Pit-1/GHF-1 and GH expression in the MCF-7 human breast adenocarcinoma cell line

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Abstract

GH expression in mammary tumors has been related to the increase and spreading of cell proliferation. Using the MCF-7 human breast adenocarcinoma cell line, it has been demonstrated that autocrine GH-stimulated mammary carcinoma cell proliferation decreased the apoptosis rate and enhanced cell spreading. Surprisingly, no data are available about the presence of Pit-1 (the main pituitary regulator of GH) or GH expression in this cell line.

Using RT-PCR, Western blot and immunohistochemistry, we have demonstrated the presence of both mRNA coding Pit-1 and GH as well as Pit-1 and GH protein in the MCF-7 cell line. These data could imply that Pit-1 may be an adequate target to inhibit breast cell proliferation.

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Introduction

Breast cancer is the most common malignancy, accounting for 31% of all cancers in women. It is known that human breast cancer cells secrete a number of growth factors and cytokines that can be involved in the control and spreading of breast cancer cell proliferation. In recent years, the role of pituitary hormones, namely prolactin and growth hormone (GH), in the pathogenesis of human breast cancer has received considerable attention. Although the presence of GH receptor has been reported, it is not known whether GH and Pit-1 gene expression is present in human breast cancer cell lines.

Pit-1/GHF factor-1 (GHF-1) is a pituitary-specific transcription factor that includes in its structure a homeo-domain characteristic of a superfamily of developmental regulatory proteins (Bodner *et al.* 1988, Ingraham *et al.* 1988). The presence of an additional domain, conserved in Pit-1 and the proteins OCT-1, OCT-2 and UNC-86 gave rise to the term POU domain which characterizes this family of homeodomain proteins (Rosenfeld 1991). Pit-1 plays an essential role both for cell differentiation during organogenesis of the anterior pituitary in mammals (Simmons *et al.* 1990, Castrillo *et al.* 1991) and as a transcriptional activator for pituitary gene transcription (Lefevre *et al.* 1987, Nelson *et al.* 1988). Pit-1, in co-ordinate action with additional factors, is responsible for the specification, expansion and survival of three specific

cell types: somatotropes, lactotropes and a subset of thyrotropes, during anterior pituitary development (Li *et al.* 1990, Lin *et al.* 1994), as well as for the transcriptional regulation of target promoters (i.e. GH, prolactin, β -subunit of thyrotropin (TSH), GH-releasing hormone receptor genes and its own gene) (Lefevre *et al.* 1987, Nelson *et al.* 1988, Chen *et al.* 1990, Li *et al.* 1990, McCormick *et al.* 1990, Lin *et al.* 1992, 1994). Mice with inactivating mutations or deletions of the Pit-1 gene fail to generate somatotropes, lactotropes and thyrotropes and consequently exhibit anterior pituitary hypoplasia and dwarfism (Li *et al.* 1990), demonstrating the importance of Pit-1 in the ontogeny of the pituitary gland. Expression of Pit-1 transcripts and protein is highly regulated, and the presence of Pit-1 protein is correlated both temporally and spatially with activation of the GH gene during pituitary development (Dollé *et al.* 1990).

Thus, at the pituitary level, Pit-1 is absolutely necessary as a transcription factor to pituitary GH gene expression, but GH gene transcription is also present in extrapituitary tissues (i.e. mammary glands). The presence of GH mRNA in the mammary gland of the cat, dog and human (Mol *et al.* 1995a,b) has been demonstrated. GH receptor mRNA is present in both human breast cancer cell lines and tissues (Sobrier *et al.* 1993, Decouvlaere *et al.* 1995) and both the mRNA and the GH receptor protein were demonstrated in the human normal and neoplastic breast (Mertani *et al.* 1998). Moreover, some authors have found

elevated GH levels in sera as well as increased plasma insulin-like growth factor (IGF) levels in patients with breast cancer, suggesting a possible relationship between GH/IGF-I and breast cancer (Emerman *et al.* 1985, Peyrat *et al.* 1993).

However, the role of Pit-1 on GH expression in non-pituitary tissues is unclear. Pit-1 and GH co-expression in human placenta (Bamberger *et al.* 1995, Schanke *et al.* 1997), in hemopoietic and lymphoid tissues as well as in HL-60 and RAJI leukemic cells (Delhase *et al.* 1993a) has been demonstrated, suggesting that, like the pituitary, Pit-1 controls GH gene expression. On the contrary, in canine normal mammary tissues, no detectable Pit-1 mRNA was found despite the presence of GH gene transcripts before and after treatment with progestins. Interestingly, GH-expressing mammary tumors showed Pit-1 expression (Lantinga-van Leeuwen *et al.* 1999).

