Biochimie xxx (2011) 1–7

Contents lists available at SciVerse ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Exposure to cadmium chloride influences *astrocyte-elevated gene-1* (*AEG-1*) expression in MDA-MB231 human breast cancer cells

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ARTICLE INFO

Article history:
Received 22 July 2011
Accepted 16 October 2011
Available online xxx

Keywords:
Cadmium
Breast cancer
AEG-1
NF-κB
Gene expression

ABSTRACT

It is known that cadmium (Cd) is able to regulate gene expression, drastically affecting the pattern of transcriptional activity and intracellular signalization in normal and pathological human cells. We have already shown that Cd exerts a cytotoxic effect on neoplastic MDA-MB231 cells from the human breast, which is characterized by the onset of a "non-classical" apoptotic kind of death, impairment of mitochondrial activity and drastic changes in gene expression pattern. In the present study, employing a combination of conventional and differential display-PCR techniques, immunocytochemical, ELISA and Western analyses, we extended the knowledge on the transcriptional modulation exerted by the metal demonstrating that in MDA-MB231 cells 5 μM $CdCl_2$ treatment for 96 h selectively down-regulates *astrocyte-elevated gene-1* (*AEG-1*) and reduces the accumulation of its protein product which appears to be associated with the internal cytomembranes and also present in the nucleoplasm. In addition, due to the acknowledged role of AEG-1 in the intranuclear shuttling of NF-κB p65 subunit, we also showed that $CdCl_2$ treatment determines the decrease of p65 amount in nuclear extracts and the down-regulation of the NF-κB downstream genes *c-fos* and *c-jun*, thus providing a new contribution to the comprehension of the intracellular molecular mechanisms implicated in Cd-breast cancer cell interactions.

1. Introduction

Cadmium (Cd) is an industrial and environmental pollutant widely used in the industry of plastics and as a component of batteries, also released as air contaminant from fertilisers and, more prominently, in the form of wastewater. Cd is not essential for the human body and has no known useful biological functions. It is classified in group I of carcinogens by the International Agency of Research on Cancer (see http://monographs.iarc.fr/ENG/Classification/index.php) and may play a role in both cancer initiation, by activating oncogenes *via* β-catenin [1], and progression, although the Cd-induced ability to promote apoptosis not only in normal cells but also in tumoral cytotypes was also demonstrated [2]. The intracellular targets of Cd, once imported through the voltage-sensitive Ca^{++}/Mg^{++} channels of the plasmalemma, are the Zn-binding domains of several metalloproteins; by consequence, Cd interferes with or prevents Zn-dependent cellular functions from being completed [3]. Increasing evidence indicate that Cd is capable to regulate gene expression thereby drastically affecting the pattern of transcriptional activity in both prokaryotic and lower/upper eukaryotic cells, in normal and pathologic conditions [e.g [4–7].]. Dealing with breast cancer, the data obtained with the estrogen receptor (ER) α- and β-positive MCF-7 cell line indicate that exposure to $CdCl_2$ promotes cell proliferation and triggers the expression of several estradiol-responsive genes such as *progesterone receptor*, *cathepsin D* and *pS2*, conversely restraining that of *ER* [8]. This indicates that the metal exerts an estrogen-mimetic effect (thus gaining the attribute of "metalloestrogen") based upon the formation of a high-affinity complex with the hormone binding domain of ERα and the stimulation of estrogen response elements (ERE) present in gene promoters, and the parallel activation of Akt, ERK1/2 and PDGFRα kinases [9]. On the other hand melatonin, which prevents the DNA-binding of the estrogen-ER complex, was found capable to inhibit such effect of $CdCl_2$ on MCF-7 cells [10]. More recently, Yu et al. [11] provided evidence of the involvement of the transmembrane estrogen receptor G-protein coupled receptor 30 (GPR30) in Cd-promoted proliferation of the ER-negative and GPR30- positive SKBR3 cell line.

Abbreviations: ER, estrogen receptor; ERE, estrogen response elements; GPR30, G-protein coupled receptor 30; IC_{50}, 50% inhibitory concentration; DD, differential display; AEG-1, astrocyte-elevated gene-1; FCS, foetal calf serum; SM, semi-quantitative-multiplex; PBSB, phosphate-buffered saline containing 1% bovine serum albumin; ROS, reactive oxygen species.

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doi:10.1016/j.biochi.2011.10.009

Using the highly-tumorigenic ERα- and GPR30- negative MDA-MB231 cell line as an alternative breast cancer model system, we have demonstrated that 96 h-exposure to $CdCl_2$ results in a decrease of cell proliferation with a 50% inhibitory concentration (IC_{50}) of 5 μM, a concentration similar to levels encountered in occupational exposure [12]. We also showed a concurrent effect of the metal on the expression level of selected genes including those coding for members of stress response-, mitochondrial respiration- and apoptosis-related factors. Comparison was made with the pattern of gene expression by the immortalized epithelial cell line HB2, obtained from non-tumoral breast, in response to the same metal concentration and time of exposure, which did not affect cell viability and proliferation with respect to controls. Additional results concerned the significant increase of mitochondrial respiratory activity, the impairing of intra-mitochondrial protein import process and the accumulation of reactive oxygen species in $CdCl_2$-treated MDA-MB231 cancer cells [13–16].

