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FAST TRACK

HDGF Is Dephosphorylated During the Early Steps of Endothelial Cell Apoptosis in a Caspase-Dependent Way

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Abstract We were looking by a proteomic approach for new phospho-proteins involved during the early steps of the TNF + cycloheximide (CHX)-induced apoptosis—preceding mitochondrial membrane permeabilization—of endothelial cells (BAEC). In the present study, we observed on the autoradiography from 2D gel of ^{32}P-labeled samples a string of proteins undergoing a complete dephosphorylation after 1 h of stimulation with TNF + CHX—while mitochondrial membrane permeabilization was observed after 3 h—identified the different spots by mass spectrometry as one and only protein, HDGF, and confirmed the identity by western blot. The intensity of the 2D phosphorylation pattern of HDGF was correlated with the amount of apoptosis induced by TNF + CHX and TNF or CHX alone and this event was inhibited by the Caspase specific inhibitor zVADfmk. Moreover the TNF + CHX-treatment did not affect the nuclear localization of GFP-HDGF. Taken together, our data suggest an involvement of HDGF during the initiation phase of the apoptotic process downstream from an initiator Caspase and a regulation of this protein by phosphorylation in the nucleus. J. Cell. Biochem. 104: 1161–1171, 2008.

Key words: TNF; endothelial cells; apoptosis; HDGF; 2D; proteomic

Apoptosis is a highly regulated and organized cellular death process, which is essential for the homeostasis of tissues, and critical for the deletion of autoreactive lymphocytes, virus-infected cells and tumor cells [Williams, 1991].

The proteases caspases are considered as the major elements activated during this process. The caspases are classified in two groups, depending on the level in the cascade at which they are activated: the initiator (Caspase-2, -8, -9, -10 and -12) and the effector caspases (Caspase-3, -6 and -7) [Loeffler and Kroemer, 2000].

During the apoptosis induced by TNF (TNFSF2) through interaction with the death receptor family member TNF-RI (TNFRSF1A), the subsequent formation in the cytosol of the death-inducing signaling complex (DISC) [Hsu et al., 1996] leads to the activation of Caspase-8 [Medema et al., 1997] which cleaves Bid [Li et al., 1998], a Bcl family member. Truncated Bid is myristoylated [Zha et al., 2000], which allows its translocation to the mitochondria.

Abbreviation used: TNF, tumor necrosis factor-α; CHX, cycloheximide; BAEC, bovine aortic endothelial cell; OA, okadaïc acid; DISC, death-inducing signaling complex; MMP, mitochondrial membrane permeabilization; zVADfmk, *N*-benzyloxycarbonyl-Val-Ala-Lys-fluoromethyl ketone; HDGF, hepatoma-derived growth factor; kDa, kiloDalton; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; MS, mass spectrometry; GFP, green fluorescent protein.

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The mitochondrial membrane permeabilization (MMP), common to many—if not to all—apoptogenic stimuli, is then observed [Loeffler and Kroemer, 2000]. Proteins as cytochrome *c* and other pro-apoptotic factors (i.e., Smac/DIABLO and HrtA2/Omi) are released in the cytosol to activate mainly the effector caspases. The cytosolic co-localization of cytochrome *c* and pro-Caspase-9 allows their interaction with the protein Apaf-1 to form the apoptosome [Li et al., 1997], while Smac/DIABLO [Du et al., 2000; Verhagen et al., 2000] and HrtA2/Omi [Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002; Verhagen et al., 2002] antagonize the activity of inhibitor of apoptosis proteins (IAPs). Caspase-9 next activates Caspase-3 and -7 [Slee et al., 1999] and Caspase-3 is the major effector of the alterations observed during the execution phase [Slee et al., 2001].

We have previously demonstrated that TNF is able to induce apoptosis of endothelial cells (EC) [Robaye et al., 1991]. However, massive death of these cells requires protein synthesis inhibition [Suarez-Huerta et al., 2000]. Such observation suggests that BAEC display constitutive protective mechanism(s) and/or that TNF triggers both pro- and anti-apoptotic signaling pathways, the latter being dominant.

Moreover, we have suggested by a pharmacological approach that the control of EC apoptosis involves protein (de)phosphorylation events [Clermont et al., 2003]. We therefore aimed to identify protein(s) undergoing modulation of their phosphorylation state during the initiation phase of apoptosis by a proteomic approach [Clermont et al., submitted]. We identify in the present report Hepatoma-Derived Growth Factor (HDGF), an endothelial mitogenic factor [Oliver and Al-Awqati, 1998], as another protein rapidly dephosphorylated in response to different apoptogenic inducers in endothelial cells. This event occurs prior to the mitochondrial membrane permeabilization but downstream from an initiator caspase, presumably Caspase-8.