The human breast adenocarcinoma cell line MCF-7 has been used to study how autocrine GH increases the rate of mammary carcinoma cell spread and cell proliferation and transcriptional activation (Kaulsay *et al.* 1999, 2000). However, the expression of Pit-1 as well as GH in the MCF-7 cell line has not been previously reported. For this reason, and in order to clarify the mechanisms involved in these GH-Pit-1-mammary tumor relationships, we have investigated the presence of Pit-1 and GH expression in the MCF-7 cell line using RT-PCR, Western blot and immunohistochemistry.

Materials and Methods

Cell culture and RNA isolation

MCF-7 human breast adenocarcinoma cell line was obtained from the European collection of cell cultures (Salisbury, Wilts, UK). Stock culture was grown in 90 mm Petri dishes using Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine in an air-carbon dioxide (95:5) atmosphere at 37 °C. Confluent cells were washed twice with phosphate-buffered saline (PBS) and harvested by a brief incubation with a trypsin-EDTA solution (Sigma, St Louis, MO, USA) in PBS. Total RNA isolation was performed with TRIzol reagent (Gibco-BRL, Life Technologies, Grand Island, NY, USA). Briefly, total RNA was extracted with chloroform (0.2 ml/ml homogenate), precipitated with isopropanol, washed with 75% ethanol, and dissolved in diethylpyrocarbonate-treated, Rnase-free water. RNA concentration and purity were determined by absorbance.

RT-PCR analysis

cDNA synthesis was generated under the following conditions: 2 µg total RNA was incubated for 50 min at

37 °C, 15 min at 42 °C and 5 min at 95 °C with 400 units M-MLV reverse transcriptase (Gibco-BRL) in the buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol and 0.5 mM spermidine) with 2 mM of each deoxynucleotide triphosphate, 20 units RNase inhibitor (RNasin RNase inhibitor; Promega, Madison, WI, USA) and 500 ng random primers (Promega) in a total volume of 40 µl.

Six microliters of the cDNA generated under the above-mentioned conditions were amplified by PCR using 2 units Taq polymerase (Promega) in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 and 1.5 mM MgCl₂, with 0.2 mM of each deoxynucleotide triphosphate and 25 pM each of two oligonucleotide primers, to a total volume of 50 µl. The samples were denatured at 94 °C for 1 min, annealed at 53 °C or 60 °C for 1 min (Pit-1 and GH respectively) and extended at 72 °C for 1 min, for 35 cycles, with an extension step for 10 min at 72 °C in the last cycle. The amplified product was separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. Each experiment was carried out at least three times, with similar data being obtained in each of them.

Primer sequences

Primer sequences for Pit-1 PCR amplification were as follows: primer A (5'-GCAACTCTGCCTCTGATA ATG-3') was a 21 mer corresponding to nucleotides 186–206 in exon 1 of Pit-1 cDNA and primer B (5'-CCACCAATTTACTTTTCCGCC-3') was an antisense 21 mer corresponding to nucleotides 460–480 in the third exon of Pit-1 cDNA. The PCR product obtained is 295 bp in length. Primer sequences for GH PCR amplification were as follows: primer A (5'-CCGACAC CCTCCAACAGGGA-3') was a 20 mer corresponding to nucleotides 314–334 in the third exon of GH cDNA and primer B (5'-CCTTGTCATGTCCTTCCTG-3') was a 20 mer corresponding to nucleotides 638–658 in the fifth exon of GH cDNA. The PCR product obtained is 344 bp in length.

Western blot analysis

MCF-7 cells were lysed at 4 °C in 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EGTA, 1.5 mM MgCl₂, 1% SDS, 10% glycerol, 1% Triton X-100, 10 mM sodium orthovanadate, 4 mM phenyl-methylsulfonyl fluoride and 50 µg/ml aprotinin) and sonicated. Cell lysate was then centrifuged at 14 000 g for 5 min at 4 °C and the resulting supernatant was collected, and protein concentration was determined by the Bradford method. Pit-1 and GH were then immunoprecipitated with a monoclonal anti-Pit-1 antibody (Transduction laboratories, Lexington, KY, USA) or a monoclonal

