Summary. Glucocorticoids play a major role in attenuation of the inflammatory response and they are useful in the primary combination chemotherapy of breast cancer, since in vitro studies have demonstrated an antiproliferative effect in human breast cancer cells. In contrast, it was recently shown that glucocorticoids protect against apoptotic signals evoked by cytokines, cAMP, tumour suppressors, and death genes in mammary gland epithelia. Their actions are mediated by intracellular receptor (GR) that functions as a hormone-dependent transcription factor; however, no previous studies have been focused on GR expression in different pathologies of the human breast, and the possible relationship with that of mineralocorticoid receptor (MR) and COX-2. Also, the role of these proteins on tumoral breast epithelial cells remains unclear. Therefore, we examined GR, MR and COX-2 expression by immunohistochemistry and Western blot techniques in 142 samples of human breast obtained by total or partial mastectomy. We found that the percentage of positive patients presenting nuclear immunoreaction to GR decreased with tumor development, while all samples analyzed showed cytoplasmic immunoreactions to MR. All positive samples to COX-2 antibody showed cytoplasmic location, a higher immunoreaction being observed in benign breast diseases than in carcinomatous lesions. Thus, breast cancer progression is associated with the accumulation of GR in the cytoplasm of tumoral cells and the decrease of COX-2 expression.

Key words: GR, MR, COX-2, Breast cancer

Introduction

The hormonal regulation of breast cancer cell growth and survival is multifaceted and very complex (Nass and Davidson, 1999) due to the potential for interaction or cross talk between the different classes of hormones, especially the ability of certain hormones to regulate the abundance of the receptors for other hormones (Traynor, 1995). Activated steroid receptors affect many genes that are involved in cellular metabolism and often affect the transcription of other steroid receptors (Dickson and Lippman, 1988). One of these receptors is the glucocorticoid receptor (GR) which operates on the mechanisms of cellular differentiation and induces apoptosis in certain cells (Lee et al., 1988; Hulkko and Zilliacus, 2002). Taking into account these properties, glucocorticoids have been used in clinical oncology for more than three decades and are useful in the primary combination chemotherapy of breast cancer (Rubens et al., 1988), since in vitro studies have demonstrated an antiproliferative effect in human breast cancer cells (Osborne et al., 1979; Zhou et al., 1989; Hundertmark et al., 1997). In contrast, it was recently shown that glucocorticoids protect against apoptotic signals evoked by cytokines, cAMP, tumour suppressors, and death genes in mammary gland epithelia, hepatocytes, ovarian follicular cells and fibroblasts (Amsterdam et al., 2002). One of the functions of glucocorticoids is to reduce the production of prostanoids by inhibiting the expression of COX-2 (Half et al., 2002). These proteins catalyze the conversion of arachidonic acid to prostaglandins, which have been detected at elevated levels in cultured human breast cancer cells as well as in invasive human breast cancers (Rolland et al., 1980; Karmali et al., 1983; Schrey and Patel, 1995; Liu and Rose, 1996). However, there are multiple contradictory
data on elevated COX-2 (an inducible isoform of cyclooxygenases) expression in breast neoplasia (Half et al., 2002; Ma et al., 2004). Thus, Half and co-workers (2002) encountered that the apparently normal breast epithelia adjacent to the tumour site expressed COX-2 in 81% of analyzed cases; whereas Watanabe et al. (2003) reported a low level of COX-2 expression in 50% of normal epithelia surrounding ductal carcinoma in situ, but in only 15% of normal epithelia surrounding invasive disease. Moreover, it is well established that prostaglandin production is greater in human breast tumours than benign tumours or normal breast tissues (Rolland et al., 1980; Boland et al., 2004).

Not only glucocorticoids but also mineralocorticoids and its cognate receptors are involved in this regulation of COX-2 expression (Zhang et al., 1999). Both receptors mediate biological responses to adrenal corticosteroids and synthetic ligands, and although many characteristics of GR are applicable to mineralocorticoid receptor (MR), there are significant differences between both receptors regarding their physiological effects in the target tissues (Bhargava and Pearce, 2004). Although the regulation of COX-2 by mineralocorticoids was described in rat renal medulla (Zhang et al., 2002), this regulation has not yet been identified in human breast cancer.

Multiple controversial data have promoted the development of this study to evaluate, in different breast lesions by immunohistochemistry and Western blot analysis, the expression profile of glucocorticoid receptor together with that of mineralocorticoid receptor and COX-2, in order to improve our knowledge of the relationship of these proteins in the development and progression of breast cancer and their possible influence in endocrine therapies responsiveness.

