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The Effect of Hypoxia on the Expression of 150 kDa Oxygen-regulated Protein (ORP 150) in HeLa Cells

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Key Words
ORP 150 • Hypoxia • HeLa cells

Abstract
Correct protein folding is an important factor, for the translocation of newly synthesised proteins to specific subcellular compartments, extracellular matrix or to biological fluids. This process is regulated by a group of specific proteins, referred to as chaperones. Many stress conditions, such as oxygen or glucose deprivation, slow down the folding process and cause accumulation of unfolded/misfolded proteins in the cell. Molecular chaperones are induced in these conditions; with some named as oxygen-regulated proteins (ORPs). These bind to unfolded / misfolded proteins to facilitate correct assembly. ORP 150 is the subject of this study. Hypoxia results in an enhancement of ORP 150 expression in several tumour cell lines cultured *in vitro*. HeLa cells grown in hypoxic conditions (despite an intensive expression of ORP 150) demonstrate higher rates of apoptosis in comparison to those cultured in normoxic conditions. Furthermore, the inhibition of ORP 150 synthesis by transfection of these cells with a specific siRNA resulted in an intensification of apoptosis, as indicated by specific markers of this process; the enhancement of poly ADP-ribose protein cleavage and the increase in Bim protein expression. We conclude from our study that the increase in ORP 150 synthesis protects the cells against the pro-apoptotic effect of hypoxia.

Introduction

The endoplasmic reticulum (ER) is the site of synthesis and maturation of secretory pathway proteins, which include resident proteins of the endocytic and exocytic organelles, as well as surface and secreted proteins. Approximately one-third of all cellular proteins are translocated into the lumen of the ER with its unique oxidizing and Ca^{2+}-rich environment, where post-translational modification, folding and oligomerisation of nascent proteins occur. The folding process is regulated by a group of proteins, referred to as chaperones. ER molecular chaperones and folding enzymes associate with the newly synthesized proteins to prevent their aggregation

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and help them to fold and assemble correctly [1, 2]. Correct folding is essential for translocation of protein molecules to specific subcellular compartments, the extracellular matrix or to biological fluids [3].

Many stress conditions such as oxygen or glucose deprivation reduces the folding process which results in the accumulation of unfolded/misfolded proteins within the cell. Molecular chaperones are induced in these conditions, bind to unfolded/misfolded proteins, and help them to be folded or refolded correctly [3]. An integral component of the cellular response to environmental stress is the expression, usually by de novo protein synthesis, of stress-associated polypeptides termed oxygen-regulated proteins (ORPs) [4, 5].

Cellular adaptations to environmental oxygen deprivation constitute an important protective pathway for the host response to ischemia [6]. One essential component of this response includes the increased dependence on anaerobic metabolism as an energy source [7]. Enhanced glycolysis is facilitated by hypoxia-mediated up-regulation of the noninsulin-dependent glucose transporter-1 and several enzymes involved in glycolytic metabolism [8], by a transcription factor referred to as hypoxia-inducible factor-1 (HIF-1) [9]. HIF-1 is a heterodimer composed of an oxygen-regulated α-subunit (HIF-1α) and a constitutively expressed β-subunit (HIF-1 β). Normally HIF-1 α expression is tightly regulated by oxygen pressure and, thus, in most cases HIF-1 α is undetectable in normoxic cells [10] due to its rapid degradation through the ubiquitin / proteasome degradation pathway [11, 12].

The cellular biosynthetic response to hypoxia also involves the expression of several ORPs [4, 8], one of which has a molecular weight 150 kDa, termed ORP 150. This is part of the ER machinery that imports proteins from the secretory pathway into the ER, and together with other chaperones such as 78- and 94- kDa glucose regulated proteins (GRP 78 and GRP 94) ORP 150 assists in the folding and assembly of secretory and membrane proteins [13]. The expression of ORP 150 becomes increased in a range of pathologic situations such as brain ischaemia [14], atherosclerotic plaques [15] and malignant tumours [16]. The expression of ORP 150 in cultured human cells is essential for their survival under prolonged hypoxia [5]. Such data suggest that ORP 150 contributes in the cellular response to environmental stress.

