Enzyme-Linked Immunosorbent Assay for Pharmacological Studies Targeting Hypoxia-Inducible Factor 1α

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Hypoxia-inducible factor 1 (HIF-1) activates the transcription of a wide range of genes related to oxygen delivery and metabolic adaptation under hypoxic (low-oxygen) conditions. HIF-1 is, in fact, a heterodimer of two subunits, HIF-1α and HIF-1β. The only analytical methods available for measuring HIF-1α levels in tumors are immunohistochemistry and Western blotting. Immunohistochemistry has the advantage of allowing the identification and direct examination of HIF-1α-expressing cells, but has the intrinsic limitation, as for Western blotting, of being nonquantitative. We developed and validated an enzyme-linked immunosorbent assay (ELISA) approach to measure HIF-1α levels in cultured tumor cell lines in vitro. HIF-1α was expressed in thirteen tumor cell lines grown under hypoxic conditions; however, the levels differed strongly between cell lines. These data point to intrinsic differences between cell lines for the induction of HIF-1α under hypoxic conditions. The ELISA developed in the present study is thus an interesting alternative to other analytical methods used to measure HIF-1α protein levels and should be useful in preclinical pharmacological studies targeting HIF-1α.

MATERIALS AND METHODS

Main reagents. Full-length glutathione S-transferase (GST)-HIF-1α fusion protein was provided by George Simos (Larissa, Greece).

Antibodies. Monoclonal anti-HIF-1α antibody (clone H129.67) was from NOVUS BIOLOGICALS (ABCAM Cambridge G-B). This antibody was raised against a GST fusion protein containing amino acids 432 to 528 of human HIF-1α.

Monoclonal anti-HIF-1α Ab-1 (clone OZ12) antibody without bovine serum albumin was from Neo Markers, Inc. (MICROM, Francheville, France).

Monoclonal anti-HIF-1α Ab-2 (clone OZ15) antibody without bovine serum albumin was from Neo Markers, Inc. (MICROM, Francheville, France).

These two antibodies were raised against amino acids 530 to 826 of the human HIF-1α protein. The epitopes of the Neo Markers Ab-1 and Ab-2 are different. The amino acid sequence of the region concerned is different from that of the others members of the HIF family (HIF-2α and HIF-3α).

Polyclonal anti-HIF-1α antibody was produced in the rabbit against amino acids 530 to 826 of the human HIF-1α (17). (Under hypoxic conditions, no HIF-1α protein was detected following transfection with siRNA against HIF-1α in HeLa cells.)

Polyclonal anti-rabbit immunoglobulin G (heavy and light chain) horseradish peroxidase antibody was from PROMEGA (Charbonnières, France).

Other reagents. Immulon 2 96-well plates for ELISA were from DYNATECH (POLYLABO, France). O-Phenylenediamine (OPD), white crystals, was from...
SIGMA (France). Protease inhibitor cocktail (a mixture of water-soluble protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic, and metallo-proteases) and phosphatase inhibitor cocktail (a mixture of inhibitors that will inhibit acid and alkaline phosphatase as well as tyrosine protein phosphatases) were from SIGMA (France).

**Cells in culture.** Several tumor cell lines were previously developed and characterized in our Institute (CAL17, CAL33, CAL60, CAL165, and CAL166 of head and neck origin). The MCF7 cell line of breast origin was provided by J. Pouyssegur. Hep-2 and Detroit 562 cell lines of head and neck origin and HT29 of colonic origin were purchased from the American Type Culture Collection.

**Cell culture and hypoxic conditions.** Thirteen human tumor cell lines of different origins were investigated in order to evaluate their HIF-1α levels in normoxic and hypoxic conditions. Cells were grown in 10-cm diameter dishes in a humidified incubator (SANYO, Japan) at 37°C in an atmosphere containing 21% oxygen and 5% CO2 (normoxic conditions). Cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50,000 U/ml penicillin and 80 µM streptomycin. Subconfluent cells were incubated under hypoxic conditions (1.2% oxygen and 5% CO2) as follows: one dish of each cell line was incubated for up to 4 h in a sealed “Bug-Box” anaerobic workstation (Ruskin Technologies, Jouan, France). In parallel one dish of each cell line was incubated under normoxic conditions.