Materials and methods

Material

Breast samples used in this study were collected from 142 patients (from 35 to 91 years of age) diagnosed between 1998 and 2000 and they had a follow-up of 60 months, by the Pathology Service of the Hospital Príncipe de Asturias of Alcalá de Henares. Primary glandular breast lesions were classified as follows: 24 cases of benign proliferative diseases, including ductal and lobular hyperplasia, apocrine metaplasia, fibroadenoma, and fibrocystic changes; 38 carcinomas in situ (CIS); 80 infiltrative carcinomas: 46 ductal (IDC) and 34 lobular (ILC). Removal of tissues and the study of archives samples were carried out with the consent of patients and/or their relatives and permission of the Ethics Committee of the Hospital. The hormonal receptor status of infiltrative carcinomatous lesions was determined together with the pathological diagnosis by Príncipe de Asturias Hospital Pathology Service. None of these patients received radiotherapy, hormonal therapy or chemotherapy before surgery.

Each specimen was divided into two approximately equal portions: one portion was processed for immunohistochemistry (10% formalin fixed and paraffin embedded), and another portion was frozen in liquid nitrogen and maintained at -80°C for Western blot analysis.

Methods

Immunoblotting

For Western blot analysis, tissues were homogenized in the extraction buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 1 mM 1,4-Ditio-L-threitol, and 0.2 M ethylenediamine tetracetic acid, pH 8) in addition to a cocktail of protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 10 µg/ml of leupeptin and 1 mg/ml of aprotinine) and phosphatase inhibitors (200 mg/ml of sodium fluoride and 50 mg/ml of sodium orthovanadate) in presence of 0.5 % Triton X-100 and 0.1% of SDS. Homogenates were centrifuged for 10 min at 10000 rpm at 4°C. Supernatants were mixed with an equivalent volume of SDS buffer (10% SDS in Tris/HCl pH 8, containing 50% glycerol, 0.1 mM 2-mercaptopethanol and 0.1% bromophenol blue). Then the mixture was denatured for 4 min at 96°C, and aliquots of 50 µg protein were subjected to SDS-PAGE (9% acrylamide gel for GR and 12% for MR and COX-2). Following SDS-PAGE, proteins were transferred to nitrocellulose membranes (0.2 mm) in the transfer buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS and 20% methanol) for 4h at 250 mA constant current. Briefly, the blots were blocked in 5% Blotto (Santa Cruz Biotechnology CA, USA) for 1 hour. After blocking, blots were incubated with primary antibodies: mouse monoclonal GR (1:200) (Novocastra Laboratories LTD, UK; Santa Cruz), mouse monoclonal MR (1:4000) (Affinity Bioreagents Golden, CO; Santa Cruz), and rabbit polyclonal COX-2 (1:1000) (Santa Cruz). All of them diluted in blocking solution 1:9 overnight at room temperature. After extensive washing with TBS/Tween 20, the membranes were incubated with biotinylated immunoglobulins diluted in blocking solution 1:9, rabbit anti-mouse for GR and MR, or swine anti-rabbit for COX-2 (Dako, Barcelona, Spain) at 1:2000 and after an extensive wash, the sheets were incubated with streptavidin-peroxidase (Zymed Laboratories Inc. South San Francisco, CA, US) at 1:10000 dilution in the same blocking solution 1:9. Finally, the membranes were developed with an enhanced chemiluminescence (ECL) kit, following the procedure described by the manufacturer (Amersham, Buckinghamshire, UK). Blots were stripped and re-probed with an anti-human β-actin monoclonal antibody (Amersham) to control for equal sample loading.

Immunohistochemistry

Sections, 5-µm-thick, were deparaffinised in xylene
and rehydrated using graded ethanol concentrations. Following deparaffination, sections were hydrated and those destined to be labelled using the streptavidin-biotin-peroxidase method were incubated with 3% hydrogen peroxide for 20 min at room temperature to inactivate endogenous peroxidase. Antigen retrieval was achieved by the pressure cooking method for 30 sec. in 0.1 M citrate buffer (pH 6). After rinsing in Tris-buffered saline (TBS), the slides were incubated with blocking solution (3% normal donkey serum (NDS) plus 0.05% Triton in TBS for GR, and 20% NDS plus 5% bovine serum albumin (BSA) in TBS for MR and COX-2) for 45 min to prevent non-specific binding of the first antibody. Afterwards, the sections were incubated overnight at room temperature, with the primary antibodies (GR 1:20, MR 1:400, COX-2 1:150) in their respective blocking solution diluted 1:9.