In light of the observed biological activity of $CdCl_2$ on MDA-MB231 cells, the present study was aimed to extend the knowledge on the transcriptional modulation exerted by the metal through differential display (DD)-PCR assay of cDNA samples obtained from cells grown in either control conditions or in the presence of 5 μM $CdCl_2$ for 96 h, in order to identify additional genes whose expression level is modified. Control and $CdCl_2$-treated HB2 cells were assayed as non-tumoral counterpart to check the expression level of the genes under study. The data obtained indicate that in MDA-MB231 cells $CdCl_2$ treatment selectively down-regulates *astrocyte-elevated gene-1* (*AEG-1*), which encodes for a protein product known to be involved in the control of NF-κB pathway and *c-fos*/*c-jun* gene expression.

2. Materials and methods

2.1. Cell culture

The MDA-MB231 breast cancer cell line was routinely grown in RPMI 1640 medium plus 10% foetal calf serum (FCS), and antibiotic/antimycotic mixture (100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/L amphotericin B) (Invitrogen, Carlsbad/CA, USA), at 37 °C in a 5% CO_2 atmosphere.

The HB2 breast epithelial cell line (courtesy of Cancer Research, UK) was routinely grown in high glucose-DMEM medium plus 10% foetal calf serum (Invitrogen, Carlsbad, CA/USA), 5 μg hydrocortisone/ml (Sigma, St.Louis, MO/USA), 10 μg bovine insulin (Sigma) and antibiotic/antimycotic mixture at 37 °C in a 5% CO_2 atmosphere.

For Cd treatment, cells were plated in the presence of 5 μM $CdCl_2$ and grown for different time lapses between 24 and 96 h, as already reported [14,15].

2.2. RNA extraction and reverse transcription

Isolation of total RNA from trypsinyzed control and treated MDA-MB231 cells was carried out with Trizol reagent (Sigma, St.Louis, MO/USA). Before the reverse transcription, the total RNA were treated with RQ1 RNase-free DNase (Promega, Madison,WI/USA) and its quality and integrity checked through agarose gel electrophoresis in denaturing conditions. Enrichment of samples for mRNAs, as required for DD-PCR, was achieved by treatment with Terminator™ 5′-Phosphate-Dependent Exonuclease (Epicentre, Madison, WI/USA), following manufacturer's instructions. The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) in the presence of 100 ng random 6-mer primers (Sigma), 50 U RNase inhibitor (Promega) and 0.5 mM each of dNTPs; reverse transcription was carried out for 60 min at 42 °C, followed by treatment with 2 U RNase H (USB, Cleveland, OH/USA) for 20 min at 37 °C.

2.3. Differential display (DD)-PCR

For differential expression analysis, DD-PCR experiments were performed as already reported [e.g [17,18].] using the arbitrary 10-mer primers designed by Sokolov and Prockop [19], in combinations of two. The PCR amplification was carried out using 25 pmoles of each of two primers, 1–2 μl of the cDNA template and 3.6 U of AmpliTaq DNA Polymerase, Stoffel fragment (Perkin Elmer, USA), in 50 μl of the appropriate reaction mixture. The thermal cycle used was a denaturation step of 94.5 °C for 3 min, followed by 45 cycles of 94.5 °C for 1 min, 34 °C for 1 min, 72 °C for 1 min and a final extension of the product for 10 min at 72 °C. After PCR amplification, 8 μl of the amplification products were electrophoresized in a non-denaturing 6% polyacrylamide gel in a sequencing apparatus at constant 55 W and the band pattern visualized *via* silver staining. Evaluation of band size was performed with SigmaGel software (SPSS, USA). For re-amplification of the differentially-displayed band, the silver-stained gel was exhaustively washed with double-distilled water and the band of interest carefully scratched from the gel with a sterile syringe needle and used as template for PCR amplifications performed as described in the following section. Several cycles of amplification and electrophoresis were repeated until a single pure band was visualized in the gel and eluted using Ultrafree DA filter columns (Millipore, Bedford MA/USA). The purified PCR product was submitted to sequencing by BMR Genomics (Padova, Italy) and DNA sequence similarity was searched with the BLAST algorithm [20] available on-line.