MATERIALS AND METHODS

Bovine Aortic Endothelial (BAE) and COS-7 Cells Culture

Endothelial cells were obtained from aortas of freshly slaughtered cows and cultivated as previously described [Van Coevorden and Boeynaems, 1984; Clermont et al., 2003]. Briefly, cells harverested after a collagenase (250 U/ml) treatment are cultured in 9 cm Petri dishes for 3 days in Minimum Essential Medium-D-Valine supplemented with FCS (5%, v/v), 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. This MEM-D-Valine medium prevents the growth of smooth muscle cells [Marcum et al., 1986]. The culture was pursued in complete medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with FCS (5%, v/v), Ham's F-12 (20%, v/v) and antibiotics as describe above). Cells were collected for amplification at confluence using classical trypsin treatment and seeded for the experiments after passage 2 or 3 in the appropriate culture plates at a specific cell concentration (see the different methods below).

COS-7 cells were cultured in Petri dishes in complete medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with FCS (10%, v/v) and antibiotics as describe above). Cells were collected using classical trypsin treatment and seeded in the appropriate culture plates for the experiments at a specific cell concentration (see the corresponding method below).

Specific cell concentrations have been chosen so that at the end of the experiments, the cells were in a non-confluent state.

Quantification of DNA Degradation

3×10^5 cells were seeded in 35 mm culture plate in complete culture medium. The next day, the cellular DNA was labeled by a 24-h incubation of the cell with [^{3}H]thymidine (1 μCi/ml, 49 Ci/mmol). After 2 washes with DMEM, the cells are treated or not with the tested agents. At the end of incubation, the culture medium was collected and the cells were scraped and lysed on ice in 1 ml of cold 5 mM Tris–HCl pH 8.0, 20 mM EDTA and 0.5% Triton X-100. In order to separate DNA fragments from intact chromatin, the lysates were submitted to a 13,000*g* centrifugation at 4°C. The radioactivity contained in the culture medium (med.), the 13,000*g* supernatant (sup.) and the pellet (pel.) was counted by liquid scintillation. The percentage of DNA degradation was calculated as follows:

$$\% \text{ of DNA degradation} = \frac{\text{dpm med.} + \text{dpm } 13,000g \text{ sup.}}{\text{total dpm}}$$

where total dpm (degradation per minute) = dpm med. + dpm sup. 13,000*g* + dpm pellet 13,000*g*.

Measurement of the Mitochondrial Transmembrane Potential

3×10^5 cells were seeded in 35 mm Petri dishes in complete culture medium. After 48 h, the cells were treated or not with the tested agents. At the end of the incubation time, the cells were harvested by trypsinization, washed with PBS buffer and incubated 15 min at 37°C in the presence of 50 mM of 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Molecular Probe). $DiOC_6(3)$ is a fluorescent dye and a positively charged molecule that permeates through the plasma membrane. At low concentrations (as used in this experiment), it accumulates in mitochondria due to the large negative membrane potential and consequently does not accumulate when the mitochondrial membrane potential is disrupted. At the end of incubation, cells were analyzed by FACscan analysis (Becton Dickinson). Cells were excited at 488 nm and fluorescence was collected on FL1 channel at 530 nm. Ten thousand cells were analyzed, and forward and side angle scatters were used to gate and exclude debris.

2D-PAGE of Phospho-Proteins

3×10^5 cells were seeded in 3.5 cm Petri dishes as described above. Two days after, the cells were washed twice with DMEM without $NaH_2PO_4 \cdot H_2O$ The cellular phospho-protein were labeled 2 h with 500 μCi/ml [^{32}P]orthophosphate (370 MBq/ml, 10 mCi/ml) in poor-phosphate medium (Dulbecco's Modified Eagle Medium (DMEM) without $NaH_2PO_4 \cdot H_2O$ supplemented with FCS (5%, v/v), Ham's F-12 (20%, v/v) and antibiotics as described above) prior to apoptotic stimulation. At the end of incubation, the cells were washed twice with a NaCl 0.9% solution and scraped in 130 μl thiourea lysis buffer (2 M thiourea, 7 M urea, 2% w/v CHAPS, 1% (w/v) DTT, 2% (v/v) Pharmalyte® 3-10 and 0.1% (w/v) Pefabloc® proteinase inhibitor). The proteins were submitted to 2D-PAGE as described by Gorg et al. [2000]. Her protocol is available online at http://www.weihenstephan.de/blm/deg/manuals.htm. Briefly, isoelectric focusing was performed in rehydrated 180 mm long IPG strips (narrow pH gradient pH 4.0–5.0) (Amersham Biosciences) on MultiPhor II (reswelling solution: 2 M thiourea, 6 M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT and 0.5% (v/v) Pharmalyte® 3-10). After cup loading (please refer to Gorg's manual "The Laboratory Manual: Two-Dimensional Electrophoresis of Proteins using Immobilized pH Gradients" available online (http://www.weihenstephan.de/blm/deg/manuals.htm) for detailed comparison of the different loading methods) of the same amount of radio-labeled proteins in each gel (classically 8×10^5 cpm, as determined by the Siekevitz method [Siekevitz, 1952]), the proteins were submitted to electrophoresis at 20°C for 30 min at 150 V, 1 h at 300 V, 1 h at 1,500 and 3,500 V to the steady state for a total of 42,000 Vh. IPG strips were then equilibrated for 2×15 min in 10 ml of a solution containing Tris–HCl buffer (500 mM, pH 8.5), 6 M urea, 30% (w/v) glycerol, 1% (w/v) SDS. DTT (1%, w/v) was added to the first, and iodoacetamide (5%, w/v) to the second equilibration step. The IPG strips were rinsed with the running buffer and then set on 12.5% polyacrylamide gels (1 mm × 180 mm × 180 mm). The second dimensional gels were run at 10°C and started at 120 V for 5 min, then the voltage was raised from 230 to 360 V. After classical fixation and drying, the gels were submitted to autoradiography on Hyperfilm β-max for the same exposure time and developed in parallel.