**Preparation of cell extracts.** The different cell lines, cultured in 10-cm diameter dishes (∼8 × 10⁶) and incubated in normoxia or hypoxia, were lysed on ice with 1 ml of cold lysis buffer 50 mM Tris-HCl pH 7.5 containing 1% Triton X-100, 100 mM NaCl, 5 mM EDTA, 40 mM β-glycerophosphate, 50 mM NaF, 10% protease inhibitor cocktail and a 10% phosphatase inhibitor cocktail. lysates were transferred to Eppendorf tubes, incubated for 15 min at 4°C and centrifuged for 15 min at 17,000 × g at 4°C. The supernatant was collected, a fraction was taken to determine the protein concentration (Bradford method), rapidly frozen in liquid nitrogen and stored at −80°C for later use.

**Western blotting.** HeLa cells incubated under the same hypoxic conditions as described in 2.3, were lysed in SDS sample buffer. The protein concentration was determined using the Lowry assay and 50 ng of whole-cell extracts were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7.5% for HIF-1α detection) and electrothermically transferred onto a polyvinylidene difluoride membrane (Millipore). Immune-reactive bands were visualized with the ECL system (Amersham Biosciences).

**General ELISA principles.** The HIF-1α assay is a solid-phase enzyme immunoassay (“sandwich” ELISA). Wells of 96-well plates were coated with a monoclonal anti-HIF-1α antibody. Three different monoclonal antibodies were compared (from NOVUS and Neo Markers, Inc., see above). Wells were washed with PBS-Tween 20 (0.1%) and 100 µl of cell extract added [5 to 200 µg of total cell protein from tumor cell lines diluted in an assay solution (100 µm phosphate buffer pH 7.2, 0.12% Triton X-100, 1% skimmed milk)]. The HIF-1α protein present in cell extracts binds to the monoclonal antibodies coated on the wells during the indicated incubation period. Unbound material is removed by washing the plates with PBS-Tween 20 (0.1%). A polyclonal anti-HIF-1α was then added to the wells. Unbound material was removed by washing the plates and an anti-rabbit IgG horseradish peroxidase-conjugated antibody was then added. After further washing, OPD (horseradish peroxidase substrate; 30 mg OPD in 10 ml of citrate-phosphate buffer pH 5.5, 0.02% H2O2) was added and incubated for 30 min in the dark. The enzymatic reaction was stopped by addition of sulfuric acid 2N and the intensity of the resulting color was read at 492 nm using a Labsystems spectrophotometer.

**Antibodies.** All experiments were performed in 96-well ELISA plates. Wells were coated overnight, at room temperature, using the different monoclonal antibodies alone or in combination at different concentrations ranging from 0.5 to 1 µg/well.

The polyclonal antibodies were diluted in the assay solution. Tests were performed with several dilutions of the polyclonal anti-HIF-1α ranging from 1/10000 to 1/100000 and dilutions of the polyclonal anti-rabbit IgG-horseradish peroxidase ranging from 1/2500 to 1/500 000. To minimize cross-reaction with monoclonal antibodies, 10% of mouse serum was added in the solution with the latter antibody.

**Measurement of the HIF-1α protein level.** SDS-PAGE and silver staining of the GST-HIF-1α protein and bovine serum albumin, as standard, showed at 20 µg of the GST protein corresponds to 1 µg of bovine serum albumin. Comparison, by Western blot analysis, of the GST-HIF-1α protein and a protein extract obtained from HeLa cells cultured under hypoxic conditions (1.2% O2) for four hours, showed that 50 µg of the cellular protein extract corresponded to 0.5 µg of GST-HIF-1α and thus the latter, contains approximately 35 ng of native HIF-1α protein. The results of the ELISA assay performed on 50 µg of the same protein extract allowed us to conclude that 1 ng of HIF-1α protein corresponded to a specific optical density (OD) of 0.030.