2.4. Conventional and semi-quantitative "multiplex" (SM) PCR

Conventional PCR analysis was performed using 1 U RedTaq DNA polymerase (Sigma)/μl, 200 μM each of dNTPs, 1 μl of the cDNA template obtained from total RNA and 2.5 μM of the following primers obtained from Invitrogen: AAAACAAAACTGCGGACACC (AEG-1 forward), TTTATTCCTCGGCTGCAGAT (AEG-1 reverse), AGCTCCCTCCGG (*c-fos* forward), CTACGAGGCGTC (*c-fos* reverse), CCCCAAGATCCTGAAACAGA (*c-jun* forward), CCGTTGCTGGACTGGATTAT (*c-jun* reverse). The thermal cycle used was a denaturation step of 94 °C for 3 min, followed by 45 cycles of 94 °C for 30 s, the appropriate annealing temperature for 30 s, and 72 °C for 30 s. A final extension of the product was performed for 5 min at 72 °C. PCR products were analysed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. For SM-PCR we followed a published protocol [e.g [14,21].] in which the species of interest is co-amplified with 18S cDNA. The intensities of the band of interest, normalized for those of 18S, were plotted as a function of cycle number and exponential regression equations fitted to the curves were used to calculate the number of cycles necessary to reach a normalized intensity threshold value = 1 for each sample. The relative difference in abundance between two samples was taken as 2^n where n is the difference between the numbers of cycles required by the samples to reach the threshold. PCR products from triplicate experiments were visualized by 2% agarose electrophoresis in the presence of ethidium bromide and, when required, band intensity evaluated with SigmaScan software (SPSS).

2.5. Total protein extraction and Western blot

As elsewhere reported [16], trypsinyzed control and treated cells were homogenized in protease inhibitor-containing lysis buffer. Protein concentrations were evaluated by Bradford method,

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and 30 μg of samples analyzed by 10% acrylamide SDS–PAGE. The molecular masses were evaluated by comparison with a set of standard proteins (PageRuler™ Prestained Protein Ladder, Fermentas). Then, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham) and the filters incubated either for 1 h with anti-actin, rabbit polyclonal antibody (1:250 final dilution, Sigma) or overnight with anti-AEG-1 antibody from rabbit (final dilution: 1 μg/ml; code 40-6500, Invitrogen). After exhaustive washing, the filters were incubated for 1 h with the alkaline phosphatase-conjugated secondary antibody (final dilution: 1:7500), and stained with BCIP/NBT (Sigma). The colorimetric signals were analyzed by SigmaScan software (SPSS, USA), and then normalized with the intensity of the band obtained with anti-actin antibody reaction.

2.6. Nuclear protein extraction and NF-κB p65 quantitation

Nuclear proteins were obtained from trypsinized control and treated cells using the Nuclear extraction kit (Panomics/Affymetrix). Protein concentrations were evaluated by Bradford method and then samples submitted to evaluation of NF-κB p65 amount in nuclear extracts using the Transbinding™ NF-κB assay (Panomics), following manufacturer's instructions.

2.7. Immunocytochemistry

For the analysis of AEG-1 intracellular localization, control and treated cells, seeded into 4-well chamber slides (Iwaki), were washed with pre-warmed complete phosphate-buffered saline containing 1% bovine serum albumin (PBSB) and fixed with 4% paraformaldehyde in PBS for 15 min. After washing with Ca^{++}/Mg^{++}-free PBSB, cells were permeabilized with 0.1% Triton X-100 in PBSB for 15 min and incubated for 1 h with anti-AEG-1 antibody from rabbit (final dilution in 10% FCS-RPMI: 20 μg/ml; code 40-6500, Invitrogen), and for 1 h with FITC-conjugated secondary antibody. After washing, nuclei were counterstained with propidium iodide and cells were observed under an Olympus FV-300 confocal laser scanning microscope equipped with Argon (488 nm) and Helium/Neon (543 nm) lasers with a PlanApo 60 x 140 oil immersion lens and scanned at 1024 × 1024 pixel resolution. Images were analyzed, measured and processed by the SigmaScan (SPSS) and Image Pro Plus (Media Cybernetics) softwares.

3. Results

3.1. $CdCl_2$ down-regulates AEG-1 in MDA-MB231 cells

In order to supplement the catalogue of $CdCl_2$-dependent genes and search for putative molecular markers linked to the lethal phenotype induced by the metal, in a first set of experiments cDNA samples obtained from enriched mRNA preparations of MDA-MB231 cells, both control and treated for 96 h with 5 μM $CdCl_2$, were submitted to DD-PCR assay as described. In the present study, among the diverse DD bands visualized after silver staining of the gel, we focussed on one 183 bp-sized band obtained utilizing the BS52/BS55 combination of arbitrary primers, which was present only in the electrophoretic lane corresponding to the cDNA preparation from control cells (Fig. 1A). The differentially-displayed cDNA fragment was purified by excision from the gel and several cycles of PCR and electrophoresis, and submitted to sequencing. Using BLASTN software, homology (98.5% identity, Z-score = 674.9 bits, expect = 1e-29) was found in the GenBank database between the sequence obtained and that of region 1620-1803 of the coding sequence for *Homo sapiens AEG-1* (Acc. nr. AF411226.1), a.k.a. *LYRIC*, *3D3* and *metadherin* (http://www.ncbi.nlm.nih.gov/gene/92140).

