PR in one histological section, because the second (and/or third) antibody of the ER-ICA-kit cross-reacts with the primary anti-PR-antibody. Even though antibody systems raised in different species were used, non-specific staining could not be avoided. Consequently simultaneous immunohistochemical double-staining techniques using different detection systems are not suitable for a specific demonstration of both receptors in one section with these commonly used antibody systems. Since cross-reactivity could not be avoided with the antisera used, the only way to evaluate specifically stained structures was to eliminate those grey values which were exclusively generated by cross-reactivity in negative controls, thus permitting a reliable evaluation of only specifically stained structures in the final images. However, it is possible that nuclei with a very weak specific immunoreactivity are eliminated, because their grey values are less intensive than those generated by the strongest cross-reactivity.

The application of computer-assisted image processing can be generally useful, when intense unspecific staining seriously interferes with a reliable interpretation of immunohistochemical double stainings (Bonkhoff and Bock, unpublished work).

The immunohistochemical double staining technique described here combines several advantages of both simultaneous and sequential staining methods. The simultaneous incubation of the primary antisera against both receptors prevents the PR from losing its antigenic epitopes during the demonstration proceedings of the ER, and the sequential demonstration of both immunoreactions allows the removal of the colored end product generated in the first staining sequence, thus avoiding

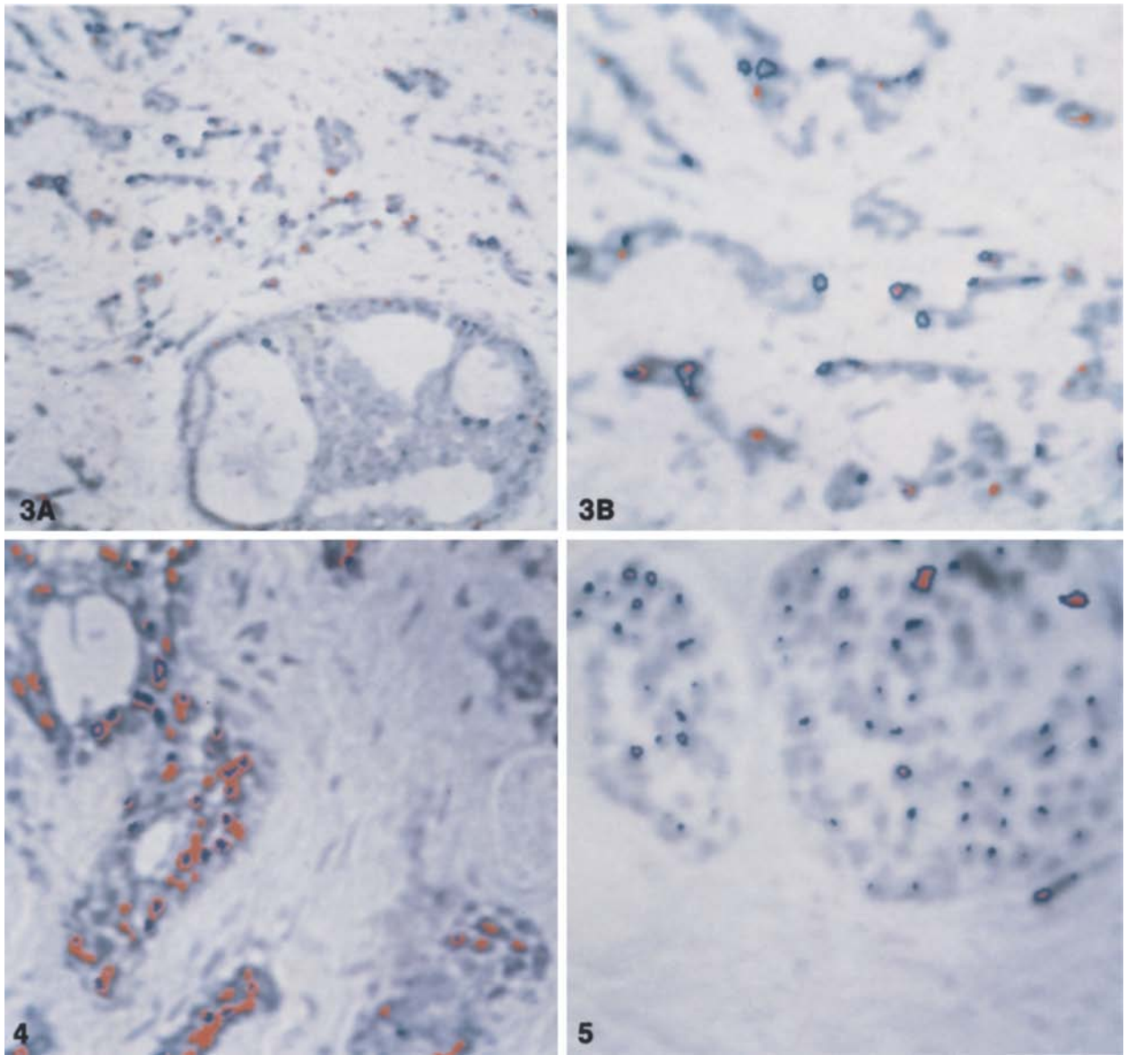

Fig. 3. (**a**) Final image. Two-color representation of the specifically stained structures of the 1. and 2. object image in the 3. object image (bleu contour: ER; red areas: PR). (**b**) Magnification of the left upper quadrant of (**a**) (bleu contour: ER; red areas: PR). Notice that three types of tumour cells with different receptor expression patterns can be distinguished: (1) Tumour cells which coexpress both the ER and the PR in their nuclei. (2) Tumour cells that express the ER only. (3) Tumour cells that express the PR only. All three expression patterns are represented in about the same number of tumour cells. The immunobiochemical assay in this tumour revealed 203 fmol/mg protein for the PR and 40 fmol/mg protein for the ER

Fig. 4. Final image (bleu contour: ER; red areas: PR). The majority of tumour cells coexpress the ER and the PR in their nuclei. In only a few cells does the PR occur without the ER. Notice that the PR:ER ratio (382:79 fmol/mg protein) is similar to that in the tumour illustrated in the Fig. 3a and 3b

Fig. 5. Final image (bleu contour: ER; red areas: PR). In most of the tumour cells the ER is expressed independently of the PR. Only a few tumour cells stain positive for the PR. All of these cells coexpress the ER. The immunobiochemical assay in this tumour revealed 139 fmol/mg protein for the ER and 76 fmol/mg protein for the PR