The specific OD was calculated by subtracting the background OD (0.120) from the values directly obtained from the spectrophotometer. All results were expressed as ng of HIF-1α protein.

Cell extracts were prepared under the above indicated conditions; prepara-

**RESULTS AND DISCUSSION**

**Assay optimization.** The assay was optimized to obtain the highest specific OD. Optimal coating was obtained by incubating overnight, at room temperature, with an equimolar mixture of the two anti-HIF-1α monoclonal antibodies from Neo Markers, Inc. (clones OZ12 and OZ15) at a final concentration of 0.5 µg/100 µl/well. Wells were washed and placed for 1 h in the presence of the assay solution. After further washing with phosphate-buffered saline-Tween 20 (0.1%), the plates were ready for immediate use. The antigen was the native HIF-1α protein extracted from cell lines as described in the methods section. The optimal signal was obtained with an incubation volume of 100 µl. The highest signal was obtained with overnight incubation at 4°C. Under these conditions the OD was linear up to 50 µg of total protein/well (Fig. 1). This assay concentration was chosen for all subsequent experiments.

The two polyclonal antibodies were tested in combination at different concentrations and at different incubation temperatures in the same experiment. The optimal OD was obtained with the polyclonal anti-HIF-1α 1/5000, incubated overnight at 4°C, then incubated with the polyclonal anti-IgG 1/30000 for 2 h at room temperature, in a final volume of 100 µl per well for each antibody. These conditions provided an optimal OD of 1.0 to 1.2 corresponding to 35 ng of HIF-1α (sample with 50 µg of protein) and a background OD around 0.120.

**Assay characteristics.** A typical curve obtained for serial dilutions, with the incubation buffer, of the HIF-1α protein from hypoxic control cells is shown in Fig. 1. The graph depicts a classical ELISA curve with a linear phase up to 50 µg of proteins followed by a saturation phase from 50 to 200 µg of protein.

The assay thus represents a valuable alternative to immuno-

blotting for more precise quantitative analysis of HIF-1α expression in experiments performed under O2-regulated conditions.

HIF-1α in extracts of the LOVO cell line, incubated in hypoxia (1.2% O2, 5% CO2, for 4 h) was diluted with progressively increasing quantities of a cell extract of the same cell line incubated in normoxia. The total amount of protein per well was kept constant at 50 µg. A plot of the amount of HIF-1α protein versus dilution of a hypoxic cell extract is shown in Fig. 2. This figure shows a good linear regression (r² = 0.974) indicating that the measured specific OD is directly linked to the amount of HIF-1α protein present in the incubation medium.

The intra and interassay reproducibilities were globally satisfactory. Data were obtained from frozen aliquots of cell extracts obtained from a control preparation: variability of the amount of HIF-1α protein (50 µg of total protein in extract) was 5.12% for intraassay (n = 5 aliquots of a hypoxic prepa-
ration of HeLa cells, mean = 27.30 ± 1.40 ng) and 11.22% for intraassay (n = 5 aliquots of a normoxic preparation of the same cell line, mean = 6.80 ± 0.77 ng).

Taking into account the different batches of the anti-HIF-1α monoclonal antibodies from Neo Markers, Inc. (n = 4) and cellular extracts from different preparations of hypoxic HeLa cells (n = 5), the overall interassay variability (50 µg of total protein extract) was 30.1% (n = 20 aliquots of a hypoxic preparation of HeLa cells, mean = 29.3 ± 8.8 ng).