Our experiments have demonstrated that hypoxia effectively induces ORP 150 expression in HeLa cells, suggesting that this protein may play an important role in the cytoprotective response to environmental stress. In order to check this hypothesis we have submitted HeLa cells to transfection with specific ORP 150 siRNA, a procedure expected to block the translation of ORP 150 mRNA. The effect of this induced ORP 150 deficiency has been studied in relation to cell apoptosis.

Materials and Methods

The cell lines

The following cell lines - provided by Novartis, Basel, Switzerland - were used: HeLa cells (human cervical adenocarcinoma), A 2058 cells (human melanoma), HCT 116 cells (human colon carcinoma), BT 474 cells (human breast carcinoma), HEK 293 cells (human embryonic kidney), MCF 7 cells (human breast cancer).

Culture media

The High Glucose Dulbecco's modified Eagle's medium (High Glucose DMEM) and OptiMEM (serum-reduced medium) were provided by Invitrogen (San Diego, USA). The High Glucose DMEM was supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The OptiMEM was used for transfection procedure only. It was not supplemented with additional components.

Reagents

Deferoxamine (Sigma, St. Louis, USA), Cobalt chloride (Fluka, Buchs, Switzerland), Passive lysis buffer (Promega, Madison, USA), BCA Protein Assay Kit (Novagen, Darmstadt, Germany), Anti-ORP 150 (IBL, Gunma, Japan), Anti-KDEL (StressGen Biotechnologies Corp., Victoria, Canada), Anti-HIF-1α (Transduction Laboratories, Lexington, USA), Anti PARP, (Cell Signaling Technology, Beverly, USA), Anti-Bim (Calbiochem, San Diego, USA), Anti-β-tubulin (Sigma, St. Louis, USA), Horseradish peroxidase-coupled secondary antibody; anti-mouse IgG (Sigma, St. Louis, USA), ECL chemiluminescence system (Santa Cruz Biotechnology Inc., Santa Cruz, USA), PMSF, (Sigma, St. Louis, USA) and siRNA of the sequence TTT CAG GGT CAC GAT CAC Cgg (Novartis, Basel, Switzerland) were used.

Cell cultures

All the cells were cultured in DMEM containing glucose at 4.5 mg/ml (High Glucose DMEM). The cells were seeded at a density of 2.5 - 3.0 x 10^5 per ml of culture medium and grown on six-well plates, in CO_2 incubator, at 37°C.

Induction of hypoxia in cell cultures

The cells (5 x 10^5 in 2 ml of medium) were seeded in six-well plates and incubated until they achieved confluence (about 72 h). The High Glucose DMEM was removed and replaced with 2 ml of the same fresh medium. Control cell cultures were kept in normoxic conditions whereas the test cells were incubated in hypoxic conditions, as described in procedures A or B.

A. Physiological hypoxia was evoked by 24 h incubation of cells in atmosphere containing a reduced (to less than 2 %) oxygen concentration. The BBL GasPak Pouch system (Becton-Dickinson) was used to reduce oxygen concentration..

B. Chemical hypoxia was evoked by 24 h treatment of cells with 0.2 mM deferoxamine or 1 mM $CoCl_2$ introduced to the culture medium. Deferoxamine induces HIF-1 activity by inhibiting HIF-1α hydroxylation, whereas $CoCl_2$ binds to the oxygen dependent degradation domain (ODDD) of HIF-1α and inhibits the von Hippel-Lindau Protein (pVHL)-binding to HIF-1α subunit [17]. No cytotoxic effects of these compounds (at least at concentrations used in our experiments) were observed [18, 19].

Sodium dodecyl sulphate /Polyacrylamide gel electrophoresis (SDS/PAGE)

The cells were harvested, washed with cold PBS and solubilised in lysis buffer. The lysate was centrifuged at 10 000 x g for 10 min at 4°C to remove insoluble cellular debris. Protein concentration was determined with the BCA Protein Assay Kit. Samples of cell lysates, containing 30 µg of protein, were subjected to SDS-PAGE, as described by Laemmli [20]. The electrophoresis was run for 40 – 45 minutes using 7.5 % polyacrylamide gel and constant voltage (220 V).