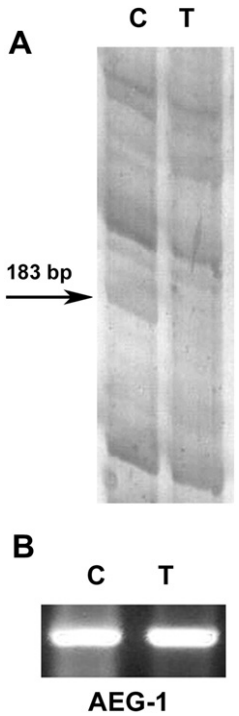

Fig. 1. Identification of *AEG-1* as a differentially-expressed gene in $CdCl_2$-treated MDA-MB231 cells. A) DD-PCR of cDNA preparations from control (C) and $CdCl_2$-treated (T) MDA-MB231 cells. The arrow points to a band of about 183 bp, selectively displayed in the control sample. Detail of a 6% sequencing PAGel, silver staining. B) Conventional PCR amplification of the product of *AEG-1* cDNA in control (C) and $CdCl_2$-treated (T) cells. Cycle number was 33. Agarose gel 2%, ethidium bromide stain.

We then checked the differential expression of *AEG-1* by PCR amplification of different cDNA samples from control and $CdCl_2$-treated MDA-MB231 cells in the presence of AEG-1 specific primers. As shown in Fig. 1B, in a preliminary assay we found an amplification band of the expected size (218 bp) after 33 PCR cycles of both samples, suggesting that expression of *AEG-1* was switched-on with and without exposure of cells to $CdCl_2$. The identity of the PCR product was confirmed by sequencing (data not shown). Then, for semi-quantitative evaluation of the expression levels, the cDNA preparations were submitted to SM-PCR as described. Fig. 2A shows a plot of normalized data for *AEG-1* versus cycle number, with exponential curve fits. Calculation of the relative difference in transcript abundance revealed that treatment of MDA-MB231 cells with 5 μM $CdCl_2$ for 96 h promoted the down-regulation of *AEG-1* by about 8.5-fold, thus validating the DD-PCR findings. In a parallel assay, non-tumoral HB2 epithelial breast cells were submitted to the same exposure to the metal and the cDNA preparations from control and treated samples analyzed by SM-PCR for *AEG-1*, once found an amplification band of the expected size in both experimental conditions (not shown). As shown in Fig. 2B, differently from MDA-MB231 cancer cells, $CdCl_2$ treatment did not exert any effect on *AEG-1* expression levels, thereby suggesting a possible tumor-specificity of Cd-mediated modulation of such transcriptional activity.

Subsequently, Western blot assays were performed to check whether the down-regulation of *AEG-1* expression in $CdCl_2$-treated MDA-MB231cells was coupled with a reduction in the intracellular accumulation of the corresponding protein product. As shown in Fig. 2C, following exposure to the metal the quantity of immunorevealed AEG-1 decreased by about 4.4-fold versus untreated controls.

We then checked the kinetics of Cd-dependent down-regulation of *AEG-1* in MDA-MB231 cells. To this purpose, preparations of

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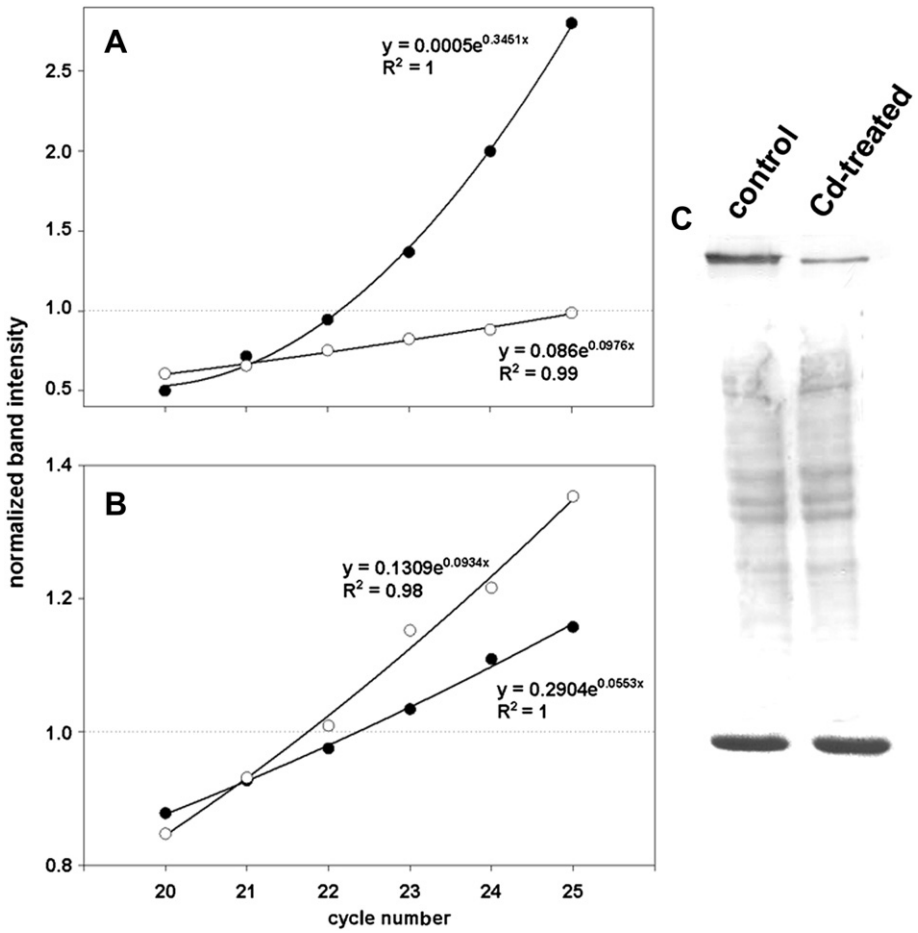

