Effect of epidermal growth factor in HLA class I and class II transcription and protein expression in human breast adenocarcinoma cell lines

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Summary The spontaneous expression of HLA class I and class II molecules in two human breast carcinoma cell lines (MCF7, T47D) and their modulation during epidermal growth factor treatment are reported. Transcription was analysed by Northern blot and hybridisation with HLA class I and class II cDNA specific probes. The expression of cell surface determinants was examined by internal protein labelling with 35S-methionine, immunoprecipitation with monoclonal antibodies specific for HLA class I or class II, followed by isolation of the immune complex on protein A-Sepharose; at least a quantification of glycoproteins was performed by chromatofocusing. Glycoprotein quantification showed a significant increase in HLA class I and class II (DR) antigen expression after stimulation by epidermal growth factor (0.02 µg ml⁻¹) in the two cell lines, when compared with untreated cell controls. However, with epidermal growth factor treatment of MCF7 and T47D cells, low increases in the amounts of HLA class I and class II RNA were obtained. These differences between in expressed antigens and correspondent RNA amounts would be explained by the fact that EGF in these two cell lines acts more in post-transcription for HLA class I and class II antigens.

HLA class I and II molecules regulate the overall immune response by mediating interactions among various immunocompetent cells. Class I molecules serve as restriction elements for T-cell-mediated cytoxicity (Zinkernagel, 1979) and class II molecules are required for the presentation of the antigen to the helper T cell (Benaceraff, 1988). In normal tissues, HLA class I antigens (Ag) are expressed by most nucleated cells (Ploegh et al., 1981), while HLA class II have a more restricted pattern of expression. They were first reported to be associated with the hematopoietic and immune lineage and, more recently, have been described in a variety of normal cells, either resting or activated (Radka et al., 1986).

In tumoral tissues, HLA class I and class II expression is extremely variable, but of importance. The presence of HLA class I molecules is inversely correlated with the degree of tumorigenicity in some animal models (Pfleumraier et al., 1985), and HLA class II are known to play a role in the interaction between the host’s immune system and tumour cells (Ferrone, 1982).

In the breast gland, while normal resting mammary tissue expresses class I but not class II antigens, these class II are found on glandular cells during lactation, where they are expressed on the milk fat globule membranes (Newman et al., 1980). Class II may be induced by exogenous administration of prolactin, suggesting a hormonal regulation of Ia antigens in the mammary gland and a possible correlation with the differentiation process (Klæreskog et al., 1980; Bernard et al., 1986a, 1990).

In addition, other studies have reported the expression of HLA class II Ags on some invasive tissues of normal lymphoid origin as melanoma (Houghton et al., 1982); adenocarcinoma from the stomach, the colon, the sigmoidal anse, the rectum, the spleen, the lung and the ovary; thymic carcinoma, prostate carcinoma, breast medullary carcinoma, bladder carcinoma, cystic astrocytoma, ganglioneuroblas-toma, glioblastoma, and meningioma (Howe et al., 1981; Natali et al., 1981; Ng et al., 1983).

Epidermal growth factor (EGF) has been shown to stimulate in vitro growth of epithelial cells derived both from normal breast and mammary carcinomas and that EGF may play a role in regulating growth of breast cancer cells in vivo (Fitzpatrick et al., 1984).

In this work, we define and analyse cell-surface expression and RNA transcripts of class I and class II before and after EGF treatment to determine whether this response was variable in different tumour cell lines from the same origin.

Materials and methods

Cell lines and cultures

Two human breast carcinoma cell lines have been studied. They were derived from metastatic breast carcinomas: MCF7 (Soule et al., 1973) and T47D (Keydat et al., 1979). These cell lines were cultured in closed plastic T², flasks (Corning, New York 14830), in growth medium composed of RPMI 1640 (Gibco Europe Ltd, Renfrewshire, Scotland), buffered with 2 gl⁻¹ sodium bicarbonate, supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 UI ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin. For MCF7, cell line, 0.04 UI ml⁻¹ insulin was added to growth medium. Cells were grown in a humidified incubator with 5% CO₂ at 37°C. Cultures were refed every 2 to 3 days. Culturing (after 1 week growth) involved trypsin digestion (7.5 mg in 3 ml⁻¹ PBS for a flask), to obtain monolcellular suspensions followed by the plating of 3 × 10⁶ cells/flask. Cultures were confluent after 1 week (approximately 10 × 10⁶ cells/flask).

In assays of the EGF effects on class I and class II HLA Ag expression, cells were grown for one week (with a refeding to day 3). Immediately after cell passage, 30 ml of medium (supplemented as described above) were added with EGF to a final concentration of 0.02 µg ml⁻¹ of culture.