Immunoblotting

The proteins were transferred to PVDF membranes (Bio-Rad, Hercules, USA) and then pre-treated with TBS containing 0.05% Tween 20 and 5% defatted dry milk, at room temperature, for 2 hours. Membranes were probed with a mixture containing the following mouse monoclonal antibodies: anti-ORP 150 (1: 100), anti-KDEL (1: 1 000), anti-HIF-1α (1:250) and anti-β-tubulin (1: 10 000) at 4°C, for 16 h. After extensive washing the membranes were transferred to a secondary antibody horseradish peroxidase-coupled anti-mouse IgG (1: 5 000), and incubated at room temperature for 1 h. After washing with TBS/T or PBS/T the membranes were analysed using an ECL chemiluminescence system. Quantification was performed with the use of Kodak Image Station. The molecular weights of the investigated proteins were estimated by comparison with Bio Rad molecular weight standards.

siRNA transfection in HeLa cells

The cell cultures were transfected with a specific ORP 150 siRNA and submitted to hypoxia as described above. For transfection 2 µl oligofectamine reagent were mixed with 11 µl of OptiMEM (Invitrogen, San Diego, USA) and allowed to stand at room temperature for 10 minutes. The original siRNA preparation was diluted with OptiMEM to obtain 180 µl of 10 nM solution. These two solutions were combined, gently mixed and incubated at room temperature for 20 minutes to form the transfection mixture.

The cells were washed twice with PBS and then 800 µl of OptiMEM were added to each well. The transfection mixture was added to all cultures and the cells were incubated at 37°C for 4 hours. The transfection mixture was removed and replaced with High Glucose DMEM and cells were incubated for 24 h in either hypoxic or normoxic conditions. The media were removed, the cells were harvested, washed in cold PBS and solubilised in passive lysis buffer. The lysates were centrifuged at 10 000 x g at 4°C for 10 minutes to remove insoluble cellular debris and supernatants were subjected to SDS/PAGE and immunoblotting, as described above.

Two series of control cultures were prepared. Control A – the cultures were submitted to hypoxia (as described above) and not subjected to any transfection procedures. Control B – the cells were submitted to transfection procedures, but they were treated with scrambled, RISC-free and non-targeting siRNA and then submitted to hypoxia (as described above). In RNAi studies using these conditions above 80% knockdown of an endogenous gene was observed (Invitrogen protocol, form No. 18057N).

Western immunoblot analysis for poly ADP-ribose protein (PARP) cleavage

This procedure was used to assess the effect of hypoxia on apoptosis of HeLa cells. Adherent cells were washed once with ice-cold PBS and scraped into 1 ml of PBS containing 1mM PMSF (an inhibitor of proteolytic enzymes). Cell pellets were collected by centrifugation (at 1500 rpm for 3 min) and resuspended in 200 µl of ice-cold PBS, followed by the addition of 200 µl of extraction buffer (50mM Hepes, 150mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 5mM EGTA, 1mM EDTA, 15 mM PPI). Cell pellets were collected by centrifugation at 10 000 rpm at 4°C for 30 minutes. Equal amounts of protein lysates were resolved on a 10% SDS-PAGE gel, transferred to PVDF membrane, and probed with rabbit anti-PARP antibody (1: 5 000) followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody. The membranes were analysed using an ECL chemiluminescence system.

Western immunoblot analysis for Bim protein

The expression of Bim protein is treated as another index of cell apoptosis where it is reported to neutralise prosurvival Bcl-2 proteins [21]. The immunoblotting procedure (with the use of anti-Bim antibody (1:1 000)) was identical as that applied for the analysis of PARP cleavage (89 kDa) product.

Protein assay

Protein concentrations were determined by the BCA Protein Assay Kit according to instructions supplied by the manufacturer. Bovine serum albumin was used as a standard.

