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Cadmium Induces Fas Down-Regulation in a Human Immature T-cell Line

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Abstract. *Cadmium (Cd^{2+}) is an ubiquitous toxic metal with apoptotic and genotoxic effects, which has been involved in a variety of pathological conditions inducing disturbance of the immune system. In the present study we treated the Fas-expressed human immature T-cell line CCRF-CEM with 10μM Cd^{2+} for 6h or 24h. We found that pretreatment of the cells with Cd^{2+} for 24h inhibited apoptosis induced by Fas-ligation with the CH-11 antibody, in contrast to pretreatment for 6h. Immunocytochemical and multiplex RT-PCR analyses indicated that Cd^{2+} treatment for 24h inhibited Fas expression at the transcriptional level. To investigate that effect of Cd^{2+}, cDNA microarray analysis was applied, which indicated that the rapid induction of NF-kB, ERK5 and JAK3 genes by Cd^{2+} was the initial step resulting in Fas down-regulation. The Fas down-regulation induced by Cd^{2+} seems to be responsible for the carcinogenic and the immunomodulatory effects of that metal.*

Heavy metals have received considerable attention since they comprise a group of environmental pollutants that may alter genome and transcriptome, are linked to homeostasis and development and are involved in neoplastic diseases (1). Among heavy metals, Cadmium (Cd^{2+}) is a ubiquitous toxic metal with a long biological half-life (>25 years). Chronic exposure to Cd^{2+} has been shown to result in a variety of pathological conditions. In addition to its accumulation in different organs (liver, kidneys *etc.*), Cd^{2+} is also detected in the blood due to chronic exposure, where it is mainly associated with nucleated cells, particularly lymphocytes (2). Furthermore, it has been shown that Cd^{2+} compounds induce tumors in lungs, testes and prostate as well as haematopoietic system malignancies (3-5), while in cultured mammalian cells they induce morphological transformations, chromosomal aberrations and gene mutations (6-9). A previous study on a human T-cell line (CEM-C12) showed that Cd^{2+} exerts its toxic effect *via* apoptosis (10), while a comparative study of the apoptotic effect of Cd^{2+} in immune system's cell lines showed a differential Cd-induced apoptosis, which may disturb the immune system's normal growth and development (11).

At the cellular level, Cd^{2+} is highly reactive with the sulfhydryl groups of proteins and substitutes for zinc in certain enzymes (12). Furthermore, it rapidly increases inositol 1,4,5-triphosphate triggering Ca^{2+} mobilization from endoplasmatic reticulum, consequently stimulating protein kinase C (13, 14). In rat brain tumor cells Cd^{2+} has also been reported to activate the p38 mitogen activated protein kinase (p38 MAPK) and the extracellular regulated kinase (ERK) (15) and in porcine renal epithelial cells, the c-Jun N-terminal kinase (JNK) (16). At the molecular level, Cd^{2+} has been shown to induce mRNA levels of several genes such as *c-jun*, *c-myc*, *c-fos*, metallothionein (MT) and heme oxygenase 1 (HMOX1) (17-20). We and others have shown that in nucleated blood cells, and particularly lymphocytes, Cd^{2+} activates transcription of both metallothionein-IIA (MT-IIA) and heat shock protein 70 (HSP 70) genes in a time- and dose-dependent manner (21, 22). Therefore, after exposure to low Cd^{2+} concentrations, MT II-A is induced whereas at higher concentrations HSP 70 is induced.

On the other hand, Fas/APO-1 is a 48-kDa transmembrane glycoprotein, a member of the TNF receptor family, serving as a cell surface receptor (CD95). In many cell types of haematopoietic origin, which express Fas, ligation of the Fas receptor with its specific ligand (FasL) or with specific anti-Fas antibodies induces apoptosis, independently of macromolecular synthesis (23, 24). Fas is implicated in the activation-induced cell death (AICD) of T lymphocytes and the CTL and NK cell-mediated cytotoxicity against a variety of

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tumor and virus-infected cells. As the activation of immature T-cells during development induces apoptosis, it seems that AICD of specific T-cell populations is a fundamental mechanism for maintaining immune tolerance and cellular homeostasis during T-cell development, immune response and disease (24, 25). Therefore, the expression of Fas is a key checkpoint for cells to commit to AICD and in many cellular systems the expression of Fas is strictly controlled. Aberrant expression of Fas has been involved in diseases in which T-lymphocyte homeostasis is compromised (26). Mutations of the gene encoding Fas, resulting in non-functional protein, give rise to fatal autoimmunity and lymphadenopathy as well as to lymphoproliferative syndromes. Fas-deficient mice accumulate B-cells and have elevated levels of immunoglobulins of various classes, including autoantibodies to ssDNA and dsDNA antibodies, suggesting an involvement of the Fas system in the deletion of activated or autoreactive B-lymphocytes (23-25).

In the present study, we describe the ability of Cd^{2+} at a non-toxic concentration (10μM) to down-regulate Fas expression in a time-dependent manner on the immature T-cell line CCRF-CEM. An involvement of Cd^{2+} in protecting the cells from the Fas ligation-induced apoptosis is suggested.