Fig. 2. Selective down-regulation of *AEG-1* gene expression and accumulation of protein product in $CdCl_2$-treated MDA-MB231 cells. A) and B) SM-PCR for *AEG-1*. Representative plots of normalized data versus cycle numbers fit with an exponential curve for tumoral MDA-MB231 (A) and non-tumoral HB2 cells (B) grown in control conditions (○) or exposed to 5 μM $CdCl_2$ for 96 h (●). Treatment of MDA-MB231 cells determined the down-regulation of *AEG-1* by about 8.5-fold, whereas no effect was exerted in HB2 cells. C) Top: Western immunodetection of AEG-1 in preparations from control and $CdCl_2$-treated MDA-MB231 cells. Treatment of MDA-MB231 cells determined the decrease of AEG-1 accumulation by about 4.4-fold versus untreated controls. Bottom: Ponceau Red stain of SDS-PAGel performed to check the quality of the lysate and actin immunodetection to check protein loading.

control and treated cells after 24, 48, 72 and 96 h of incubation were submitted to SM-PCR. As shown in Fig. 3, *AEG-1* down-regulation starts already between 24 and 48 h of exposure to the metal, the expression level decrease being about 5.5-, 7- and 8.5-fold after 48, 72 and 96 h of treatment, respectively.

3.2. $CdCl_2$ determines the decrease of intranuclear AEG-1 amount in MDA-MB231 cells

In a second set of experiments we checked the intracellular distribution pattern of AEG-1 in control and treated MDA-MB231 cells, in light of the controversial literature data localizing the protein at tight junction level in association with ZO-1 and occludin [22], in the perinuclear and endoplasmic reticulum region, in the nucleoplasm [23] or exposed on the cell surface [24]. In addition, in light of the presence of nuclear localization signals (NLS) in AEG-1 sequence, which target the factor to the nucleus/nucleolus [25], we also determined the nuclear/total factor ratio in the two experimental conditions by evaluating the number of fluorescent pixels in the nuclear area, identified by propidium iodide counterstain, and in the total intracellular area. As shown in the panel of micrographs of Fig. 4A, AEG-1 appeared to be seemingly associated with the internal cytomembranes, e.g. endoplasmic reticulum and nuclear envelope, rather than being exposed on plasmalemma surface. Fluorescent signals could be recorded also in intranuclear

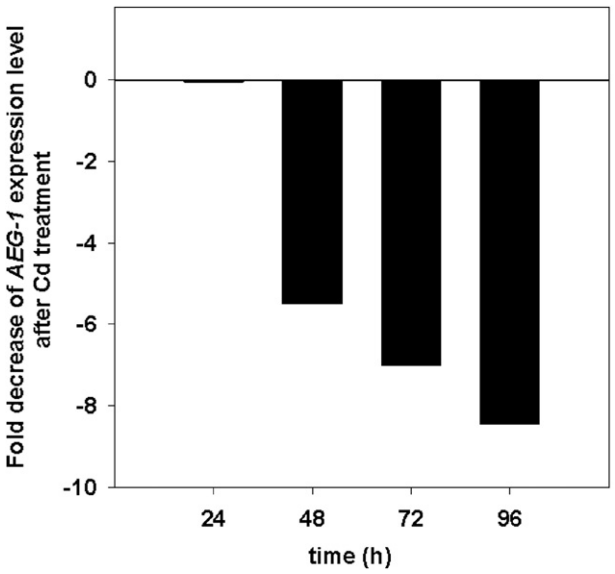

Fig. 3. Time-dependent *AEG-1* down-regulation in $CdCl_2$-treated MDA-MB231 cells. The histogram summarizes the results from SM-PCR assays on cDNA preparations obtained from control and exposed cells at different time lapses, demonstrating a decrease of gene expression of about 5.5-, 7- and 8.5-folds after 48, 72 and 96 h of treatment.