Results

The effect of physiological hypoxia on the expression of ORP 150 in various tumour cells

Fig. 1 shows the comparison of ORP 150 expression in various tumour cell lines growing in normoxic (part A) and hypoxic (part B) conditions. It is apparent that hypoxia results in an increase of ORP 150 expression in all the

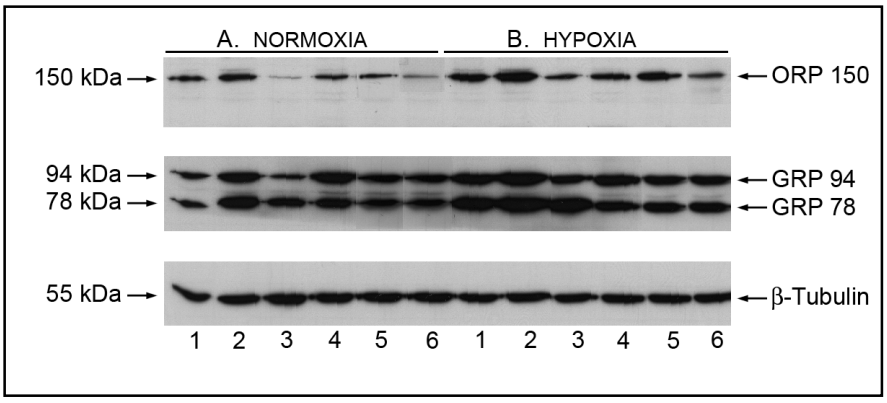

Fig. 1. Western immunoblot analysis of ORP 150 expression in various tumour cells growing in normoxic and hypoxic conditions: lane 1 - A 2058 cells, lane 2- HCT 116 cells, lane 3 - BT 474 cells, lane 4 - HEK 293 cells, lane 5 - MCF 7 cells and lane 6 - HeLa cells (upper panel). The expression of GRP 94 and GRP 78 under the same conditions is demonstrated (middle panel). All samples submitted to analysis contained 30 μg of cell lysate protein. Position of β tubulin (lower panel) is shown as a control for protein loading.

investigated cells (upper panel). Furthermore, hypoxia increased also the expression of GRP 78 and GRP 94 in the same cells (middle panel).

Figure 1 shows that increased ORP 150 expression is a common biochemical feature of the various tumour cells; our subsequent studies focused specifically on HeLa cells.

Detection of HIF-1α in HeLa cells submitted to physiological hypoxia

Figure 2 (lane 1) shows that cells grown in normoxic conditions did not demonstrate the expression of HIF-1α (a biochemical marker of hypoxia) and shows very weak expressions of ORP 150 as well as GRP 94 and GRP 78. In contrast; those cells grown in hypoxic conditions demonstrated an intense expression of both HIF-1α as well as ORP 150, GRP 94 and GRP 78 (lane 2).

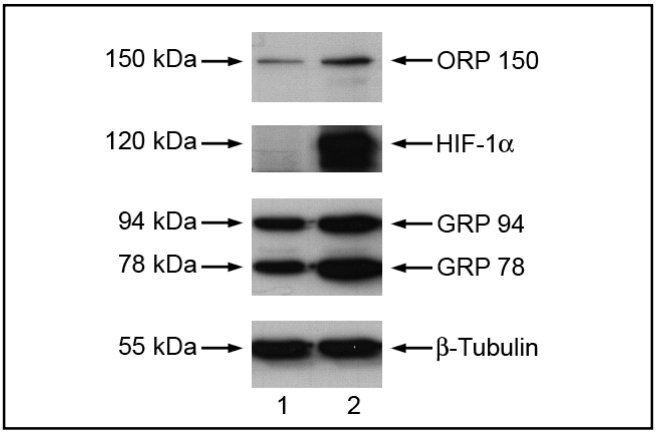

Fig. 2. Western immunoblot analysis of ORP 150 (upper panel) and GRP 94 and GRP 78 (middle panel) in HeLa cells growing in normoxia (lane 1) and physiological hypoxia (lane 2). The expression of HIF-1α expression (middle panel) under the same conditions is demonstrated. All samples submitted to analysis contained 30 μg of cell lysate protein. Position of β tubulin (lower panel) is shown as a control for protein loading.