Materials and Methods

Media and reagents. The medium for cell cultures was RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Life Tech., Paisley, Scotland), 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and 20mM HEPES buffer (culture medium) (all from Biochrom, Berlin, Germany). Cadmium chloride (Cd^{2+}) (Sigma Chem. Co., St Louis, MO, USA) was stored as a sterile stock solution (10mM, 4˚C). The CH-11 human anti-Fas monoclonal antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA).

Cell cultures. The CCRF-CEM human immature T-cell line was obtained from the ECACC (Salisbury, U.K.). Cells (3 x 10^5 cells/ml) were cultured in culture medium at 37˚C in a humidified atmosphere of 5% CO_2 in air. For each experiment, exponentially grown cells were harvested and resuspended (1 x 10^6 cells/ml) in fresh culture medium in the absence (control) or in the presence of Cd^{2+} 10μM for 6h and/or 24 h. Then, the cells were washed with normal saline, resuspended in fresh culture medium and further incubated for 24h in the absence (control) or presence of different CH-11 concentrations.

Cell viability assay. After the treatment of the cells for the indicated time without (control) or with Cd^{2+}, 1 x 10^6 cell/ml were further incubated with various CH-11 concentrations for 24h. Viable cell number and viability was assessed by the Trypan Blue exclusion assay (TB test). The results were expressed as the percentage of viable cells in the total number of cells counted.

Quantification of apoptotic cells. The detection and quantification of apoptosis was performed as previously described (27). Briefly, after the exposure of control and of Cd-treated cells (2 x 10^6cells/ml) to various CH-11 concentrations, 8μl of the cell suspension were mixed with 2μl of a fluorescent EtBr-containing dye (0.1 μg/ml Ethidium Bromide, 1.5% NP40, in PBS). This suspension was placed on a microscope slide and fluorescent-stained cells were examined with an Epi-Fluorescence Microscope (Optiphot-2, Nikon, Japan). The cells were scored and categorized as normal, apoptotic or necrotic and the results were expressed as the percentage of each cell kind to the total counted cells. For each CH-11 concentration at each time-point, more than five slides were prepared and more than 500 cells/slide were examined.

RNA isolation. After the incubation of the cells with or without Cd^{2+} for the indicated time, 4x10^6 cells were centrifuged and washed twice with ice-cold PBS. The pellets were lysed by the addition of RNAzol B (Wak-Chemie, Homburg, Germany). Total RNA was extracted by chloroform, precipitated by isopropanol and recovered by centrifugation (12,000 x g, 15 min, 4˚C). After a wash step (75% ethanol, 9,000 x g, 10 min, 4˚C) the RNA pellet was dissolved in sterile DNase, RNase-free water (Biochrom). The total RNA concentration was determined by spectrophotometry and the quality of RNA was checked by running 1μg RNA on a standard 1% denaturing formaldehyde-agarose gel.

Multiplex Reverse Transcription PCR (MPCR). As CCRF-CEM cells normally express MT (28), a custom-designed multiplex reverse transcription PCR kit that included Fas, FasL and MT primers from Maxim Biotech (San Francisco, CA, USA), was used. cDNAs for MPCR analysis were prepared from 10μg total RNA templates, using 800 units Superscript II reverse transcriptase (200 units/μl) and 5μg random hexameric primers (3μg/μl) (all derived from Invitrogen) to a final volume of 56μl, according to the manufacturer's instructions. The initial RNA template was degraded by the addition of 10 units RNase H (10 units/μl) (Clontech, Palo Alto, CA, USA) and incubation at 37˚C for 15 min. The produced cDNA was cleaned by Quick Clean Purification Resin (Clontech), precipitated with sodium acetate/absolute ethanol (-20˚C, 1h) and collected by centrifugation (21,000 x g, 20 min, 4˚C). After a washing step (70% ice-cold ethanol) it was resuspended in sterile DNase, RNase-free water (Biochrom) and its quality and quantity were determined by spectophotometry. One hundred ng of the produced cDNA were used per MPCR reaction, in a final volume of 50μl and the reaction was carried out, as recommended by the manufacturer, in a Thermal Cycler 480 (PerkinElmer Life Sciences, Norwalk, CT, USA). MPCR products were fractionated and analyzed electrophoretically in a 2% agarose gel containing 1μg/ml EtBr. Differences in gene expression were determined by comparing the band intensity against that of GAPDH, which was included in the kit and used as a reference housekeeping gene. For each set of reactions, a positive control included in the kit and a negative control (no DNA) were also amplified.

cDNA microarrays. For the microarray analysis, cDNA was produced and labeled by an indirect two-step procedure, using the Atlas Glass Fluorescent Labeling Kit (Clontech). Twenty μg of the previous isolated total RNA was used for the cDNA synthesis and the simultaneous incorporation of aminoalyl-dUTP, following the manufacturer's instructions strictly. The produced cDNAs were dissolved in 10μl 2-fold fluorescent labeling buffer provided in the kit and an equal volume of Cy-3 or Cy-5 (5mM in DMSO) (Amersham Pharmacia Biotech Export Gmbh, Glyfada, Greece) was added. The labeling reaction was performed at room temperature for 30min. The labeled cDNA was precipitated with sodium acetate/absolute ethanol (-20˚C, 2h), recovered by