Purification of the Phospho-Proteins a–e (Cellular Fractionation)

2.25×10^7 BAE cells were seeded in 24.5 × 24.5 cm Petri dishes as described above. After 48 h, the cells were scraped in the culture medium with a rubber policeman and collected by gentle centrifugation. The cell pellet was lysed by 5 cycles of freeze/thawing in 1 ml of 40 mM Tris–HCl pH 7.4 containing Pefabloc® (50 μg/ml), leupeptin (50 μg/ml), NaF (5 mM), Na_3VO_4 (100 μM) and okadaic acid (100 nM). After centrifugation (4,500*g*), the proteins in the supernatant were precipitated by trichloroacetic acid (TCA, 10%, w/v) in the presence of deoxycholate (DOC, 0.015%, w/v) for 15 min. After a 13,000*g* centrifugation, the pellet was rinsed once with acetone and dried. Precipitated proteins were solubilized in 175 μl of 2D lysis solution and submitted to 2D-PAGE (cup

loading on IPG strips pH 4.0–5.0; running conditions on MultiphorII: 1 h at 150 V, 5 h at 300 V, 16 h at 1,500 and 3,500 V to the steady state of 85 kVh for the first dimension).

Silver Staining

The protocol was modified from the protocol described by Heukeshoven and Dernick [1988] and is described by Gorg in her manual "The Laboratory Manual: Two-Dimensional Electrophoresis of Proteins using Immobilized pH Gradients" available online (http://www.weihenstephan.de/blm/deg/manuals.htm).

In-Gel Digestions and Mass Spectrometry Analysis

After gel electrophoresis and protein silver staining, the spots were excised from 12 bidimensional gels and processed for tryptic digestion, based on the procedure described previously [Shevchenko et al., 1996]. Briefly, the destained gel pieces were treated with DTT 10 mM at 56°C for 30 min and then with iodoacetamide 55 mM for 30 min at room temperature. The gel pieces were washed with 100 mM ammonium bicarbonate and shrunk with 80% acetonitrile (ACN) in water before trypsin incorporation into the gel (100 ng per condition; sequencing grade; Promega). Digestion was carried out for 10 h at 37°C. Peptides were extracted from the gel in the presence of ACN. The digested peptides (approx. 20 μl) were used for a direct peptide monoisotopic mass fingerprinting after a μZipTip (Millipore) purification. ZipTip purification was performed as follows: after rehydration of the solid phase with 80% ACN in water, the C18 resin was equilibrated with 5% ACN in water plus trifluoroacetic acid (TFA) 0.2% and the digested peptides were adsorbed with 20 repetitive pipettings. Peptides were eluted directly in 1.5 μl of 2,5-dihydroxybenzoic acid 2 mg/ml and α-cyano-4-hydroxycinnamic acid 10 mg/ml, plus fucose 2 mM and ammonium acetate 5 mM (matrix mix) onto a metallic target.

Mass spectrometry analysis was performed on a Q-TOF Ultima Global mass spectrometer (Micromass) equipped with a MALDI source. Ionization was achieved using a nitrogen laser (337 nm beam, 10 Hz). The instrument was externally calibrated using the monoisotopic masses of tryptic and chymotryptic peptides from bovine serum albumin. Acquisitions were performed in a V mode reflectron position. Microsequencing was performed by argon-induced fragmentation after selection of the parent ion.