The effect of chemical hypoxia on the expression of ORP 150

Fig 3 shows that both $CoCl_2$ and deferoxamine evoked symptoms of hypoxia. The control cells (lane 1) did not express HIF-1α, whereas the cells treated with $CoCl_2$ (lane 2) or deferoxamine (lane 3) demonstrated an intense expression of this protein. Control cells growing in normoxic conditions (lane 4) demonstrated a trace expression of ORP 150 whereas the action of $CoCl_2$ (lane 5) or deferoxamine (lane 6) resulted in an increased induction of ORP 150 expression by the HeLa cells.

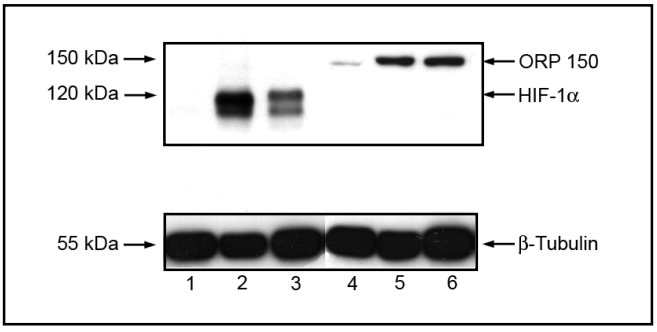

Fig. 3. Western immunoblot analysis of ORP 150 expression in HeLa cells submitted to chemical hypoxia. The HIF-1α expression is shown, as a marker of hypoxia, in normoxic cells (lane 1), in cells treated with $CoCl_2$ (lane 2) and those treated with deferoxamine (lane 3). The expression of ORP 150 is shown in normoxic cells (lane 4), in cells treated with $CoCl_2$ (lane 5) and in those treated with deferoxamine (lane 6). All samples contained 30 μg of cell lysate protein. Position of β tubulin is shown as a control for protein loading.

The effect of siRNA transfection on the expression of ORP 150

Fig. 4A (upper panel) shows that normoxic control HeLa cells (lane 1) and those treated with non-targeting siRNA (lane 2) demonstrated a weak expression of ORP 150. By contrast, transfection of the cells with siRNA specific to ORP150 mRNA resulted in a decrease of this

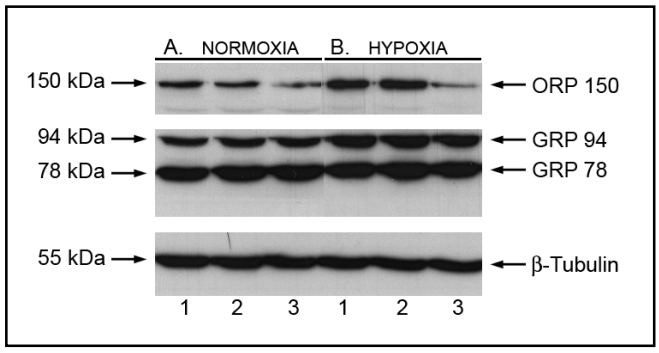

Fig. 4. Western immunoblot analysis of ORP 150 (upper panel) synthesised in control HeLa cells and in those submitted to transfection with specific ORP 150 siRNA in normoxic (Fig.4A) and hypoxic (Fig.4 B) conditions. Lane 1 – non-transfected cells, lane 2 – cells treated with non-targeting siRNA, lane 3 - cells transfected with siRNA. The expression of GRP 94 and GRP 78 (middle panel) is shown as a control for the specificity of transfection. All samples contained 30 μg of cell lysate protein. Position of β tubulin (lower panel) is shown as a control for protein loading.