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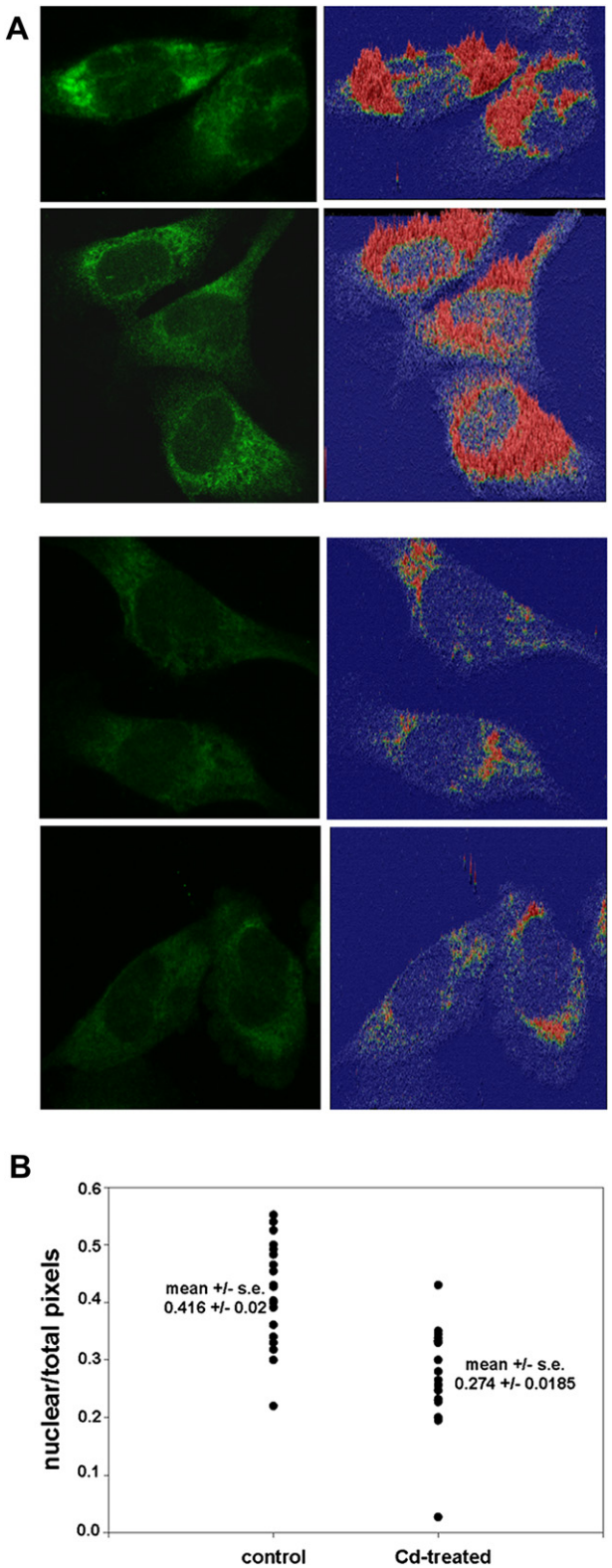

Fig. 4. Intracellular localization of AEG-1 in control and $CdCl_2$-treated MDA-MB 231 cells. A) Left: Representative fluorescence micrographs of control (top panel) and $CdCl_2$-treated cells (bottom panel) immunostained for AEG-1 intracellular distribution pattern. Right: Image Pro-Plus software-assisted elaboration of the same micrographs showing fluorescence intensity as green spots (low intensity) and red peaks. The fluorescent signal is seemingly associated to internal cytomembranes and also present in the nucleoplasm in both preparations, the intensity being drastically reduced in $CdCl_2$-exposed cells. B) Graph reporting the ratio between the number of fluorescent pixels, representative of AEG-1, in the nucleus and those in the whole cells. As expected, the mean value in case of exposure to $CdCl_2$ was lower, being about the 65% of that of control preparations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

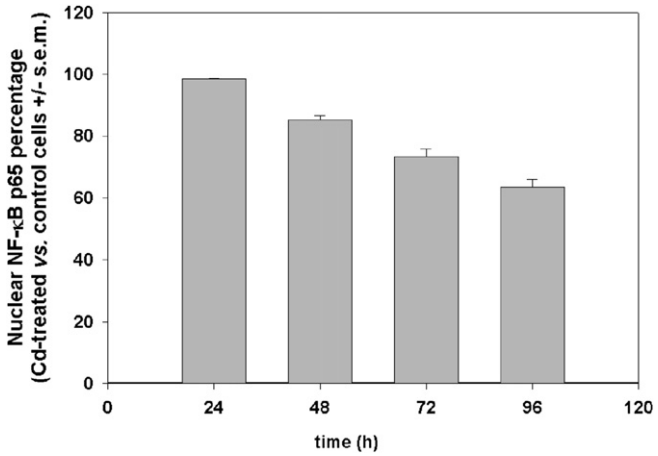

Fig. 5. Evaluation of the amount of NF-κB in nuclear extracts from control and $CdCl_2$-treated MDA-MB231 cells. The histogram summarizes the results obtained by ELISA assays demonstrating the time-dependent decrease of intranuclear NF-κB accumulation following cell exposure to $CdCl_2$ by about 15, 26 and 36% after 48, 72 and 96 h of treatment.

localizations. As expected, when MDA-MB231 cells were exposed to $CdCl_2$ the intensity of the fluorescent signal decreased in both the cytoplasm and the nucleus in accordance with the already-reported down-regulation of AEG-1 mRNA and protein. Moreover, when we compared the number of nuclear versus total fluorescent pixels in the two experimental conditions we found a decrease of the ratio in Cd-treated cells (Fig. 4B), thereby indicating that the down-regulation was coupled also to a reduced intranucleoplasmic import of the protein.