Western Blot Analysis of Proteins

The western blot experiment was performed classically, as previously described [Clermont et al., 2003]. 3×10^5 cells were seeded in 35 mm Petri dishes in complete culture medium. Two days after, the cells were treated or not with the tested agents. At the end of the incubation, cells were scraped in the culture medium with rubber policeman, collected by gentle centrifugation. The cell pellets were solubilized in Leammli buffer. The protein concentration for each sample was determined using the method of Minamide and Bamburg [1990]. The same amount of proteins (classically 50 μg) for each condition was submitted to an electrophoresis onto a 10% SDS–PAGE. Proteins were then transferred overnight at 60 V and 4°C onto a PVDF membrane, using 20 mM Tris, 154 mM glycine, 20% (v/v) methanol, as transfer buffer. The membrane was then incubated 1 h in the blocking solution (50 mM Tris–HCl, 0.15 M NaCl, 0.1% (v/v) Tween 20 and 5% (w/v) BSA) and incubated for 4 h in the presence of rabbit anti-HDGF antibody (serum, 1/10,000) (production of anti mHDGF/GST pk AK SA6121 antibody has been previously described [Abouzied et al., 2004]) or for 1 h in the presence of mouse monoclonal anti-beta actin antibody, clone AC-15 (1/10,000) (Sigma–Aldrich) in the working solution (50 mM Tris–HCl, 0.15 M NaCl, 0.1% (v/v) Tween 20 and 1% (w/v) BSA). Following 3×10 min washes in the working solution, the membrane was incubated with protein A - HRP (0.5 ng/ml) (Sigma–Aldrich) or goat anti-mouse IgG peroxidase conjugate (1/8,000) (Sigma–Aldrich). After 3×10 min washes, the immunodetection was realized by the ECL + Western blotting analysis system and autoradiography.

Plasmid Construction and Cell Transfection

The coding sequence of bovine HDGF [Dietz et al., 2002] was subcloned by PCR using the primers cagagaattcatgtcgcgatccaaccgg and cagagcggccgccattggtggctacaggctct to introduce *Eco*RI and *Not*I restriction sites (underlined in the primers sequences). The coding sequence

was introduced into the eukaryotic expression vector pcDNA3 (Clontech Laboratories, Palo Alto, CA) digested by *Eco*RI and *Not*I and pEGFP-C2 (Clontech Laboratories) digested by *Eco*RI and *Sma*I. The constructs were checked by sequencing.

3×10^5 BAEC or 10^5 COS-7 were seeded in 35 mm Petri dishes in complete culture medium. They were grown up to 50% confluency and transfected in complete medium using FuGENE6 (Roche Diagnostics) according to the manufacturer's instructions (the ratio FuGENE/DNA was 3/1).

RESULTS

Modifications of a One Unit Bidimensional Phospho-Proteins Pattern of BAEC During Early Apoptotic Process

In order to identify protein undergoing modulation of their phosphorylation state during the initiation phase of apoptosis, [^{32}P]orthophosphate-labeled BAEC were treated with TNF + cycloheximide (CHX) up to 1 h. This time is previous to all the markers of the integration and execution phases (loss of mitochondrial transmembrane potential (Fig. 1C),