In parallel, control cells without any treatment were refed and collected at the same time with EFG-treated cells.

Raji cell line was grown in closed plastic T², flasks (Corning, New-York 14830) in RPMI 1640 supplemented with 10% foetal calf serum (Pulvertaft, 1963) in suspension under the concentration of 0.5 × 10⁶ cells ml⁻¹.

Reagents

Epidermal growth factor (EGF) (Collaborative Research Incorporated, Bedford MA 01730) with receptor grade from
mouse submaxillary glands was added to culture medium.

The monoclonal antibody (MoAb) anti-HLA class II was an anti-human DR framework (anti-OK1a) and was obtained from the Ortho Pharmaceutical Corp., Raritan, NJ, at the concentration of 2 mg ml\(^{-1}\). The MoAb anti-HLA class I was specific to human β2-microglobulin (HLA-ABC-m) and was obtained from Silenus, Victoria, 3122 Australia.

### Probes

HLA class II locus specific probes were derived from cDNA clones: DRα (Wake et al., 1982) (500 bp), DQx (Auffray et al., 1982) (500 bp). The inserts were radiolabelled by the random priming technique (Boehringer Mannheim). HLA class I (B) specific probe was derived from a cDNA clone, pAS3-6 (Coppin et al., 1985) (6.5 Kb). This probe was radiolabelled by Nick translation technique (Amersham).

### RNA extraction

Direct RNA extraction was performed by homogenisation of starting material in 7 ml of 0.1% SDS, 6 M urea and 3 M LiCl for 10' cells at full speed for 4 min with an ultraturrax. The homogenate was kept overnight at 4°C in ice. The RNA was pelleted by centrifugation at 4°C for 30 min at 8,000 rpm (Sorvall RC 5C). The DNA in solution was removed. The collected RNA was dissolved in sterilised distilled water. Then the homogenate was treated for 5 min at 65°C, gently vortexed, and sodium acetate pH 5 was added to 0.1 M final concentration. Then, RNA was separated from proteins by two phenol extractions and precipitated with 2.5 vol ethanol at −20°C. The RNA purification was improved with a second ethanol precipitation with 0.3 M sodium acetate pH 5 (Auffray, 1980).

### Northern blot analysis of RNAs

Equal amounts of total RNAs (25 μg) were denatured at 65°C for 5 min and quickly cooled to room temperature, then electrophoresed on a 1% agarose gel containing 3% formaldehyde in buffer pH 8.3 containing 0.4 M boric acid and 0.2 M EDTA. They were then transferred onto a gene screen membrane (Hybond N+, Amersham) using 20 x SSC buffer. After transfer, RNAs were fixed to the membrane by a brief alkaline treatment. Northern blots were pre-hybridised at 42°C for 4 h in a solution containing 4 x SSC buffer, 0.005 M NaPO₄ buffer pH 6.5, 0.2% SDS, denatured Salmon sperm DNA (10 μg ml⁻¹), 50% formamide, 0.05% Denhardt's solution. Hybridisation was performed overnight at 42°C in the same solution with 30% dextran sulfate and 32P-labelled probe. Membranes were washed three times in 2 x SSC and 0.5% SDS for 15 min at 42°C, then three times in 0.5 x SSC and 0.1% SDS for 15 min at 42°C. The 32P-DNA bound to the filters was visualised by autoradiography at −80°C using Hyperfilm TM–MP (Amersham) and intensifying screens (Dupont, Wilmington, DE). All intensity bands were quantified and related to their radioactivity with a computerised densitometer Allen Bradley Servovision (Rockwell Int. Cy.), using the Expert PVS 20805 software programme.

### Radiolabelling of cells

Internal labelling with 35S-methionine was performed in flask when culture was at confluence, corresponding to a density of approximately 106 cells/flask. Before labelling, the medium was removed and each flask received 3 ml of the sterile culture medium described above, with 100 μCi 35S-methionine (366 mCi/mM; Amersham International plc, England). The mixture was incubated at 37°C in a 5% CO₂ incubator for 5–6 h. At the end of this time, label incorporation was stopped by adding 10 ml cold PBS. The cells were then gently washed in PBS at 4°C (Bernard et al., 1986b).

### Preparation of Nonidet P-40 extracts

Washed 35S-methionine cells were solubilised in the culture flask by 3 ml of 0.5% Nonidet P-40 (NP-40; Sigma, Chemical Company, St-Louis, NO, USA) in Tris-buffered saline (150 mM NaCl, 50 mM Tris, 0.02% NaN₃, pH 7) and incubated for 15 min at 4°C. The insoluble material was removed by centrifugation at 30,000 g for 30 min. The extracts collected were used immediately or stored at −80°C.