Figure 1 Detection of Pit-1 and GH mRNA in MCF-7 human breast adenocarcinoma cell line. mRNA from the MCF-7 cell line was used as a template for cDNA synthesis and then amplified by PCR using specific primers for Pit-1 and GH. (A) Ethidium bromide staining of PCR product corresponding to the Pit-1 cDNA. Lane 1: molecular weight marker (1000 bp DNA ladder). Lane 2: RT-PCR product from human pituitary used as control. Lane 3: uncleaved RT-PCR product from the MCF-7 cell line. Lane 4: RT-PCR product from the MCF-7 cell line cleaved with Mbo II. (B) Ethidium bromide staining of PCR product corresponding to the GH mRNA. Lanes 1, 2 and 3: as above in (A). Lane 4: RT-PCR product cleaved with Bgl II.

anti-human GH (hGH) antibody (Sorin, Italy) and incubated overnight at 4 °C. Protein-G-Sepharose (30 µl) was then added and incubated for 45 min. The samples were centrifuged at 14 000 *g* for 3 min and the pellet was washed five times with HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol and 0.1% Triton X-100). Pit-1 and GH were then resuspended in 2 × SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 min. The samples were subjected to 12% or 15% SDS-PAGE electrophoresis (for Pit-1 and GH respectively). Proteins were transferred for 2 h at 4 °C to nitrocellulose membranes. Nitrocellulose membranes were blocked with 0.1 g casein in PBS with 0.1% Tween 20 (PBST) for 1 h at room temperature. Blots were then immunolabeled overnight at 4 °C with: (1) polyclonal anti-Pit-1 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and (2) polyclonal anti-hGH antibody (a

kind gift from Dr A F Parlow, NIDDK, Bethesda, MD, USA). After five washes for 5 min each in washing buffer (PBST), membranes were incubated with goat anti-rabbit IgG (1:5000) alkaline-phosphatase-conjugated second antibody, using CSPD[®] as substrate (Tropix, PE Biosystem, Bedford, MA, USA) for 1 h at room temperature. The membranes were further washed five times for 5 min each time in PBST before immunolabeling. Immunolabeling was detected by placing the blot with standard X-ray film according to the manufacturer's instructions (Tropix).

Immunohistochemistry

One hundred microliters of MCF-7 cells in suspension were put on autoclaved 3-aminopropyl-triethoxysilane-coated slides at 37 °C for 30 min. Slides were placed in Petri dishes with DMEM overnight at 37 °C. The medium was then removed and the slides were air dried. After that, MCF-7 cells were fixed by the following procedure: 15 min in 10% buffered formalin, 5 min in PBS (0.01 mol/l phosphate buffer, pH 7.4, containing 0.15 mol/l NaCl), 4 min in −20 °C methanol, 2 min in −20 °C acetone and 5 min twice in PBS. Pit-1 (X-7) affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.; raised against a polyhistidine fusion protein construct containing sequences corresponding to full length Pit-1 of rat origin) or polyclonal antibody to GH (Dakopatts, Glostrup, Denmark) were used. The antigens were retrieved by microwaving at 750 W for 10 min in 0.01 M trisodium citrate buffer, pH 6.0. The streptavidin-biotin complex (SABC) immunohistochemical method was used and the sections were consecutively incubated in: (1) 3% hydrogen peroxide (Merck, Darmstadt, Germany) in distilled water for 10 min in order to block endogenous peroxidase; (2) polyclonal antibody anti-Pit-1 at a dilution of 1/10 for 1 h at room temperature or polyclonal antibody to GH at a dilution of 1/100 for 1 h at room temperature; (3) biotinylated goat antibodies to mouse/rabbit immunoglobulins (Duet kit; Dakopatts) at a dilution of 1/100, for 30 min; (4) streptavidin-biotin-peroxidase complex (Duet kit; Dakopatts) prepared according to the protocol provided by the manufacturer, for 30 min; and (5) 3,3'-diaminobenzidine-tetrahydrochloride (DAB) solution prepared by dissolving one DAB buffer tablet (Merck) in 10 ml distilled water for 10 min. Between steps, the sections were washed twice for 5 min with PBS and after step 5 with distilled water. All dilutions were made in PBS. This buffer was added with 0.1% bovine serum albumin (Sigma Chemical Co.) for dilution of the primary antibodies (step 2) and with 1.5% normal goat serum (Dakopatts) for the biotinylated antibodies (step 3). No counterstaining was carried out.