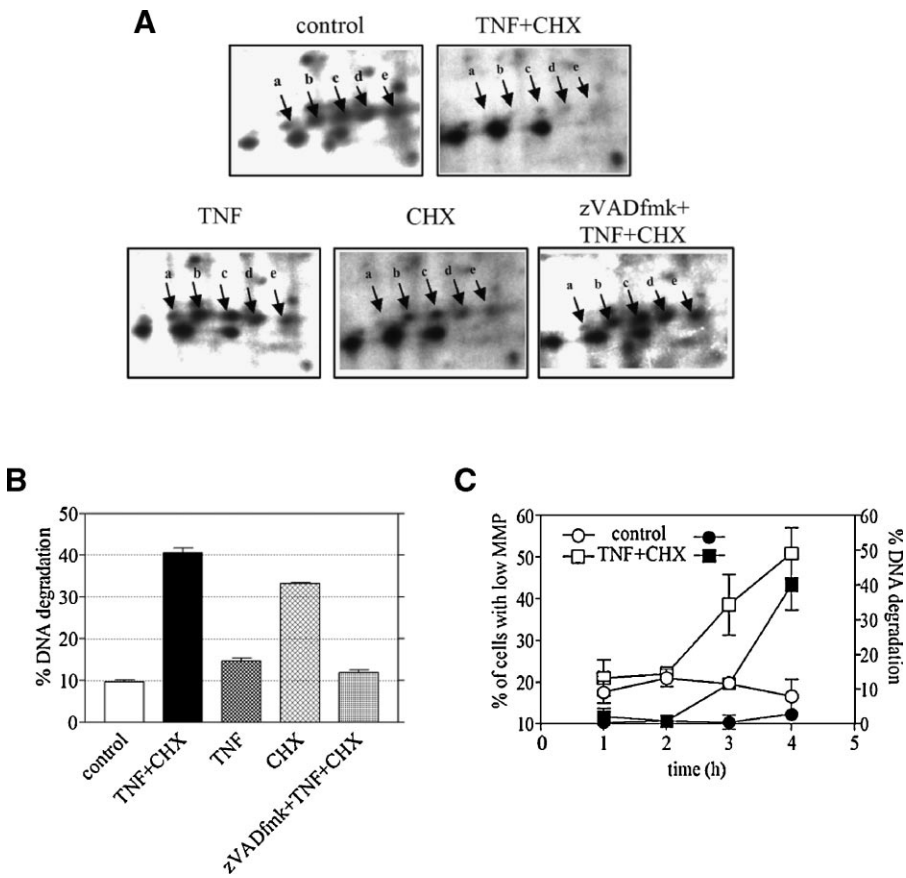


Fig. 1. Modification of the intensity of the phospho-spots a–e. **A**: Intensity of the phospho-spots a–e (2D-PAGE, pH gradient 4.0–5.0) in BAEC treated with TNF + CHX, TNF, CHX and zVADfmk + TNF + CHX. BAE cells were treated for 1 h with TNF (30 ng/ml) + CHX (2 μg/ml), TNF (30 ng/ml) or CHX (2 μg/ml) or incubated during the second hour of ^{32}P labeling with zVADfmk (20 μM) before the treatment for 1 h with TNF (30 ng/ml) + CHX (2 μg/ml). Experimental MW: 40 kDa; experimental pI: 4.61 (a), 4.65 (b), 4.69 (c), 4.73 (d), and 4.76 (e). This experiment is representative of two others. **B**: DNA degradation of BAE cells treated with TNF + CHX, TNF, CHX and zVADfmk + TNF + CHX. BAE cells were treated or not for 6 h with TNF (30 ng/ml) + CHX (2 μg/ml), TNF (30 ng/ml) or CHX (2 μg/ml) or incubated with zVAD-fmk (20 μM) for 1 h before the treatment for 6 h with TNF (30 ng/ml) + CHX (2 μg/ml). Each value represents the mean ± SD of triplicate measurements from an experiment representative of at least two others. **C**: Kinetic of loss of mitochondrial membrane potential and DNA degradation of BAE cells treated with TNF + CHX. BAE cells were treated or not for the indicated times with TNF (30 ng/ml) + CHX (2 μg/ml). The DNA degradation (black symbols) is quantified in the same experiment in parallel to the measurement of the loss of mitochondrial membrane potential (white symbols). Each value represents the mean ± SD of triplicate measurements from an experiment representative of at least two others.

caspase-3 activation and phosphatidylserine (PS) exposure (data not shown)) associated to TNF + CHX-induced apoptosis of BAEC. Proteins were then submitted to a bidimensional (2D)-PAGE and the phospho-proteins were detected by autoradiography. In the present publication, we present results using IPG strips with narrow pH gradient 4.0–5.0. We detected five major spots (spots a–e) exhibiting a reduced intensity in the TNF + CHX-treated cells pattern compared to this of control condition (Fig. 1A). Interestingly, these spots were not detectable when using IPG strips covering larger pH gradients (data not shown).

Identification of the Corresponding Proteins

We introduced a simple purification step before 2D electrophoresis to engage a sufficient amount of the candidate proteins in the electrophoresis: the cytosolic fraction is separated from the particular one by centrifugation after cell lysis. The candidate proteins were recovered in the cytosolic fraction (see Materials and Methods Section). The candidate proteins were then excised from the 2D gel and analyzed by mass spectrometry.

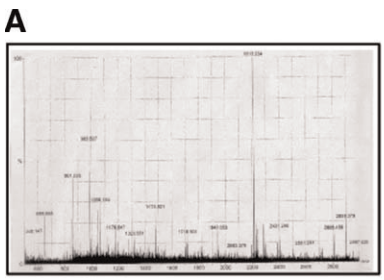
We obtained different microsequences for each excised spot after the mass spectrometry analysis (Fig. 2C). The microsequences from those proteins matched to the sequence of only one protein, the bovine Hepatoma-Derived Growth Factor (HDGF). This identification was confirmed by a western blotting experiment (Fig. 2D): the immunodetection performed after 2D electrophoresis revealed five spots (Fig. 2D) exhibiting identical MW (40 kDa) and pI (from 4.61 to 4.76) as those of the spots observed on the 2D gel autoradiography (Fig. 1A).

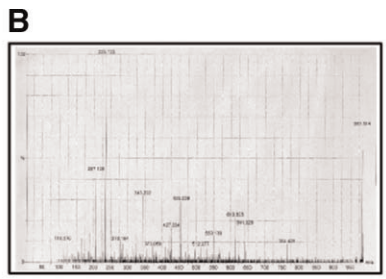
Characterization of the Modification of HDGF in BAEC

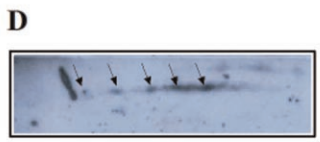
The post-translational modification of HDGF can be considered as an early event: the disappearance of those spots was quasi complete after 1 h of TNF + CHX stimulation (Fig. 1A) while the mitochondrial membrane permeabilization is only observed after 3 h in our experimental system (Fig. 1C). By silver staining of the gels, we observed that the intensity of the five silver spots was not affected by the TNF + CHX-treatment while the spots observed on the autoradiography decrease in intensity (Fig. 3A). Moreover, the immunodetection of HDGF after classical SDS–PAGE in control condition revealed one signal at 40 kDa, whose intensity was not significantly affected by 1 h of TNF + CHX treatment (Fig. 3B).