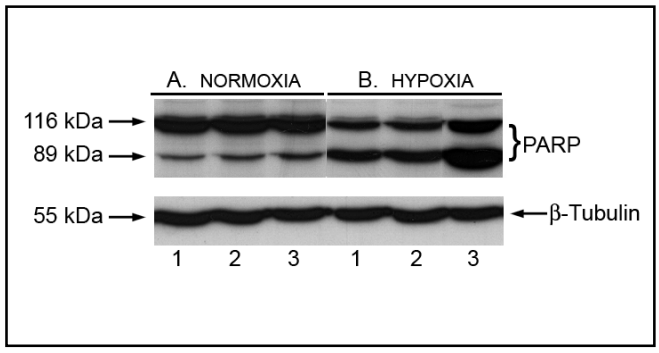

Fig. 5. Western immunoblot analysis of PARP (116 kDa) and PARP cleavage product (89 kDa) in control HeLa cells and in those submitted to transfection with specific ORP 150 siRNA under normoxic (part A) and hypoxic (part B) conditions. Lane 1 – non-transfected cells, lane 2 – cells treated with non-targeting siRNA, lane 3 - cells transfected with siRNA. All samples contained 30 μg of cell lysate protein. Position of β tubulin (lower panel) is shown as a control for protein loading.

expression (lane 3). Fig 4 B (upper panel) shows that hypoxia resulted in increased ORP 150 expression both in control cells (lane 1) and in those treated with non-targeting siRNA (lane 2). Transfection of the cells with siRNA specific to ORP 150 mRNA resulted in the disappearance of ORP 150 expression (lane 3) under hypoxic conditions.

Fig 4 shows that the effect of transfection was specific to ORP 150 since the expression of other proteins such as GRP 94 and GRP 78 did not change, both in normoxic (Fig 4A, middle panel, lanes 1, 2, 3) and hypoxic conditions (Fig. 4B, middle panel, lanes 1, 2, 3).

The effect of siRNA transfection on apoptosis of HeLa cells

The cleavage of poly ADP-ribose protein (PARP) is an index of cell apoptosis. Degradation of PARP (116 kDa) results in the appearance of 89 kDa product, which may be detected by Western immunoblot analysis. Fig. 5A shows that normoxic control HeLa cells (lane 1) and those treated with non-targeting siRNA (lane 2) contained a low amount of PARP degradation product (89 kDa), similar to that produced by cells transfected with siRNA specific to ORP 150 mRNA growing in normoxic conditions (lane 3). However, Fig 5 B shows that hypoxia resulted in increased PARP degradation both in control cells (lane 1) and in those treated with non-targeting siRNA (lane 2); but transfected of HeLa cells with siRNA specific to ORP150 mRNA showed a further increase of PARP degradation (lane 3).

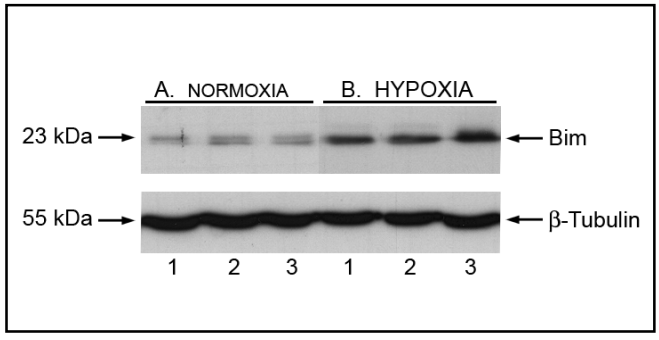

Fig. 6. Western immunoblot analysis of Bim expression in control HeLa cells and in those submitted to transfection with specific ORP 150 siRNA under normoxic (part A) and hypoxic (part B) conditions. Lane 1 – non-transfected cells, lane 2 - cells treated with non-targeting siRNA, lane 3 - cells transfected with siRNA. All samples contained 30 μg of cell lysate protein. Position of β tubulin is shown as a control for protein loading.

Fig. 6 A shows that normoxic control HeLa cells (lane 1) and those treated with non-targeting siRNA (lane 2) demonstrated a weak expression of Bim. Transfection of the cells with siRNA specific to ORP 150 mRNA did not change the expression of Bim in the cells growing in normoxic conditions (lane 3). In contrast, Fig 6 B shows that hypoxia resulted in an increase in Bim expression both in control cells (lane 1) and in those treated with non-targeting siRNA (lane 2); but the transfected cells showed an even further increase of Bim expression (lane 3).