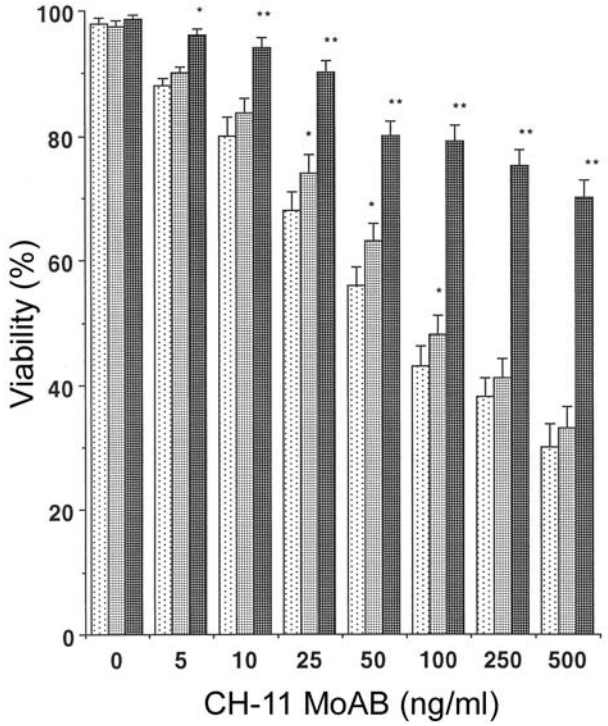

Figure 1. *Effect of CH-11 MoAb on Cd^{2+}-treated CEM cells. Cells were cultured in absence (control) or presence of 10μM Cd^{2+} for 6h or 24h and subsequently incubated for 24h without or with different concentrations of the human anti-Fas CH-11 MoAb. Cell viability was assessed by TB test as described in Materials and Methods. : control, : cells cultured for 6h with Cd^{2+}, : cells cultured for 24h with Cd2+. Results are mean + S.E.M. of six independent experiments. P values are *p<0.05 or **p<0.005 for cells treated with Cd^{2+} as compared to control .*

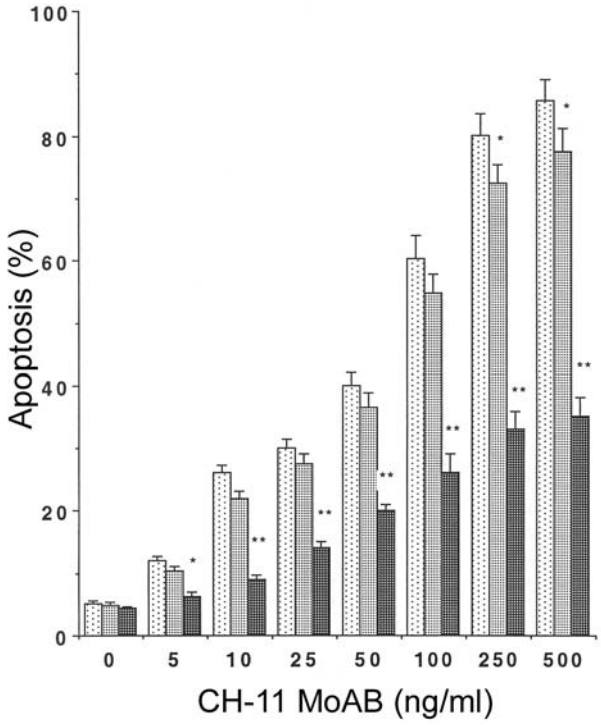

Figure 2. *Quantification of apoptosis in cells cultured in absence (control) or presence of 10μM Cd^{2+} for 6h or 24h and subsequently incubated for 24h without or with different CH-11 anti-Fas MoAb. : control, : cells cultured for 6h with Cd^{2+}, : cells cultured for 24h with Cd^{2+}. Results are mean±S.E.M. of six independent experiments. P values are: *p<0.05 or **p<0.005 for cells treated with Cd^{2+} as compared to control.*

centrifugation (21,000 x g, 20min), washed with 70% ethanol and finally dissolved in water (Clontech). The quality and quantity of the labeled cDNA were determined by spectrophotometry, while the efficacy of the labeling reaction (labeling ratio between 10 to 20) for Cy-3 and Cy-5 was calculated by A260/(A550-A750) and A260/(A650-A750), respectively.

The Atlas Glass Human 1.0 Microarray cDNA microchip (Clontech) was used. The microchip includes 1081 human cDNA fragments (80mers), nine housekeeping genes, negative and positive controls (www.clontech.com/atlas/atlasglass/index.html). For the normalization of the results, 10μg (determined by spectrophotometry) of each Cy-labeled cDNA probe was used. The microchip was hybridized for 18h at 50˚C, washed according to the manufacturer's instructions and finally scanned using the ScanArray 4000XL scanner (PerkinElmer Life Sciences). Data from each fluorescence channel were stored as separate images and analyzed by the use of the QuantArray Analysis Software (PerkinElmer Life Sciences). The quantification was based on the intensity of each spot as a fixed circle, after the subtraction of the crosslinking fluorescent excitation. After data normalization, provided by the analysis software, the fluorescent intensities per slide of positive controls and housekeeping genes in each of the scanned channels were almost equal (fluorescent intensity ratio per channel compared to control =1). Thus, genes with a fluorescent intensity ratio over 1.7 compared to untreated cells (control) were considered as up-regulated (29).