Relationship of the Modification of HDGF With Apoptosis

In order to establish the potential relationship between the modification of HDGF and apoptosis, we studied the effect of CHX, another







C

Spot	m/z	Microsequence	Protein database search report (accession number)	Residues in bovine HDGF in primary structure	Theoretical MW (kDa)	Theoretical pI	Experimental MW (kDa)	Experimental pI
b	983.53	GYPHWPAR	Hepatoma-derived growth factor (Q9XSK7)	22-29	26.60	4.70	40	4.65
c	1824.99	EGATLEGERPLPVEAEK	Hepatoma -derived growth factor (Q9XSK7)	181-197	26.60	4.70	40	4.69
d	1819.98	GFSEGLWEIENNPTVK	Hepatoma -derived growth factor (Q9XSK7)	81-96	26.60	4.70	40	4.73

Fig. 2. Identification of the phospho-proteins a–e. The mass spectrometry analysis was performed as described in Materials and Methods Section. **A**: Mass pattern of the peptides resulting from the tryptic digestion of protein b. **B**: Microsequence pattern of the peptide from the protein b with a *m/z* of 983.53. **C**: The microsequences were compared to the non-redundant protein databases by a BLAST. **D**: Immunodetection of bovine HDGF on Western blot after 2D-PAGE (pH gradient 4.0–5.0). About 1 mg of proteins were submitted to electrophoresis and working dilution of anti-HDGF antibody was 1/10,000 and protein A-HRP was 1/20,000. Experimental MW: 40 kDa; experimental pI: 4.61, 4.65, 4.69, 4.73, and 4.76. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

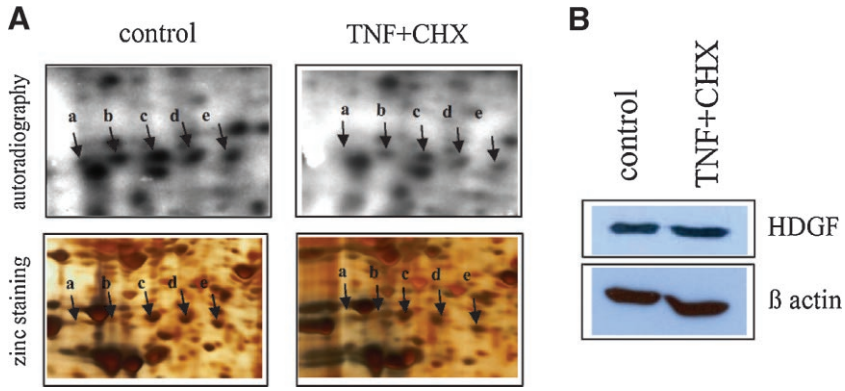


Fig. 3. Effect of TNF + CHX on the expression level of HDGF. **A**: 2D pattern (pH gradients 4.0–5.0) of proteins a–e. BAE cells were treated or not for 1 h with TNF (30 ng/ml) + CHX (2 μg/ml). About 2 mg were engaged in the electrophoresis. At the end of the 2D electrophoresis, the gels were stained and then submitted to an autoradiography. **B**: Immunodetection of bovine HDGF on Western blot (SDS–PAGE). BAE cells were seeded as described in Materials and Methods Section. The cells were treated or not for 1 h with TNF (30 ng/ml) + CHX (2 μg/ml). 50 μg of proteins for each condition were submitted to electrophoresis and working dilution of anti-HDGF antibody was 1/10,000 and protein A-HRP was 1/20,000 and anti-beta actin antibody was 1/10,000 and anti-mouse-HRP was 1/8,000.

apoptogenic stimulus: CHX alone induced both apoptotic DNA fragmentation of BAEC (Fig. 1B) and a reduction of intensity of the HDGF phospho-spots (Fig. 1A). Okadaic acid had similar effect (not shown). On the other hand, TNF alone that was a poor apoptotic inducer in the absence of CHX (Fig. 1B), did not affect significantly the phosphorylation state of HDGF (Fig. 1A).

When BAEC were pre-incubated with the general caspase inhibitor zVADfmk, both TNF + CHX-induced DNA fragmentation (Fig. 1B) and HDGF dephosphorylation (Fig. 1A) were completely abolished.

Effect of the Dephosphorylation of HDGF on the Cellular Localization

As the nuclear localization of HDGF has been correlated to the mitogenic—presumably anti-apoptotic—function of this protein [Everett et al., 2001; Kishima et al., 2002], we studied the effect of the TNF + CHX-treatment on its localization. BAEC were transfected with a vector coding for the chimeric protein GFP-HDGF (see Materials and Methods Section). The day after transfection, the cells were treated with TNF + CHX for 1 h. As shown in Figure 4A, the fluorescence in control cells expressing GFP-HDGF was strictly observed in the nucleus for each fluorescent cell, meanwhile the cellular localization of the fluorescence was not affected by the TNF + CHX-treatment (because of a very low transfection efficiency of the primary cultured BAE cells, about 50 cells per well were expressing the GFP or GFP-HDGF). This suggests that the dephosphorylation does not affect the localization of this protein.