3.3. Nuclear NF-κB p65 translocation and c-fos and c-jun expression levels are decreased in MDA-MB231 cells exposed to $CdCl_2$

It is known that AEG-1 promotes the degradation of IκB, the inhibitor of NF-κB, thereby inducing the activation of the transcription factor, and that it interacts physically with NF-κB p65 subunit and translocates into the nucleus with the latter, thus contributing to its activation. By consequence, AEG-1 has been proven to actively modulate the expression of a number of NF-κB downstream genes, including *c-fos* and *c-jun* [26,27].. In a subsequent set of experiments, we have therefore evaluated whether the observed AEG-1 down-regulation at transcript and protein level was coupled to a decrease in the amount of nuclear NF-κB p65. To this purpose, nuclear protein extracts from control and $CdCl_2$-treated MDA-MB231 cells were subjected to ELISA assay for this subunit of the transcription factor. The histogram in Fig. 5 reports the results obtained from triplicate experiments, indicating that, starting from 48 h of exposure to $CdCl_2$, the amount of p65 imported into the nucleoplasm decreased by about 15, 26 and 36% after 48, 72 and 96 h of treatment, respectively.

Then, we checked whether Cd treatment was capable to modify the expression levels of two NF-κB-responsive genes, i.e. *c-fos* and *c-jun*, through SM-PCR, and compared the results obtained with MDA-MB231 and HB2 cell lines which, as already demonstrated, displayed a down-regulated or unaltered *AEG-1* expression, respectively, when exposed to 5 μM $CdCl_2$ for 96 h. The histograms in Fig. 6 show that in MDA-MB231 cells a down-regulation of *c-fos* and *c-jun* expressions can be recorded at 72 h of exposure to the

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metal (about −3.3 fold for both genes) and it remains steady after 96 h from treatment (about −3.2 and −3.5 fold, respectively), whereas $CdCl_2$-treated HB2 cells exhibited only a weaker decrease of *c-fos* expression level (about −1.5 fold) at 96 h of exposure.

4. Discussion

It is known that Cd is able to induce a reprogramming of gene expression in many different cytotypes, including MDA-MB231 breast tumor cells, and present results identify a new key protagonist in the complex scenario of $CdCl_2$-induced cytotoxic effect, i.e. AEG-1 a gene homologue to mouse 3D3 and rat LYRIC, located at chromosome 8q22 and initially identified as up-regulated in HIV-infected or gp120- and TNFα-treated astrocytes [23,28]. *AEG-1* encodes for a single pass transmembrane protein with predicted Mr of 64 kDa that contains a bipartite NLS between aminoacids 79-91, 432-451 and 561-580 [29]. Interestingly, Brown and Ruoslahti [24] cloned *AEG-1* as *metadherin* from a highly-malignant mammary carcinoma and reported the existence of an extracellular lung-homing domain that allow cell binding to lung endothelium thereby playing a key role in metastasis control. More recently, AEG-1/metadherin was found to be involved in esophageal, hepatic and prostatic carcinogenesis and in the modulation of the tumorigenic properties of neuroblastoma cells [30–32]. Lee et al. [33] have also produced evidence on *AEG-1* as a downstream transcriptional target for activated Ha-*ras*, due to the multiple Sp1 binding motifs and high GC content of its promoter, *via* the intracellular signal cascade PI3K/AKT/GSK3β/c-Myc, the latter binding the E-box elements present in the region −356/−302 of the promoter. A cooperation between Ha-*ras* and AEG-1 in the induction of the phenotypic transformation of human and rat non-neoplastic cells has been demonstrated [28,34].