Immunocytochemistry. Expression of Fas protein was detected using the human anti-Fas monoclonal antibody C-20 (Santa Cruz Biotech. Inc, Santa Cruz, CA, USA). Cells (1 x 10^6cell/ml) were incubated without (controls) or with Cd^{2+} for the indicated time intervals and the cell suspensions were cytocentrifuged (1000 rounds/min, 5 min) with a Cytospin 2000 (Sandon Inc, Pittsburgh, PA, USA). The slides were air dried, fixed for 10 min in Zabonis fixative (Ylem, Rome, Italy) and washed twice with Tris-buffered saline (pH 7.6) prior to labeling. Immunocytochemical investigations were performed by the biotin-streptavidin-alkaline phosphatase technique using the Sandon's Kwik Kit, in the Sequenza coverplate immunostaining system (Sandon). The antibody was diluted 1:50 and applied for 1h at room temperature. The reaction was revealed by fast red chromogen, the slides were counterstained with hematoxylin and mounted with glycerol gelatine. Positive cells appeared red. At least five fields from each slide were examined. Negative controls were made by omitting the primary antibody.

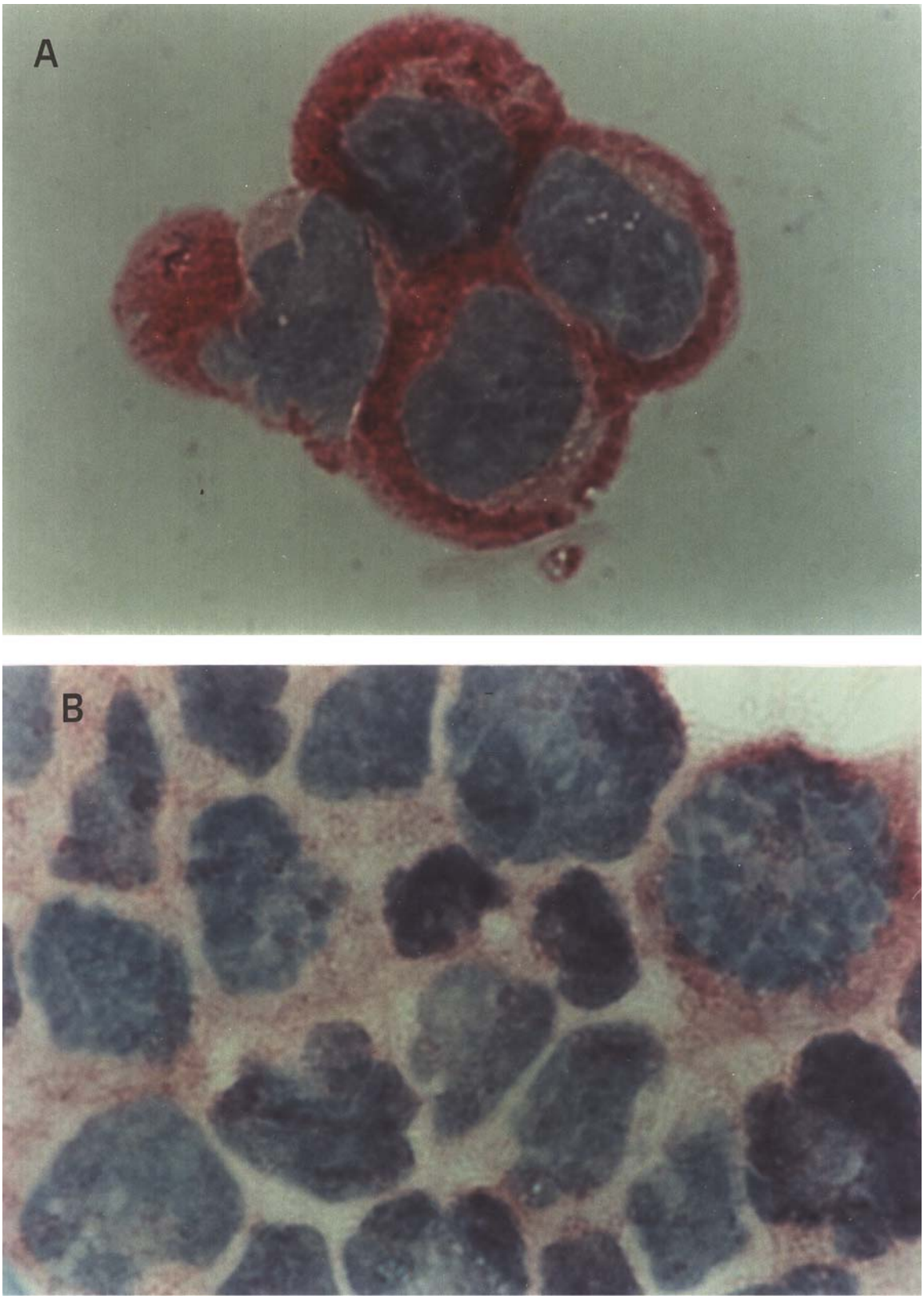

Figure 3. *Immunocytochemical detection of Fas in CEM cells cultured in absence (A) or presence of 10μM Cd^{2+} for 24h (B). Positive cells appeared red. One representative experiment out of six is shown (magnification x1000).*