This strictly nuclear localization of HDGF was surprising according to our observation that HDGF was recovered in the cytosolic fraction after cell lysis and fractionation of the sample (see above and Fig. 4B). We therefore investigated this discrepancy using COS-7 cell line because the transfection efficiency was too low when using our primary cultured endothelial cells, harboring about 3% of expressing cells (data not shown). In a same cell culture, we observed that the chimeric GFP-HDGF protein was nuclear in all the fluorescent cells (Fig. 4C), but after cell lysis and fractionation of the samples, we observed by western blotting that GFP-HDGF was recovered in the soluble fraction (Fig. 4D, lines 3 and 6). We also observed the same distribution when normal, untagged, HDGF is expressed in these cells (Fig. 4D, lines 2 and 4).

DISCUSSION

During the initiator phase of apoptosis, TNF in the presence of synthesis inhibitors lead to the activation of Caspase-8 and subsequent translocation of tBid to the mitochondria. Caspase-8 activation is considered as the initiation and a major step leading to the apoptotic cell death. However, TNF can also activate other different signaling pathways including

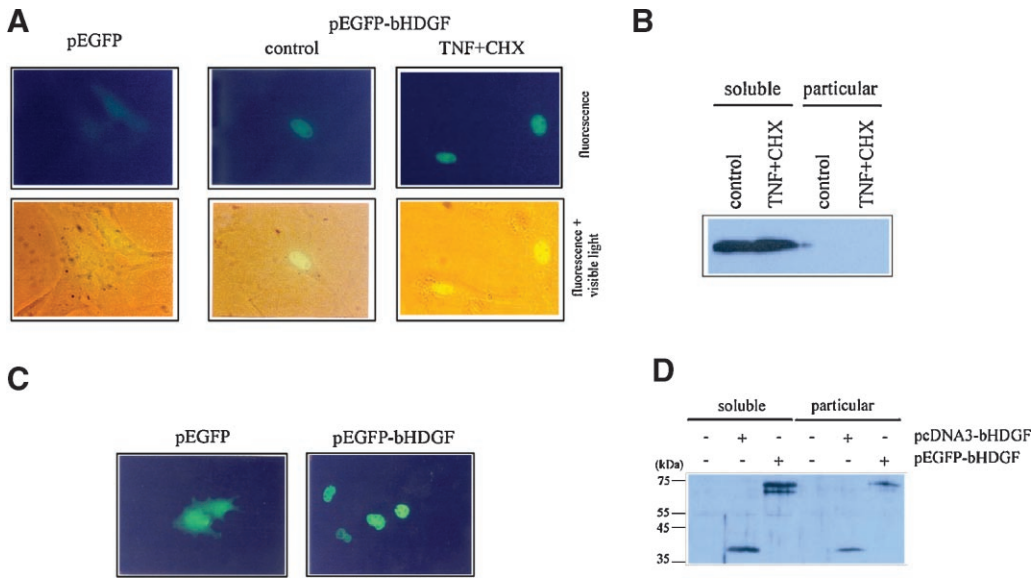


Fig. 4. Cellular localization of GFP-HDGF. **A**: Effect of TNF + CHX on the cellular localization of GFP-HDGF in BAEC. BAEC cells were transfected by pEGFP-bHDGF. The next day, the cells were treated or not for 1 h with TNF (30 ng/ml) + CHX (2 μg/ml) and observed under microscopy. This experiment is done in triplicates and is representative of two experiments. **B**: Sub-cellular distribution of HDGF in BAEC. The BAEC were treated or not for 1 h with TNF (30 ng/ml) + CHX (2 μg/ml). After lysis and fractionation, 50 μg of proteins by condition were submitted to an electrophoresis and HDGF is revealed after immunodetection (working dilution of anti-HDGF antibody was 1/10,000 and protein A-HRP was 1/20,000, experimental MW: 40 kDa). This experiment is representative of two experiments. **C**: Cellular localization of GFP-HDGF in COS-7. COS-7 cells were transfected by pcDNA3-bHDGF or pEGFP-bHDGF. The next day, the cells were observed under microscopy. **D**: Sub-cellular distribution of HDGF in COS-7. After lysis and fractionation, 50 μg of proteins by condition were submitted to an electrophoresis and HDGF is revealed after immunodetection (working dilution of anti-HDGF antibody was 1/10,000 and protein A-HRP was 1/20,000). This experiment is representative of two experiments.

MAP kinase p38 and JNK pathways [Clermont et al., 2003]. We have shown by a pharmacological approach that the p38 kinase could be involved in an anti-apoptotic pathway in the endothelial cell (EC) [Clermont et al., 2003] while the activation of JNK could be required for the activation of Caspase-8 [Deng et al., 2003]. We have therefore hypothesized that different undescribed proteins regulated by (de)phosphorylation could act during the initiation phase of the EC apoptosis.