To avoid the use of streptavidin (and then to be sure that we were not revealing endogenous biotin), the

Figure 2 Western blot analysis of immunoreactive Pit-1 and GH in MCF-7 cell line. Cell extracts obtained from the MCF-7 cell line were prepared and subjected to SDS-PAGE, and Western blot analyses were performed as described in Materials and Methods. (A) The blot was incubated with the polyclonal Pit-1 antiserum. The major 31 and 33 kDa immunoreactive bands are indicated by arrows. Lane 1: molecular weight marker (10–200 kDa prestained protein ladder). Lane 2: human pituitary extract used as control. Lane 3: MCF-7 cell line extract. (B) The blot was incubated with the polyclonal GH antiserum. The immunoreactive GH band is indicated by an arrow. Lane 1: MCF-7 cell line extract. Lane 2: human recombinant GH used as control. Lane 3: human pituitary extract also used as control.

immunohistochemical detection of GH was also automatically performed (TechMate 500; Dako Carpinteria, CA, USA) using the Dako EnVision staining procedure that substitutes steps 3 and 4 with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (30 min).

Controls for specificity of immunohistochemistry included using alternately PBS in place of one of the incubation steps or incubation with the primary antibodies preadsorbed overnight at 4 °C with the immunogen peptides Pit-1 (Santa Cruz) and GH (Pharmacia Iberia, San Cugat del Vallés, Spain).

Results

Detection of Pit-1 and GH mRNA by RT-PCR

PCR amplification of cDNA prepared from MCF-7 human adenocarcinoma cells gave rise to a 295 bp PCR product corresponding to human Pit-1 (33 kDa) (Fig. 1A, lane 3) and a 344 bp PCR product corresponding to hGH (Fig. 1B, lane 3). In order to verify the identity of PCR products, we performed restriction enzyme cleavage. PCR products have a characteristic and unique restriction site localized between the primers. The restriction enzymes used were Mbo II for Pit-1 and Bgl II for GH product. Mbo II cleaves the product at position 405 (corresponding to the Pit-1 cDNA) obtaining 219 bp and 76 bp fragments. Bgl II cleaves the PCR product at position 551 (corresponding to the hGH cDNA) obtaining 237 bp and 107 bp fragments. These patterns were visualized on ethidium bromide staining of 2% NuSieve (BMA, Rockland, ME, USA) agarose gel electrophoresis (Fig. 1A,

lane 4 and B, lane 4). The PCR products were also sequenced to confirm the identity of Pit-1 and GH cDNAs using the fmol DNA cycle sequencing system from Promega. The sequences were identical to the pituitary Pit-1 and GH (data not shown).

Detection of Pit-1 and GH protein by Western blotting

Two major Pit-1 immunoreactive bands were readily visible with the MCF-7 extracts (Fig. 2A, lane 3). These bands, which arise from alternative translation initiation codon usage of Pit-1 mRNA, have been referred to previously as 31 and 33 kDa (Voss *et al.* 1991). Western blots also clearly showed similar immunoreactive bands from human pituitary gland extracts used as control (Fig. 2A, lane 2).

GH was also detectable in MCF-7 cells by Western blotting. The results of anti-hGH-antibody binding revealed a 22 kDa protein band corresponding to the approximate molecular weight of hGH protein (Fig. 2B, lane 1). We also observed a strong band corresponding to the recombinant GH (Fig. 2B, lane 2) and the human pituitary gland extracts (Fig. 2B, lane 3) used as positive controls.

We also performed Western blots for Pit-1 and GH protein detection using Saos-2 (human osteosarcoma cell line) and Hek-293 (human embryo kidney cell line), but were unable to find any meaningful expression of either of them (data not shown).

Detection of Pit-1 and GH by immunohistochemistry

MCF-7 cells showed positivity for Pit-1. Immunoreactivity appeared as a punctate signal identified in the nuclei of

MCF-7 cells (Fig. 3a). No immunoreactivity was observed when the primary antibody was preadsorbed with the homologous antigen (Fig. 3b).

Immunostaining for GH was localized in the cytoplasm of MCF-7 cells. Both methods, SABC and EnVision, showed a weak positivity in MCF-7 (Fig. 3c and d). No

immunoreactivity was observed when the primary antibody or other incubation steps were substituted by PBS (Fig. 3e) or when the primary antibody was preadsorbed with the homologous antigen (Fig. 3f).