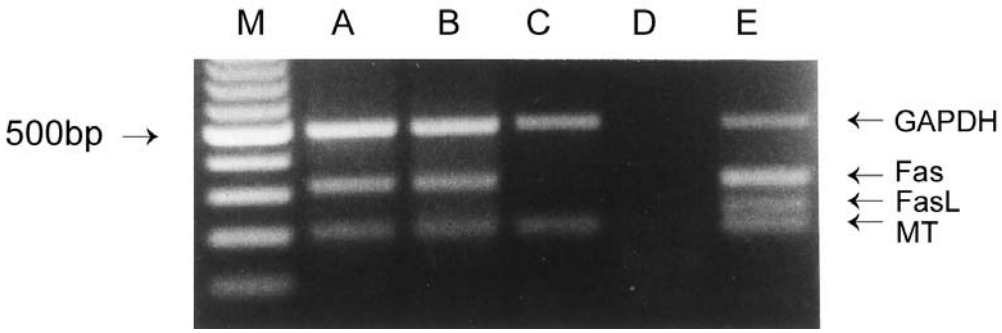

Figure 3. *C. Analysis for Fas transcription by multiplex reverse transcription PCR. A: RNA from cells cultured without Cd^{2+} for 24h (control), B: RNA from cells cultured with 10μM Cd^{2+} for 6h, C: RNA from cells cultured with 10μM Cd2+ for 24h.M: marker, D: negative control (No-DNA), E: positive control. One representative experiment out of four is shown.*

Statistical analysis. Results obtained from at least 6 independent experiments were analyzed using the Student's *t*-test for paired variables and they were considered statistically significant at a $p<0.05$.

Results

In the first set of experiments CCRF-CEM human immature T-cells were cultured in the absence (control) or presence of 10μM Cd^{2+} for 6h or 24h. The cells were subsequently incubated for 24h without or with different concentrations of the human anti-Fas CH-11 MoAb and cell viability was assessed. We found that CH-11 induces a dose-dependent decrease in cell viability in the control cell culture as previously described (Figure 1) (30). In contrast, in CCRF-CEM cells treated with 10μM Cd^{2+} for 6h, an inhibition of cell death induced by CH-11 was observed, which was more significant after the 24h-treatment of the cells. The differential sensitivity of Cd^{2+}-treated cells to CH-11 reflects on the different LD_{50} (n=6) of the above cell cultures. Thus, for untreated cells (control) the LD_{50} was 73ng/ml CH-11, for the cells treated with 10μM Cd^{2+} for 6h the LD_{50} was 93.2ng/ml CH-11, while for the cells treated for 24h, the LD_{50} was more than 500ng/ml CH-11, indicating that these cells were resistant to CH-11-induced cell death.

It was previously reported by Landowski *et al.* (31) that CCRF-CEM cells express Fas and thus ligation of the Fas receptor by the CH-11 MoAb induces cell death by apoptosis. To explore whether the resistance of Cd^{2+}-treated cells to CH-11-induced cell death was due to the inhibition of apoptosis, the untreated and Cd^{2+}-treated cells were incubated with different CH-11 concentrations for 24h and apoptosis was measured. We found that treatment of cells with 10μM Cd^{2+} for 6h resulted in a minor inhibition of apoptosis induced by CH-11, whereas a significant inhibition of CH-11-induced apoptosis was observed after the treatment of the cells for 24h with Cd^{2+} (Figure 2).

To determine whether the resistance to Fas-mediated apoptosis after the treatment of CEM cells with Cd^{2+} for 24h is due to alterations in Fas expression, we examined the expression of Fas by immunocytochemistry. Thus, we found that the untreated and the cells treated with Cd^{2+} for 6h expressed Fas. In contrast cells treated with 10μM Cd^{2+} for 24h were found negative for Fas expression (Figure 3A and B). In order to determine whether that suppression of Fas expression in Cd^{2+}-treated cells occurred at the transcriptional level, MPCR analysis for Fas and FasL mRNAs of untreated and treated with Cd^{2+} cells was performed. Figure 3C indicates that treatment of the cells with 10 μM Cd^{2+} for 24h induces inhibition of Fas transcription, while treatment of the cells with the same Cd^{2+} concentration for 6h, as well as treatment of the cells with 20μM Cd^{2+} for 6h or 24h, did not influence Fas transcription. Also, Figure 3C indicates that the treatment of CEM cells with Cd^{2+} did not influence the level of FasL mRNA.

Finally, to determine the possible pathway(s) which leads to Fas down-regulation we performed cDNA microarray analysis. We found that treatment of the cells with 10μM Cd^{2+} for 6h resulted in up-regulation of 21 genes compared to untreated cells (control), while treatment for 24h resulted in up-regulation of 19 genes (Table I) compared to control. Among these, 11 genes (Table I, genes 1-11) were found up-regulated after 6h of treatment with Cd^{2+} and they remained so up to 24h; 10 genes (genes 10-21) were found up-regulated only after 6h of treatment, indicating a transient up-regulation, while 8 genes (genes 22-29) were found up-regulated only after 24h of treatment, indicating a later up-regulation.