The threshold of sensitivity of this method defined as twofold the background of the assay was 4 ng. HeLa cells were incubated in standard hypoxic conditions for different times up to four hours and HIF-1α protein was detected by ELISA at each incubation time. The plot of HIF-1α protein obtained as a function of the time of incubation is given in Fig. 3. It shows that HIF-1α levels are directly linked to the incubation time in hypoxia. The amount of HIF-1α in HeLa cells was further examined after four hours in hypoxia followed by a return to normoxia at two different temperatures (37°C and 4°C) for the indicated times. As shown in Fig. 4, at 37°C, the HIF-1α level decreased regularly and was no longer measurable after 40 min. In contrast, no significant modification in HIF-1α levels was observed at 4°C. These data indicate that the ELISA test is able to detect modifications in the amount of HIF-1α protein when regulated by environmental changes in oxygen.

Comparison of the ELISA with a semiquantitative Western blot analysis (corresponding lanes) showed a very good correlation ($r^2 = 0.963$, Fig. 5). This result strengthens our confidence in the ELISA developed in this study.
Biological observations obtained with the assay. HIF-1α levels determined with the ELISA in thirteen tumor cell lines incubated either under hypoxic or normoxic conditions are shown in Fig. 6. The amount of HIF-1α protein detected in normoxia was very low and for the majority of cell lines was below the threshold level of the assay. In contrast, HIF-1α levels in cell lines incubated in hypoxia increased considerably for most cell lines, though substantial differences were observed between cell lines. These data point to intrinsic differences between cell lines for the induction of HIF-1α under hypoxic conditions. Thus, an important part of the contrasting levels of HIF-1α in human tumors may be due to intrinsic tumor characteristics implying different mechanisms of regulation for HIF-1α (16). These mechanisms may concern the control of HIF-1α degradation governed by the rate of hydroxylation and/or of acetylation of HIF-1α which leads to its capture by VHL and final degradation (9–10, 20).

Recent studies have pointed out the prognostic value of tumor oxygenation or HIF-1α expression (1, 4, 5, 8, 11). Increased HIF-1α tumor levels are potentially associated with more aggressive tumors (3). A valuable alternative to the determination of angiogenic factors like HIF-1α in tumors could be their detection in patients’ serum (22). A study by Ugurel and coworkers suggests that angiogenic factors like VEGF are useful predictive serum markers for survival (22). On the other hand, the measurement of angiogenic factors in serum can be of interest for the evaluation of the effects of angiogenic treatments (13) and particularly those targeting HIF-1α (24). It is known that the cellular half-life of HIF-1α is very short (17). This phenomenon is not due to an intrinsic instability of HIF-1α but to its rapid degradation by the ubiquitin-proteasomal system (17). This does not preclude the possibility of examining the presence of HIF-1α in serum which could be released by tumor shedding. The development of a sensitive ELISA to explore the serum levels of this marker may prove to be of clinical relevance. It is important to note that, to this aim, the present assay should be adapted and performed in serum.

The ELISA developed in the present study allows for in vitro quantitation of the HIF-1α protein. The results highlight marked intrinsic differences between cell lines regarding their ability to produce HIF-1α under hypoxic conditions. These differences concord with the complex regulation of HIF-1α (9–10, 20) and suggest that a part of the great intersubject variability in tumor HIF-1α expression may be related to variable intrinsic capacities of the type of tumor to regulate HIF-1α levels.

Certain in vitro studies may necessitate quantification of HIF-1α expression, for example to evaluate HIF-1α expression under variable hypoxic conditions (2) or to evaluate the level of HIF-1α when targeted by siRNA (26) or by specific inhibitors (14). The screening of known small-molecules as non specific inhibitors of HIF-1α is a first step in inactivating HIF-1. Fundamental and pharmaceutical research into angiogenesis has lead to the development of new molecules that inhibit HIF-1α (7, 15). The presently described assay will be very useful in preclinical studies with new drugs targeting HIF-1α for anticancer therapy.
FIG. 6. Screening for the HIF-1α protein level in 13 cell lines incubated for four hours under normoxic or hypoxic conditions (50 μg of total cell extract protein; bars show the standard deviation of duplicates). The plotted line indicates the limit of sensitivity of the method.

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