Then, the sections were washed in TBS and incubated for 1 hour with either rabbit anti-mouse (for GR and MR), or swine anti-rabbit (for COX-2) biotinylated immunoglobulins (Dako), diluted 1:500 in blocking solution 1:9. After an extensive wash in TBS, the sections were incubated with the streptavidin-peroxidase (Zymed) for GR and COX-2 antibodies and for MR with the streptavidin-alkaline phosphatase (Zymed) complexes for 45 min at room temperature. The peroxidase and alkaline phosphatase activities were detected using the glucose oxidase-DAB-nickel intensification method and the Zymed’s AP-red solution respectively. DAB stained sections were dehydrated, cleared in xylene, and mounted in DePex (Probus, Badalona, Spain). AP-red stained samples were counterstained with haematoxylin and coverslipped using AquaTex (Merck, NJ, USA).

To assess the specificity of immunoreactions, negative and positive controls were used. As negative controls, sections of breast samples processed identically were incubated using the antibody preabsorbed with corresponding blocking peptide (purchased from Santa Cruz), or omitting the primary antibody. As positive controls, sections of rat adrenal gland and kidney were processed with the same antibody.

The staining intensity of GR, MR and COX-2 was classified into two categories: 0, negative or staining was observed in less than 10% of the cells; 1, staining was detected in more than 10% of the cells. In contrast to nuclear staining of GR and MR, the staining pattern of the extranuclear expression for these proteins was observed in two types: diffuse staining and spotted staining in the cytoplasm according to the following criteria: score 0, no staining at all; 1, a faint/barely perceptible staining; 2 and 3, a weak to moderate staining or a strong staining was observed in more than 10% of the tumour cells. Only scores 2 and 3 were assessed as positive expression. The assessment of the grade of staining was performed in a blinded way always by the same experienced investigators (I.C., M.I.A.).

### Statistical analysis

Data retrieval and analysis were made using a personal computer and a statistical program (SPSS 12.0). To perform a differential analysis of the positive patients for GR, MR and COX-2 among the different lesions of the breast, Dunn’s Multiple Comparison Test was used. In infiltrative carcinomas, univariate analysis comparing categorical variables (GR, MR and COX-2 expression and clinicopathological data -patient age, tumor grade, axillary lymph node status, estrogen and progesterone receptor status, histological type, free-time disease-) was performed using chi-square tests. We test for the presence of a linear trend when there were more than two categories of staining using the Mantel-Haenszel chi-square statistic. Continuous variables were compared using the Mann-Whitney test. For all these tests we computed p-values using an exact method due to small sample sizes. This analysis was completed with multiple regression analyses (Durbin-Watson statistic) to evaluate, in these infiltrative carcinomas, the possible dependence between GR expression and the other variables analyzed. All tests were performed in the two tail form and a value of p<0.05 was considered statistically significant.

### Results

#### Western blot analysis

To check the antibodies specificity, in some samples from each breast lesion, Western blot analysis was performed and results are shown in Figure 1. For each antibody, only a single band at the corresponding molecular weight was found: 94 kDa for GR, 116 kDa for MR, and 70 kDa for COX-2.
**Immunohistochemical characterization of samples of benign lesions**

The percentage of positive samples for GR, MR and COX-2 immunostaining is indicated in Figure 2. No background immunoreaction to GR, MR and COX-2 was observed in any of the negative controls (Fig. 3, inset). As expected, samples of rat adrenal gland and kidney showed an intense staining for these antibodies.

In the benign lesions examined, GR immunolabelling was observed in the nuclei of ductal and acinar cells (Fig. 3A). Stromal cell nuclei were immunoreactive to GR and a strong immunoreactivity was also observed in the nuclei of myoepithelial cells surrounding breast ducts. This immunostaining pattern was observed in 100% of samples studied.

All benign breast lesions showed a very strong immunoreaction to MR but uniformly distributed in the cytoplasmic compartment and in some cells around the nucleus (Fig. 3B). Also, a strong immunoreactivity in the smooth muscle of the tunica media of blood vessels and in the stromal cells was observed. COX-2 enzyme labelling was detected in 20 of 24 samples analyzed (83.4%) and this immunostaining was not uniformly distributed and only observed in some cells and preferentially in ductal formations and located in the basal and apical cytoplasm (Fig. 3C). No immunoreactivity was observed in stromal cells or in vasculature around normal breast ducts.