Discussion

The presence of Pit-1 synthesis in the pituitary is required for prolactin, GH and TSH cell commitment, differentiation and gene expression (Karin *et al.* 1990, Simmons *et al.* 1990, Steinfeld *et al.* 1991). Indeed, the inhibition of Pit-1/GHF-1 synthesis leads to a marked decrease in cell proliferation in GH- and prolactin-producing cell lines (Castrillo *et al.* 1991). The expression of Pit-1 was higher in pituitary adenomas than in normal pituitary, and this expression is restricted to GH-, prolactin- and TSH-expressing cells, suggesting a possible role of this transcription factor in the pathogenesis of pituitary tumors (Asa *et al.* 1993, Delhase *et al.* 1993b). In non-pituitary tissues, Lantinga-van Leeuwen *et al.* (1999) did not find Pit-1 expression either in normal or progesterone-induced GH expressing canine mammary gland, suggesting that GH gene expression appears to be independent of Pit-1 at least before malignant mammary transformation. In the human myeloid leukemic HL-60 cells, the presence of Pit-1 has been demonstrated, and this presence is correlated with their degree of proliferation but appears to be independent of cell differentiation (Costoya *et al.* 1998).

In our work, we have demonstrated the presence of mRNA coding Pit-1 (using RT-PCR) and Pit-1 protein (both using Western blot and immunocytochemistry) in MCF-7 human breast adenocarcinoma cells. Thus, these data demonstrated that Pit-1 is expressed in a tumoral cell line, supporting the hypothesis that Pit-1, as in canine mammary tumors or in HL-60 cells, could be related to an increased proliferation process.

The role of GH as well as the factors involved in their control in the pituitary gland is well established. However, the role of GH production in extra-pituitary tissues and the mechanisms that control this expression are currently

Figure 3 Immunohistochemical detection of Pit-1 and GH in MCF-7 cell line. (a) MCF-7 cells immunostained for Pit-1. A granular signal is shown in the nuclei (objective magnification $\times 60$). (b) Negative control for Pit-1. No signal was detected when the antiserum was preadsorbed with the homologous antigen (Nomarsky differential interference contrast; objective magnification $\times 40$). (c) MCF-7 cells immunostained for GH by the SABC method. A weak positivity was detected in the cytoplasm (objective magnification $\times 40$). (d) MCF-7 cells immunostained for GH by the EnVision method. A weak positivity is also observed (objective magnification $\times 40$). (e) Negative control for GH performed by substitution of GH polyclonal antibody with PBS (objective magnification $\times 40$). (f) Negative control for GH. No signal was found when the antiserum was preadsorbed with the homologous antigen (Nomarsky differential interference contrast; objective magnification $\times 40$). Bars: 20 μ m.

under discussion. GH and GH receptors are present in the human mammary gland (Decouvlaere *et al.* 1995, Mol *et al.* 1995a). Endocrine and autocrine GH exert a direct effect on the development and differentiation of mammary epithelia *in vitro* and *in vivo* (Feldman *et al.* 1993, Plaut *et al.* 1993) and elevated GH levels in sera have been correlated with breast cancer (Emerman *et al.* 1985). In a recent paper it has been demonstrated that treatment with GH induces mammary gland hyperplasia in aging primates (Ng *et al.* 1997), suggesting that this mammary epithelial proliferation may be a consequence of the increased levels of IGF-I induced by the high levels of GH or directly by acting through the epithelial prolactin receptor. The latter hypothesis has also been discussed in a recent paper (Wennbo & Törnell 2000).

On the other hand, it has been demonstrated that hGH can act in an autocrine manner in MCF-7 cells to promote cell proliferation and transcriptional activation, and that the increase of GH production enhances the rate of mammary carcinoma cell spreading in a JAK2-dependent manner (Kaulsay *et al.* 1999, 2000). Interestingly, these researchers demonstrated that the autocrine-produced hGH is a more potent stimulator of mammary carcinoma cell spreading than exogenously administered hGH, and this effect is mediated via the hGH receptor (Kaulsay *et al.* 2000, 2001). However, in these above-mentioned works the authors used a model system in which the hGH gene or a translation-deficient hGH gene (as negative control) is stably transfected into MCF-7 cells. The fact that, for the first time, we have demonstrated the presence of hGH in MCF-7 cells could be due to the transformation of these malignant cells. In any event, we think that the presence of hGH should be tested when using these MCF-7 cells to evaluate the effects of GH on tumor mammary proliferation or spreading.

Thus, in this work, we have demonstrated the presence of both Pit-1 and GH in the MCF-7 human breast adenocarcinoma cell line. The presence of GH, the main regulator of Pit-1 in the pituitary, in this MCF-7 cell line provides a model that should be carefully studied to see the effects of both Pit-1 and/or GH on the development, maintenance or spread of breast tumors. Although from our data we cannot demonstrate that Pit-1 regulates mammary GH expression, the presence of both Pit-1 and GH mRNA and protein together with the GH receptor in breast tumor cell line MCF-7 could be related to Pit-1 acting directly or co-ordinately with GH on cell proliferation. Further studies assessing this hypothesis are clearly merited.

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