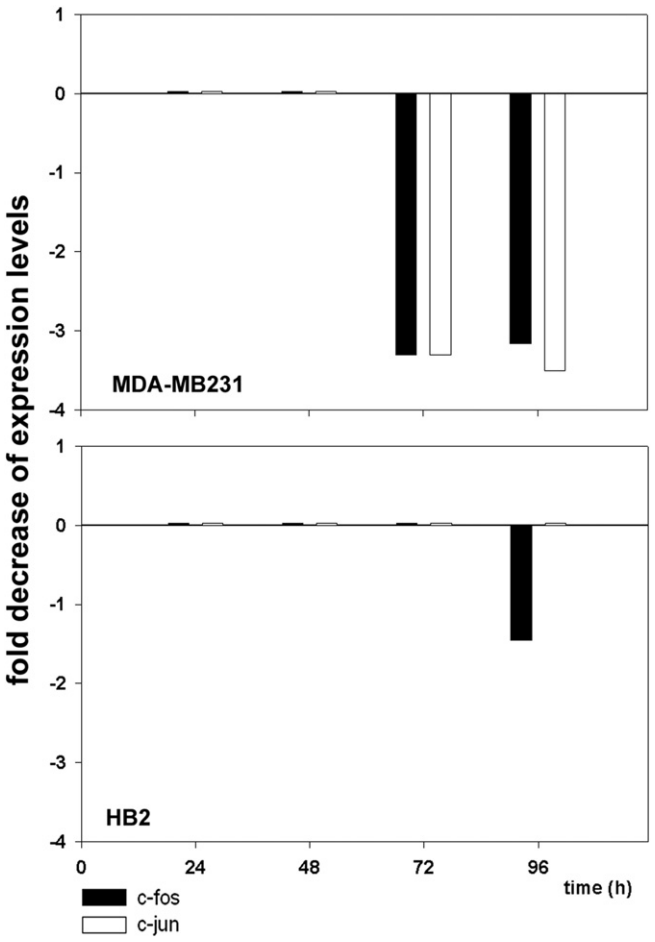

Fig. 6. Time-dependent *c-fos* and *c-jun* down-regulation in $CdCl_2$-treated MDA-MB231 cells. The histograms summarizes the results from SM-PCR assays on cDNA preparations obtained at different time lapses from control and $CdCl_2$-treated MDA-MB231 tumor cells compared to non-neoplastic HB2 cells, demonstrating a consistent decrease of both gene expressions after 72 h of treatment in MDA-MB231 cells only.

In this manuscript, we report that exposure of the highly-malignant ER-negative MDA-MB231 breast tumor cells to a concentration of $CdCl_2$ corresponding to the IC_{50} at 96 h selectively down-regulates *AEG-1*, *c-fos* and *c-jun*, whereas no such effect can be found in HB2 non-neoplastic breast epithelial cells. This finding expands the list of Cd-dependent gene expressions by the cancer cell line under investigation, which comprises so far the up-regulation of *metallothionein IA*, *–IF* and *-IL*, *hsp27*, *p38γ*, *DAP kinase*, *Receptor-interacting protein 1*, *Waf*-1, *caspase-1*, *-2*, *-6*, *-7*, *-8* and *-9*, and the down-regulation of *metallothionein IG*, *cytochrome oxidase subunit II* and *–IV*, *hsp60*, *hsc70*, *hsp75*, *grp78*, *hsp90β*, *Bcl-2*, *p38α* and *β*, thereby strongly suggesting the involvement of Cd not only in the regulation of stress response, reactive oxygen species (ROS) production and apoptosis-like death induction [13,14,16], but also in the switching-off of some NF-κB-mediated biological activities *via* AEG-1. Noteworthy, Su et al. [35] have examined the differential expression of AEG-1 by immunocytochemical analysis of a large set of paraffin-embedded breast samples including cases of usual ductal hyperplasia, atypical ductal hyperplasia, ductal carcinoma in situ of different grades, invasive ductal carcinoma, and normal breast tissues from reduction mammoplasty specimens as controls. The results obtained showed an increase of staining from normal to carcinomatous breast and also a positive correlation with patients' age, ER status, p53 status and high Ki67 index, thereby strongly supporting the concept that AEG-1 overexpression could be linked to proliferative breast lesions and contribute to breast carcinogenesis.

It is known that gene expression signatures have relevance in assessing hazards and risks of human exposure to toxic agents, including Cd, and that descriptive epidemiological studies may surely benefit from information at a molecular level. Classically, the first gene expression used as a biomarker for Cd toxicity was that of metallothionein, followed by evidence on down-regulation of ribosomal proteins and zinc-binding proteins and, more recently, on Cd effect on a panel of genes involved in inflammation, survival and apoptotic death [36]. In particular, Dakeshita et al. [37] found a significant correlation between blood/urinary Cd concentrations and the altered expression levels of seven genes encoding anti-oxidant factors (*GPX3*, *ITGAL*, *SLC3A2*) and apoptosis modulators (*BCL2A1*, *CASP9*, *COX7B*, *TNFRSF1B*), already at the lowest level of exposure. In addition, Bollati et al. [38] have reported a negative correlation between Cd exposure and the expression of miR146a, a noncoding small RNA that regulate the expression of gene networks at the post-transcriptional level, in particular known to be involved in the limitation of inflammatory responses triggered through the innate immune system. Although caution must be exercised in the extrapolation of in vitro results to the in vivo situation, also cell cultures have been used for the identification of specific gene expression patterns affected by environmentally-significant and toxic metals [[7] for review], especially in light of the progress of "omic" technologies [e.g. [39]].

5. Conclusions

In conclusion, in line with this growing list of data that merge molecular evidence with prognostic applications, the results here reported may have a twofold significance. First, they contribute to expand the knowledge of the cell biology of Cd on human cancer

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cells, by detecting a new target gene product involved in signalling and metabolism which appears to be responsible, at least in part, of the cytotoxic effect exerted by the metal at molecular level. Second, they identify a new putative marker gene, *AEG-1*, for the health risk assessment of exposure to Cd whose validation as a member of gene signature panels awaits further and more comprehensive studies.

Acknowledgments

This work was supported by the University of Palermo [R.S. ex 60%] and the Italian Ministry of the University [PRIN 2004].

We thank Ms. Simona Re and Rossella Ragusano for help in performing experiments during their laboratory internship. HB2 cells were courtesy of Cancer Research (UK).

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