**Immunohistochemical characterization of carcinomatous samples**

In samples that presented ductal and lobular carcinoma *in situ*, a positive reaction to GR, MR and COX-2 antibodies was observed in the whole tumoral architecture (Fig. 3D, E, and F respectively), regardless of whether it corresponded to a solid structure, a cribriform pattern or a papillary pattern. The percentage of positive samples for GR and COX-2 was lower in these carcinomas than in benign breast diseases, while the MR presented the same pattern of expression as in benign lesions.

In samples of infiltrating ductal carcinoma, solid clusters of tumour tissue were observed. In these clusters, the percentage of immunoreaction observed for GR was lower than that observed in benign breast lesions and carcinomas *in situ*, though its immunoreactivity was located in the nuclei, as well as in the cytoplasm (Fig. 3G). The location and percentage of immunostaining of MR was similar to that encountered in the other lesions analysed (Fig. 3H). COX-2 immunoreaction was located in the cytoplasm of most neoplastic cells, but the percentage of positive samples was reduced (Fig. 3I).

Samples of infiltrating lobular carcinoma which |
GR expression in human breast
were characterized by the presence of small cells diffusely invading the stroma showed reactivity to GR in both the nuclei and the cytoplasm of tumoral cells (Fig. 3J), though the number of positive samples was reduced. Stromal cell nuclei were also immunoreactive. Immunoreaction to MR and COX-2 was similar to that observed in infiltrative ductal carcinomas (Fig. 3K, L).

**Statistical analysis**

A Dunn’s Multiple Comparison Test carried out the significance of differences in the expression pattern of each protein studied between the different patient groups. The results of GR and MR values differed significantly between benign breast lesions and infiltrative carcinomas (p<0.001 and p<0.01 respectively). With the tumoral progression, no differences in the COX-2 expression pattern were encountered.

The Fisher’s exact test or Chi-square test between different clinicopathological data, such as the age of patients, the tumour stage, the lymph node status, estrogen and progesterone receptors status, free-time disease and the expression of GR, MR and COX-2 showed that only the cytoplasmic expression of GR presented a direct, significant statistical association with the patients’ age (p=0.0007) and with the free-time disease (p=0.013) (Table 1). To check whether the association of GR with free-time disease is secondary to the association with patients’ age, we performed an analysis of variance by ranks (Kruskal-Wallis test) observing that each variable differed from one of the others. The multiple regression analysis showed a marked direct association between the GR and ER-α expression (p=0.0079).

**Discussion**

Adrenal corticosteroids, glucocorticoids and mineralocorticoids play important physiological roles in humans and their actions are mediated by intracellular receptor molecules (GR and MR) that function as hormone-dependent transcription factors. Ligand-activated receptors modulate the transcription rates of responsive genes by either interacting with responsive elements in the promoters of these genes or influencing the activities of other transcription factors, via protein-protein interactions (Kino and Chrousos, 2004). The

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**Table 1.** Association of GR immunoexpression with expression of MR, COX-2 and several clinicopathological factors in breast infiltrative carcinomas. Fisher’s exact test (p<0.05).

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FTD: free-time disease.
specificity of the glucocorticoid receptors transcriptional activity varies widely between cell types, accounting for the diverse -and sometimes opposite- physiological effects of glucocorticoids in different tissues. Thus, glucocorticoids have been shown to promote apoptosis in lymphocytes (Willie, 1980; Schwitzman and Cidlowski, 1994), whereas in rat hepatoma epithelial cells (Evans-Storms and Cidlowski, 1994) and human mammary epithelial cells (Moran et al., 2000) are protected from apoptosis after GR activation and this activation is realized by the MKP-1 up-regulation (Wu et al., 2005). We have observed that, in the carcinoma development, the nuclear GR expression is lost in a high number of samples; this loss might be explained by the receptor nucleocytoplasmic shuttling (Madam and DeFranco, 1993), since unliganded glucocorticoid receptors are usually located within the cytoplasmatic compartment (Qi et al., 1989) and also, in patients with breast cancer, a flattened cortisol rhythm occurs (Sephton et al., 2000). Our findings are in accordance with those reported by Ioannidis et al. (1982) who detected GR expression in all mamignant breast tumours and fibrocystic disease was negative. However, Lien et al. (2006) have observed a strong expression of GR in metaplastic carcinomas and malignant phyllodes tumours, and non-metaplastic carcinomas were only positive in 3.9% of cases. These discrepancies are unlikely to be due to differences in the methodology, since a careful comparison of the procedures used by these authors revealed no important differences in the procedure for immunolabelling. A different degree of immunoreactivity might be due to the antibody used, but this would only explain the absence of cytoplasmic GR since it is particularly significant that Lien et al (2006) failed to detect the well-known presence of unliganded GR in the cytoplasm (Qi et al., 1989), a fact that suggests the antibody might only recognize DNA-bound GR receptor. On the other hand, we have studied primary, non-treated tumours, whereas we have no indication of whether the samples from the study of Lien et al (2006) came from treated or non-treated patients. It is conceivable that cancer therapy may modify the expression profile of GR. Finally, conflicting reports regarding the expression pattern of a given protein in cancer tissues are frequently published. Clearly, more studies are needed to elucidate the degree of expression of GR in pathological and non-pathological breast tissues.