Table I. *Genes up-regulated by the treatment of CCRF-CEM cells with 10μM Cadmium for the indicated time period.*

GENES	Gene Bank Acc. No #	6h	24h
1. Extracellular signal-regulated kinase 5; MAP kinase 7 (ERK5; BMK1; MAPK7)	U25278	■	■
2. Cationic amino acid transporter 3 (CAAT3)	AJ000730	■	■
3. Janus tyrosine-protein kinase 3 (JAK3)	U09607	■	■
4. Growth-arrest-specific protein 1(GAS1)	L13698	■	■
5. Neurogranin protein kinase C substrate (NRGN;RC3)	Y09689	■	■
6. Ephrin-A5 precursor (AL-1)	U26403	■	■
7. Zinc finger protein 161 (Transcription factor DB1)	D28118	■	■
8. Putative renal organic anion transporter (hROAT1)	AF057039	■	■
9. Calcium-activated potassium channel (HSK1)	U69883	■	■
10. Ca++ transporting ATPase plasma membrane isoform 2 beta (ATP2B2, PMCA)	L20977	■	■
11. Stratifin (SFN); 14-3-3 protein sigma	AF029082	■	■
12. Paired box protein 5 (PAX5;BSAP)	M96944	■	
13. DNA mismatch repair protein PMS1 (PMS1)	U13695	■	
14. Glutamate decarboxylase 67-kD (GAD67;GAD1)	M81883	■	
15. YL-1 protein (YL1)	D43642	■	
16. CCAAT displacement protein (CUTL1;CASP)	L12579	■	
17. Transcription factor 5; Heat shock transcription factor 1(TCF5; HSTF1)	M64673	■	
18. Growth arrest and DNA damage-inducible protein 45 gamma (GADD45 gamma,CR6)	AF078078	■	
19. High mobility protein isoforms I&Y (HMGIY)	M23619	■	
20. NF-kappa B P105 subunit (NF-kB P105)	M58603	■	
21. KIAA0151	D63485	■	
22. Sodium-dependent dopamine transporter (DAT)	M95167		■
23. Phosphorylase B kinase gamma catalytic subunit (PHKGT;PSK-C3)	M31606		■
24. 6-O-methylguanine-DNA methyltransferase (MGMT)	M29971		■
25. KIAA0303	AB002301		■
26. Kruppel-type zinc finger protein (ZK1)	AB011414		■
27. Leukine zipper protein Fip3p (IKBKG)	AJ271718		■
28. Superoxide dismutase 2 mitochondrial (SOD2)	X07834		■
29. Zinc ring finger protein SAG (RING SAG)	U70976		■

The results represented the genes found to be up-regulated in the cells treated with 10 μM Cd^{2+} compared to untreated cells (control), reproducibly in four independent experiments by the c-DNA microarray analysis as described in *Materials and Methods*. Stippled bars indicate the time interval in which each gene was found up-regulated.

Discussion

Heavy metals, being extremely toxic, pose an environmental threat. The mechanism producing cellular responses depends on the type of metal, its subcellular distribution, its concentration and the period of exposure. After long-term exposure with low metal concentrations, accumulation of the metal within the cell is observed to a certain threshold level before the appearance of toxic effects. During that accumulation, the metal can directly influence various processes, including enzyme and signal transduction pathways, in addition to indirect effects such as gene expression and formation of free radicals, peroxides or cytokines (32). Of the heavy metals, Cd^{2+} is among the most toxic as well as one of the best-documented human carcinogens (33). Recent studies on cadmium toxicity have shown that, *in vitro*, low Cd^{2+} concentrations trigger apoptosis, while higher concentrations induce necrosis (10) and immune system disturbance (11).

In the present study, the human immature T cell line CCRF-CEM was selected, because it has the ability to express Fas and it has been used as one of the models for the study of Fas regulation (30, 31). Furthermore, in this cell line Cd^{2+} toxicity has extensively been studied and it has been found that Cd^{2+} time- and dose-dependently influences cellular function (34). At the molecular level, low Cd^{2+} concentrations produce a different effect from that at higher concentrations. The dose-dependent effect of Cd on cellular function seems to be more complex, as these findings indicated a rapid and a late molecular effect (34, 35). We investigated the role of Cd^{2+} on Fas regulation, by treating the cells with a low Cd^{2+} concentration (10μM), at which this metal is less toxic for the cells after a 6h-or 12h- incubation (Viability (%) = 95% ± 2.3 and 92% ± 2.4, respectively, data not shown) as has been previously reported (11, 34). At the molecular level, the results obtained indicated that treatment of CCRF-CEM cells with 10μM Cd^{2+} for 24h induces inhibition of Fas function, as a result of Fas down-regulation at the transcriptional level.

Although the mechanism regulating Fas expression is not completely known, recent studies indicate that at least three different regulating mechanisms of Fas down-regulation exist, one related to the transcription inhibition of Fas and Fas mRNA production, another one to mRNA translation and Fas protein production and a last one related to the translocation of the Fas protein to the cellular membrane (36, 37). The results obtained showed that a 6h-incubation of the cells with Cd^{2+} has a minor effect on CH-11-induced apoptosis, suggesting that Cd^{2+} did not influence the Fas function. Furthermore, the MPCR analysis showed inhibition of the Fas mRNA production, after the treatment of the cells with Cd^{2+} for 24h.