### Isolation of glycoproteins by affinity chromatography

The NP-40 cell lysates were preclered by affinity chromatography on Lentil-Lectin Sepharose 4B to remove labelled proteins. Labelled glycoproteins bound to Lentil-Lectin gel were eluted with 2% α-Methyl Mannoside (Sigma) in PBS.

### Purification of HLA Ags on protein A-Sepharose CL-4B

Radiolabelled glycoproteins eluted from Lentil-lectin-Sepharose 4B were pooled and concentrated to a volume of 1 ml by ultrafiltration (Immersible CX 10, Millipore Corporation, Bedford, MA, USA). Class II or class I HLA Ags were then immunoprecipitated specifically by a 30 min incubation time at 37°C with 20 μl anti-class II or class I HLA MoAbs. The obtained immunoprecipitate was isolated from Protein A-Sepharose CL-4B (Pharmacia Fine Chemical, Uppsala, Sweden) after elution with 0.025 mM citrate buffer pH 2.6.

### Quantification of HLA Ags by chromatofocusing

Then, quantification of radiolabelled HLA Ags was performed by chromatofocusing on PBE 9–4 gel column (Pharmacia) as follows. The fractions containing the immune complex eluted from Protein A-Sepharose were dialysed against 0.025 M ethanalamine buffer, pH 9.4 and then were poured over the top of the column (1 x 30 cm), which had been equilibrated with three column volumes of 0.25 M ethanalamine, pH 9.4. Elution was carried out with Polylbuffer 9–6, pH 6 (Pharmacia) at 10 ml h⁻¹. Two ml fractions were collected with an automatic collector (Gilson). The radioactivity of each fraction was measured with a dual-channel automagamma spectrometer. When the 35S-specific activity of glycoproteins eluted from Lentil-Lectin-Sepharose 4B is known, the amount of 35S-labelled glycoproteins which bind specifically to the MoAb can be deduced (Bernard et al., 1984a,b, 1985, 1990).

### Statistical analysis

Differences in quantification by affinity chromatographies and following chromatofocusing between treated cells with EGF and untreated cells were assessed by student's t-test.

### Results

#### RNa studies

Results are shown in Figure 1. HLA cDNA probe used for class I studies detected the classical RNA band of 1.7 Kb in T47D and MCF7. After treatment with EGF (0.02 μg ml⁻¹ culture medium), the 1.7 Kb band was increased 3-fold in MCF7 cells and no difference was seen between T47D control cells and correspondent EGF-stimulated cells.

HLA cDNA probe used for class II DRα showed the expected RNA band at 1.5 Kb in MCF7 cells, which was increased 3-fold in intensity after cell stimulation with EGF. Conversely T47D untreated cells showed a very low amount of class II DRα RNA which increased not significantly after EGF stimulation. With HLA class II DQα probe, no band was detected. Then a weak induction of DQα was obtained with EGF stimulation in MCF7 cells, so that no DQα band could be detected in T47D controls nor in T47D treated with EGF. For RNA Northern blots with HLA class II and class
In, Raji cell line was used as control because they are known to express class I and class II Ags.

In contrast to these results, levels of β-actin RNA were not affected by EGF treatment (data not presented).

Modulation of HLA class I and class II antigen expression by EGF

After the internal cell labelling with 35S-methionine, the specific immunoprecipitation of HLA class I and class II (DR) Ags with correspondent MoAbs, the quantification of Ags after affinity chromatographies and chromatofocusing showed a significant increase after EGF treatment in the expression of the HLA class I and class II (DR) Ags in both studied breast tumor cell lines (MCF7 and T47D), when compared with the correspondent control group (untreated cells). Results are expressed in Table I.

With our quantification method, we found in MCF7 cell line that the expression of HLA class I Ags increased 7-fold and HLA class II (DR) Ags 10-fold when stimulation with EGF (0.02 μl ml−1 culture medium) was performed. Meanwhile, in T47D cell line, HLA class I Ags increased 3-fold, and HLA class II (DR) Ags increased 4-fold after EGF stimulation (0.02 μg ml−1 culture medium).

Discussion

This work was undertaken to determine qualitative and quantitative changes induced in HLA class I and class II molecules by EGF in two human breast adenocarcinoma cell lines: MCF7 and T47D. To this purpose, qualitative changes in HLA class I and class II RNA were realised with Northern blotting and quantification of Ags class I and class II (DR) was performed with MoAb-binding studies.