Discussion

It is known that most neoplastic tumours are poorly vascularized and not sufficiently supplied with oxygen, glucose and other nutritients. These limitations enhance cell apoptosis and inhibit tumour progression [22-24]. The evolution and survival of tumour cells in this environment depends on the selection of a cell population which on the one hand is resistant to apoptosis [25] and on the other hand, induces the formation of new blood vessels [26-28].

Environmental stress factors bring about changes in the ER known as a proteotoxic insult. This is recognised by immature proteins accumulating in the lumen of ER together with aggregation and conformational changes [29], and the induction of chaperones is often the result [30]. The chaperones inhibit the aggregation of denatured protein [31] and this protective role is crucial for cell survival and repair in response to environmental stress [30]. Because they were first identified as being induced by glucose starvation, these stress proteins are known as the glucose - regulated proteins (GRPs) [31], some of which are called oxygen regulated proteins (ORPs) [6]. Two of the principal members of this family, GRP 78 and GRP 94, are resident in the ER under normal conditions and have been strongly implicated in the processing of proteins traversing the secretory pathway [32]. In addition to these, a third GRP of approximately 150 kDa (named GRP 170 or ORP 150) was detected [33].

Although the role of ORP 150 in cellular physiology remains obscure, some important features of this protein have been described. Some observations indicate that ORP 150, like GRP 78 and GRP 94, is involved in the processing of proteins in the secretory pathway. Indeed, it is reported that ORP 150 may associate directly or indirectly with GRP 78 and GRP 94, and with immunoglobulins, suggesting that all three GRPs may function as a multimeric complex in immunoglobulin assembly in vivo [31]. Kuznetzov et al [34] have shown that ORP 150 and some other GRPs/ORPs form complexes with thyroglobulin, a major protein secreted by thyroid epithelial cells. Furthermore, ORP 150 has been shown as major calcium – binding protein in the ER [35].

The exposure of HeLa cells to hypoxic conditions results in the induction of various GRPs/ORPs, including ORP 150. One may suspect that this specific response to oxygen deprivation is a common biological phenomenon, which may be important for the survival of various cells under hypoxic conditions.

This study was designed to evaluate the role of ORP 150 in apoptosis of HeLa cells. Using assay which are considered to be recognised markers of this process. Shinjyo et al [21] reported that apoptosis is accompanied by an increase in the expression of Bim protein which binds and neutralizes some anti-apoptotic proteins such as Bcl-2 and Bcl-xL. Lazebnik et al [36] described an enhanced cleavage of PARP in apoptotic cells; the PARP protein (116 kDa) is degraded by apoptosis-activated caspase 3 releasing a specific product of 89 kDa. Thus the expression of pro-apoptotic protein Bim and the analysis of 89 kDa product of PARP cleavage were used to assess the apoptotic tendency of the investigated cells.

Hypoxia was shown to increase ORP 150 as well as GRP 78 and GRP 94 expressions in several tumour cell lines cultured *in vitro*. Further experiments performed on HeLa cells demonstrated that cells grown in hypoxic conditions (despite high GRPs/ORPs contents) demonstrate higher apoptosis in comparison to those cultured in normoxic conditions. However, the inhibition of ORP 150 synthesis by transfection of these cells with a specific siRNA resulted in an enhancement of apoptosis, as indicated by the increased PARP cleavage and Bim expression.

The ER is a critical locus for both biosynthesis and posttranslational modification of proteins. ORP 150 - as an integral part of this system - exerts a cytoprotective effect in various stress conditions [33]. It is apparent from our study that the increase in ORP 150 expression protects the cells against the pro-apoptotic effect of hypoxia.

A better understanding of the signals and pathways by which tumour cells overproduce stress proteins under conditions that might normally trigger cell death, is not only of biological interest since it might also help to identify molecular targets for the therapy of malignant diseases [13].

Abbreviations

PMSF (Phenylmethylsulphonylfluoride); EGTA (Ethyleneglycol-bis(aminoethylether)-tetraacetic acid); EDTA (Ethylene diamine tetra-acetic acid); PPI (Pyrophosphate); PVDF membrane (Polyvinylidene difluoride membrane); TBS/T (Tris Buffered Saline/ Tween 20); PBS/T (Phosphate Buffered Saline/Tween 20).

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