Our results showed that, in infiltrative lobular carcinomas, GR and ER-α are dependent on each other. In several physiological and pathophysiologic processes estrogen action is opposed to glucocorticoids. Thus, numerous breast tumour cell lines have been demonstrated to have both ER and GR (Ewing et al., 1989), observing that estrogen promotes growth, whereas glucocorticoids inhibit it (Zhou et al., 1989). Therefore, there are potential interactions for ER/GR at the level of transcription (Uht et al., 1997). Also, it has been reported that estrogen causes a significant down-regulation of GR expression in breast cancer cells leading to a substantial suppression of GR-mediated transcriptional activity (Krishnan et al., 2001).

Although we have not encountered any correlation between PR status and GR expression in carcinoma samples, several studies have reported the progesterone-like effects of glucocorticoids and mineralocorticoids (Schneider et al., 1999; Jiang et al., 2002) and both molecules cross-talk with PR to induce growth inhibition and focal adhesion in breast cancer cells (Leo et al., 2002). Moreover, in our study, no relationship between MR and GR was observed even though van Steensel et al. (1996) have shown that both receptors colocalize in specific nuclear domains and form heterodimeric complexes resulting in a pattern of gene expression different from that of either receptor alone. However, there are no data available about the biological role of the mineralocorticoid receptor in the process of apoptosis. Sasano et al. (1997) localized this receptor in breast ductal epithelial cells, and correlated its presence to the ductal differentiation of breast carcinomas. We detected a strong immunoreaction to MR in all breast lesions and this widely cytoplasmic distribution could be related to the possibility that the MR compensate the loss of GR expression as has been observed in regulating the transcription of milk protein genes among the different steps of mammary gland development (Kingsley-Kallesen et al., 2002).

One of the multiple functions of both mineralocorticoids and glucocorticoids is the regulation of COX-2 expression (Zhang et al., 1999). However, no studies of this regulation have been performed in human breast lesions. Such studies would be very interesting because non-steroidal anti-inflammatory drugs inhibit COX enzymes and are potent chemopreventive agents against some mammary models (Half et al., 2002). Thus, we have observed that the patients who seem to be the most influenced by all of these proteins are those who present benign breast disease, since a high number of patients were positive for these proteins. This observation was surprising considering that previous studies showed overexpression of COX-2 in breast malignant tumours (Half et al., 2002; Ristimäki et al., 2002; Shim et al., 2003; Boland et al., 2004; Ranger et al., 2004) and that this expression is associated with angiogenesis and lymph node metastasis (Costa et al., 2002), tumour size or grade (Zhang et al., 1999), although other studies have not encountered any association (Kelly et al., 2003; Ranger et al., 2003); however, these studies have exclusively been focused on COX-2 expression in breast cancer lesions. Half et al. (2002) have recently demonstrated that ductal carcinomas in situ (DCIS) adjacent to invasive cancer expressed higher COX-2 levels than the invasive component, suggesting that COX-2 expression could be an early event in breast carcinogenesis. Our study, extended to the analysis of benign breast diseases, revealed that overexpression of this enzyme is higher in these lesions than in breast cancer considering the percentage of samples, although
in these immunolabelled samples the number of positive cells was decreased. Together with this expression pattern, the presence of nuclear GR in these lesions suggests that the chemopreventive use of COX-2 inhibitors may have profound effects on breast cancer incidence. However, further specific studies should be performed to test this hypothesis.

The percentage of nuclear immunoreaction to GR in benign breast lesions was related to the highest percentage of COX-2 immunostaining. Breast cancer progression is associated with high accumulation of GR in the cytoplasm and lower percentage of positive samples for COX-2. This fall in immunostaining was not observed for MR, suggesting that when this receptor is activated by its corresponding ligand, it could substitute the role of GR as an inhibitor of COX-2 in the treatment of breast cancer.

In summary, our results suggest that breast cancer progression is associated with the accumulation of GR in the cytoplasm of tumoral cells and the decrease of COX-2 expression. Therefore, it could be interesting to assess the role of GR as a new marker of neoplastic transformation in the human breast.

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GR expression in human breast