In this study, we demonstrated that EGF increased only class I RNA expression in a breast adenocarcinoma cell line, MCF7, but the rates remained slower than in lymphoblastoid cells (Raji). Moreover, no change was obtained for RNA class I in T47D cells after EGF stimulation. Besides, we found an increase in class I surface-antigen expression in T47D cell line and in MCF7 cells but it was smaller in T47D cell line.

For class II MHC Ags which showed a limited tissue distribution in vivo and are involved in the presentation of Ag to T-helper cells; EGF significantly increased the class II DRα RNA and the expression of surface DR Ags in MCF7 cells. For T47D cells, after EGF stimulation, the increased change for DRα RNA and DR Ag expression are weaker than in MCF7 cells.
A different type of response in the transcription of DQα locus after EGF treatment was observed in the two cell lines. DQα RNA was induced in MCF7 cells, while no DQα RNA band could be detected in T47D cells in Northern blotting.

Moreover, this discordance in RNA between the two loci (DR and DQ) and the different cell lines may reflect a locus-independent regulation and a different degree of differentiation. It may also imply other transcriptional regulatory factors (De Preval, 1988; Yunis et al., 1989).

EGF appeared to act as an inducer of HLA class I, II (MHC) class II antigens. The functional significance of increased expression of HLA antigens in neoplastic tissues may play a role in antigen presentation.

Further, increased expression of EGF receptor has been found in a variety of tumours: glioblastomas, squamous-cell carcinomas (Merlino et al., 1984b, Ulrich et al., 1984; Libermann et al., 1985; Yamamoto et al., 1986), human breast cancer cell lines and in membrane prepared from breast cancer biopsies (Fitzpatrick et al., 1982, 1984; Lebeau & Goubin, 1987).

On the other hand, associations between HLA class I molecules and cell-surface receptors for various growth factors and hormones have been well documented (Haliotis et al., 1990). Receptors thought to associate with class I molecules are numerous but include epidermal growth factor. Shreiber et al. (1984) had used a monoclonal antibody to human class I antigens, HLA-A, B, C, to probe the interaction of these antigens with the receptor for EGF in intact cells. They showed that in two different cell types, human tumour cells and normal human fibroblasts, the binding of antibody to HLA antigens alters the display of EGF receptors, while binding of EGF to its receptors affects the binding of antibody to HLA.

In previous publications, we have already reported the hormonal modulation of HLA class II molecules for neoplastic non-hematopoietic-derived tissues such as N-nitroso-N-methyurea (NNU)-induced rat mammary carcinoma and MCF7 breast tumour cell line. An increase in the expression of HLA class II antigens was observed after treatment with prolactin, and a decrease was seen after treatment with 2α-bromoergocryptine (an inhibitor of pituitary prolactin secretion). These treatments did not modify the total prolactin receptor amounts in NNU-induced rat mammary tumours (Bernard et al., 1986b). Effectiveness of prolactin on the induction of HLA class II antigens was also demonstrated in vitro for the human breast cancer cell line MCF7 (Bernard et al., 1986d) which possesses specific prolactin receptors with a higher density than normal human mammary epithelial cell lines (Shiu et al., 1979).

Moreover, we had investigated the negative effects of cyclosporine A (an immunosuppressive agent) on the expression of HLA class II antigens, resulting from a competition between cyclosporine and prolactin to the prolactin receptors. A significant decrease was obtained in HLA class II antigen expression by NNU-induced mammary tumours of animals treated with cyclosporine A because cyclosporine A acts as an antagonist to prolactin receptors in such hormone-dependent mammary cancer (Bernard et al., 1990). Further evidence of antagonism of prolactin binding to prolactin receptors by cyclosporine A was demonstrated on a breast tumour cell line (MCF7) by measuring the decreased specific 125I-labelled prolactin-binding to prolactin receptors with increasing concentrations of cyclosporine A (Bernard et al., 1991).

At the same time, we had also studied the ovarian hormone modulation of HLA class II antigens expressed by the NNU-induced rat mammary tumour cells. The administration of 17β-estradiol was highly effective in decreasing the expression of HLA class II antigens and conversely progesterone was without any effect on expression of HLA class II antigen expression when compared with the rat control group receiving only NNU (Bernard et al., 1989).

So, a few substances do indeed influence the expression of HLA class II antigens in mammary cancers. And new evidence for a variety of disciplines supports the notion that the MHC complex influences nonimmunological functions in the regulation of cell proliferation and the malignant phenotype.

Further studies are now required to define better the regulation of HLA molecules in cancer surveillance.

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