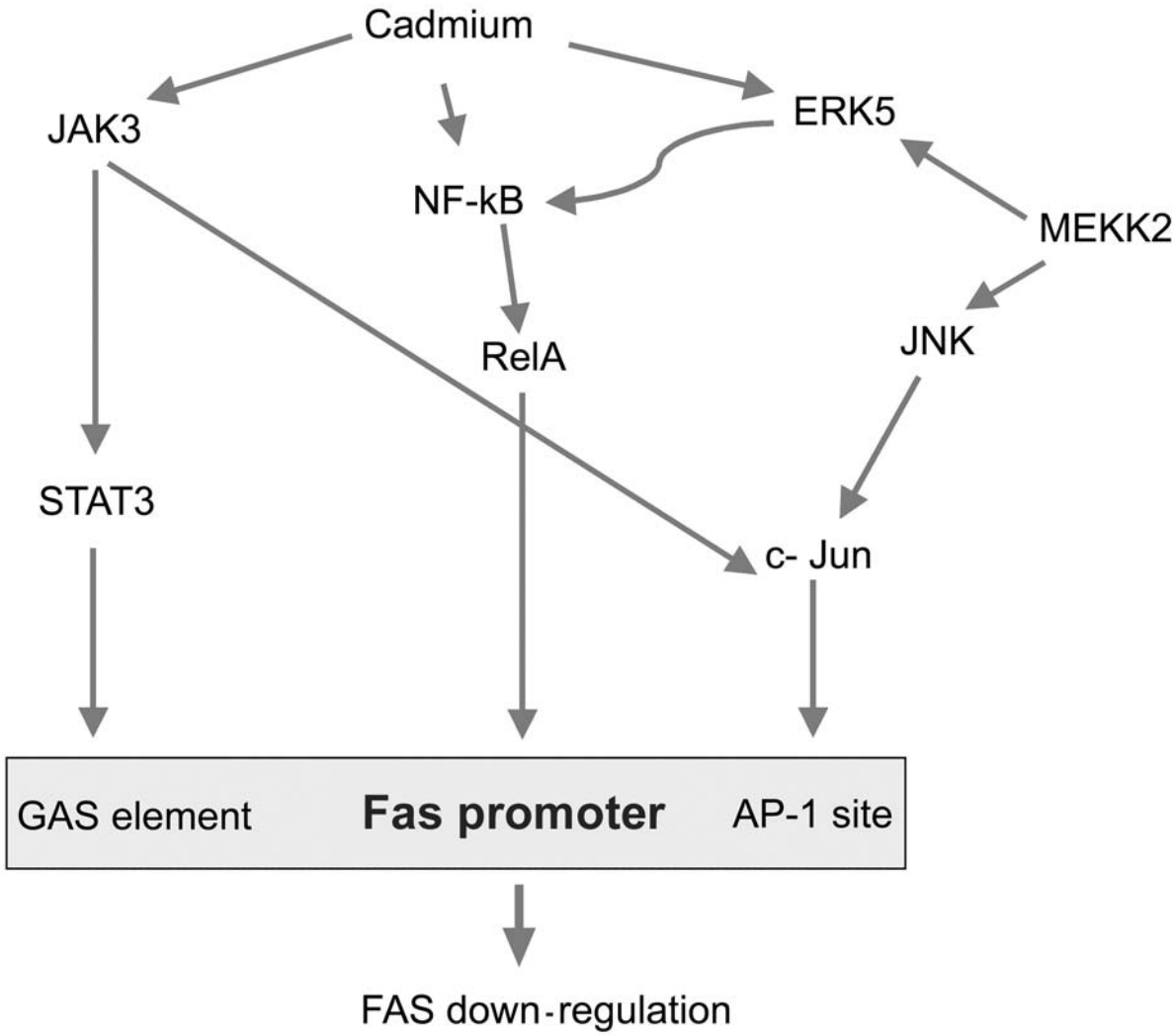

Figure 4. *Suggested mechanism of Fas down-regulation by Cadmium.*

Gene down-regulation is a complex phenomenon, which implies the decreased activity of transcription factors by inactivation or by their deliberation from the gene promoter. Furthermore, gene down-regulation appears as decreased mRNA and protein levels, implying not only the suppression of gene transcription and mRNA production but also the degradation of already existing mRNA and protein of the expressed genes. In this regard, although our results indicated that the Fas down-regulation is a later effect, appearing 24h after the addition of Cd^{2+} to the cells, it is possible that the inhibition of Fas transcription and the Fas mRNA production take place earlier, as a result of a molecular cascade rapidly induced by Cd^{2+}. Microarray analysis indicated that 21 genes were found up-regulated 6h after the addition of Cd^{2+} to the cells, 11 of which remained up-regulated up to 24h (Table I).

Regarding Fas regulation, Ivanov *et al.* have demonstrated that down-regulation of Fas expression in human melanoma-derived cell lines is mediated by Stat-3 cooperation with c-Jun (38). Stat-3 suppresses Fas expression through the GAS element of the Fas promoter, while c-Jun affects the Fas promoter through its AP-1 site. Under conditions in which Fas expression is suppressed, both Stat-3 and c-Jun were bound to the Fas promoter. Additionally it has been reported that Stat-3 could regulate gene transcription through interaction with JAK3 tyrosine kinase (39, 40), while JAK3 could also regulate c-jun activity (41). The above findings, together with the results obtained by the microarray analysis indicating that, in CEM cells, Cd^{2+} rapidly induces JAK3, suggest that a possible initial point of a pathway regulating Fas expression is the

induction and activation of JAK3. Microarray analysis also indicated that Cd^{2+} rapidly induced BMK1/ERK5 expression, suggesting that JAK3 and BMK1/ERK5 kinases expression and activation play an important role in the action of Cd^{2+}. In the same cell line, a recent study (42) showed that non apoptotic doses of Cd^{2+} phosphorylate ERK and p38 MAPK, while higher doses were required for the clear phosphorylation of JNK. In the time-course study, ERK and p38 MAPK were phosphorylated earlier than JNK. Although the targets of ERKs, JNK and p38 MAPK seem to be different, the ERK5 and JNK cascades might be activated simultaneously by MEKK2 (43). These findings suggest that Stat3 and c-Jun-mediated Fas down-regulation might be the result of different signal transduction pathways activated by Cd^{2+} (Figure 4).