a mixture of dye-stuffs. If simultaneous double staining techniques had been used, the appearance of mixed dye-stuffs generated by different chromagens would have been difficult to interpret since it might have represented either a colocalization of the antigens, or non-specific staining by cross-reactivity. In addition, the DAB-nickel technique is reputed to intensify conventional DAB-development (Hancock 1984). In contrast with other alcohol-insoluble chromagens (including DAB), the

DAB-nickel complex can be removed easily by a solution of phthalanhydride and glycerin without disturbing the demonstration of the second antigen (Bonkhoff and Bock, unpublished work), thus permitting the use of an optimal chromagen in each staining sequence.

The analysis of the topographical relationship between the ER and the PR in one histological section allows us to distinguish two types of ER in breast cancer: the first type is an ER which is coexpressed with the PR in the same tumour cell, the second is expressed in tumour cells in which the PR is not detectable immunohistochemically.

Several reports in the literature (Horwitz and McGuire 1978a, b; Romić-Stogković and Gamulin 1980; Lykkesfeld and Briand 1986; Thorpe 1987) suggested that the synthesis of PR in human breast cancer may be under the control of a mechanism involving ER. The first type of ER demonstrated by us may thus be an ER able to induce PR expression.

In tumour cells expressing exclusively ER of the second type the ER-mechanism might not be necessarily defective – PR might be present in amounts not detectable by immunohistochemistry. Furthermore, even though the PR would be absent at that moment, it could be expressed at another time. Nevertheless, it is not excluded that the second type of ER might have lost the ability to induce the PR-expression (or never had it).

According to our findings, PR is, however, not always coexpressed with ER in the same tumour cell. These morphological data are consistent with observations that in a few tumour cell lines PR is obviously not induced by estrogens (Devluschouwer et al. 1986; Lykkesfeld and Briand 1986; Reiner and Katzenellenbogen 1986). These tumour cells might have lost the ability to express ER during malignant transformation, but become able to express the PR independently of a mechanism triggered by estrogens and their receptor.

Both receptor expression patterns (the independent expression and the coexpression of the ER and the PR) are found in each tumour we studied. These morphological data suggest that the different functional relationships between both receptors, as observed in different tumour cell lines (modulation of the synthesis of PR by ER or not), can exist in the same tumour.

The question arises whether the two types of ER demonstrated by us in human breast cancer differ in their influence on tumour growth. The first type of ER that is coexpressed with PR in the same tumour cell could be the "intact" receptor which mediates estrogen-induced tumour growth. Thus, cancers expressing mainly this type of receptor would respond better to an antiestrogenic therapy than others, in which the ER is predominantly expressed without the PR.

Further prospective investigations of a large series of tumours are necessary to answer this question.

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