Furthermore, the results obtained indicated that Cd^{2+} rapidly but transiently induces NF-kB expression, suggesting another possible mechanism of Fas down-regulation, as in Jurkat cells transfection with NF-kB confers resistance against Fas-mediated apoptosis (44). Additionally, it was also reported that in fibroblasts the RelA subunit of NF-kB is important for the regulation of Fas, while in mature T cells inhibition of NF-kB results in enhanced Fas-mediated killing, suggesting that NF-kB activation suppresses Fas expression (45). Subsequently, other studies have shown that the NF-kB activation as well as its DNA binding are regulated by ERK5 (46) and/or JAK3/Stat3 pathways (47). In agreement with that, Wilson *et al.* have reported that, in Jurkat cells, activation of the ERK pathway could suppress Fas-induced apoptosis (48). Our results indicated that the rapid, simultaneous and continuous activation of both ERK5 and JAK3 are the crucial points for Fas down-regulation (Figure 4). The findings of the present study were not sufficient for the identification of the exact mechanism of Fas transcriptional down-regulation, but they suggest that the Cd^{2+} treatment of CCRF-CEM cells constitutes a good model to obtain valuable information about the pathways regulating Fas expression.

Regardless of the exact mechanism of Fas transcriptional down-regulation, the obtained results also indicated that Cd^{2+} can disturb the immune system, down-regulating Fas expression. Aberrant expressions of Fas and FasL have been involved mainly in diseases in which the lymphocyte homeostasis is compromised. Loss of function mutations of Fas or FasL resulting in systemic autoimmune diseases, characterized by splenomegaly and glumeronephritis in both mice and human, illustrated the importance of the Fas system in maintaining homeostasis, specifically in the life and AICD of lymphocytes. (49,50). AICD in T-cells is a fundamental mechanism for maintaining immune tolerance and cellular homeostasis during T-cell development, immune responses and diseases. Activated T-cells express Fas and FasL and they are sensitive to Fas-induced apoptosis, suggesting that activated T-cells undergo AICD to down-regulate the immune reaction (24). Thus, autoimmune disease in Fas-/- or FasL -/- mice involve accumulation of autoreactive T-cells because of impaired AICD. Recent studies have also shown that Fas is important for tumor surveillance by CTLs and NK cells (51). Furthermore, using a high dose of antigens, recent experiments indicated that Fas-induced apoptosis may be involved in clonal thymic deletion for thymocytes that are reactive to highly expressed antigens in the thymus (23, 45). A number of studies have significantly improved the knowledge of the pathophysiological role of Fas and FasL *in vivo*, particularly in the induction and regulation of several organ-specific autoimmune diseases (51, 52). In this regard an improperly regulated Fas/FasL system could become a serious danger for the organism, leading to selective destruction of target cells within a tissue. Finally, impaired Fas signaling is frequently observed during tumor progression and has been attributed in most cases to down-regulation of Fas expression. Loss of Fas expression has been implicated in increased resistance of tumors to apoptosis induced by chemical and physical stimuli, as well as in the acquisition of the metastatic phenotype (52).

The above, together with the results of the present study, suggest that the previously described effects of Cd^{2+} are related to the ability of that metal to induce Fas down-regulation. Consistent with that hypothesis, a number of studies has previously shown that the kidneys, liver and the haematopoietic system are the major targets of Cd^{2+} toxicity, while Cd^{2+} compounds induced tumours in the lungs, testes and prostate as well as haematopoietic system malignancies (4). Furthermore, human exposure to fumes or gases containing Cd^{2+} may lead to acute pneumonitis, pulmonary oedema, acute tracheobronchitis and emphysema (53). Concerning the immunomodulatory effects of that metal, it was found that Cd^{2+} induces apoptosis differentially in immune system cell lines (11). Furthermore, in human PBMC, at low concentrations Cd^{2+} stimulates immune responses (54, 55), while in children high Cd^{2+} body burdens are consistently associated with a dose-dependent suppression of immediate hypersensitivity and IgG levels (56), and in mice where exposure to Cd^{2+} decreases hypersensitivity responses and antigen-stimulated splenic T cell proliferation (57, 58).

In summary, the results of the present study indicated that treatment of CCRF-CEM cells with a low Cd^{2+} concentration (10μM) induces Fas down-regulation at the transcriptional level, as the final step of a mechanism initiated by a rapid and simultaneous induction of ERK5 and JAK3. Thus, the effect of Cd^{2+} on the CCRF-CEM cells may give clues to understanding the signaling pathways

leading to Fas regulation and, additionally, as Cadmium is one of the immunomodulatory metals (56, 59), using this system it could be possible to verify the signaling pathways triggered by environmental stress in immune system cells.

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