In the present report, we show by 2D-PAGE that a string of 5 phospho-proteins of 40 kDa are rapidly affected when BAEC are stimulated with TNF + CHX. We unambiguously identify by mass spectrometry each component of this spots series as HDGF (Hepatoma-Derived Growth Factor). To our knowledge, this is the first report demonstrating that HDGF is a phospho-protein. We also show that HDGF is resolved on 2D electrophoresis gel in distinct spots, which suggests different levels of post-translational modification(s) of the protein: phosphorylation or glycosylation, the latter being previously reported for this protein [Oliver and Al-Awqati, 1998]. The amount of material on 2D-PAGE was too low to determine by mass spectrometry the residues harboring the post-translational modifications nor identify them. Nevertheless, the silver staining of the gel has shown that the 2D distribution of HDGF is not altered by the TNF + CHX-treatment, while the spots observed on the autoradiography decrease in intensity. Therefore, the 2D distribution of HDGF may be due to glycosylation. Moreover, as the total amount of HDGF is not altered by the TNF + CHX treatment as shown by western blotting and silver staining, the reduction in intensity of the spots on the autoradiography cannot result from a proteolysis. Therefore, our data suggest that HDGF is dephosphorylated in response to TNF + CHX stimulation.

The dephosphorylation of HDGF seems to be associated with apoptosis as it is observed in response to different apoptogenic stimuli and is inhibited by a caspase inhibitor. If true, this event occurs during the initiation phase of the apoptotic process, as the dephosphorylation is already complete after 1 h while the

mitochondrial membrane permeabilization is only observed after 3 h in our experimental system.

HDGF has been identified as a mitogenic factor in the conditioned medium of 2 different cell lines [Nakamura et al., 1989; Oliver and Al-Awqati, 1998]. HDGF seems to be secreted, however it does not contain any signal sequence [Nakamura et al., 1994]. On another hand, this factor contains a canonical bipartite nuclear localization sequence and its nuclear localization has been correlated to its mitogenic function [Everett et al., 2001; Kishima et al., 2002]. HDGF is mitogenic to many kind of cells, including to endothelial cells [Oliver and Al-Awqati, 1998]. So, HDGF seems to performed distinct roles at the intra- and extra-cellular level and only a mitogenic effect was demonstrated. However, a very recent report strongly suggests that HDGF is involved in apoptosis [Machuy et al., 2004]. Indeed, Machuy et al. showed that the silencing of the HDGF expression inhibits the TNF + CHX-induced apoptosis in HeLa cells. However, they also observed, after a cell lysis fractionation, HDGF in the cytosolic fraction of the control cell and in the membrane fraction of the TNF + CHX-treated cells. They conclude for a cytosolic localization of HDGF and a translocation during apoptosis. They relate this translocation to the inhibition of Smac/DIABLO release from mitochondria observed when HDGF expression is blocked by siRNA [Machuy et al., 2004]. Our data are not in agreement with their conclusions. Indeed, we showed a nuclear localization of the chimeric protein GFP-HDGF in both control and TNF + CHX-treated cells. Next, we observed that HDGF is artefactually recovered in the cytosolic preparation after cell fractionation. Therefore, our findings affirm that HDGF is a nuclear protein. Moreover, as we mentioned above, previous results correlate the mitogenic function to the nuclear localization [Everett et al., 2001; Kishima et al., 2002] and HDGF is mitogenic on endothelial cells [Oliver and Al-Awqati, 1998] while our BAEC are indeed in a proliferative state.

As TNF + CHX treatment of BAEC does not affect the nuclear expression of the chimeric protein, this suggests that the dephosphorylation of HDGF does not influence its localization but could rather affect the function of this protein. The function of HDGF could not to be restricted to its mitogenic activity, as it is expressed in non-proliferative cells [Abouzied et al., 2004; Zhou et al., 2004]. Moreover, its level of expression is reduced in established radioresistant cells and its reduction was associated with reduced sensitivity to irradiation [Matsuyama et al., 2001]. Finally, our data and those from Machuy and collaborators argue for a role of HDGF as a pro-apoptotic factor. Further experiments are required to define the mechanism underlying the mitogenic and apoptotic functions of HDGF and to identify the residues harboring the different post-translational modifications. This will help us to address if only the dephosphorylation step could explain how HDGF could shift from a mitogenic function to an apoptotic one.

CONCLUSION

In the present report, we show that Hepatoma-Derived Growth Factor (HDGF) is a phosphoprotein in the endothelial cell (EC). The triggering of EC apoptosis by TNF + CHX leads to an early dephosphorylation of this protein. This post-translational modification of HDGF occurs before the mitochondrial membrane permeabilization and downstream from an initiator caspase. We also suggest that HDGF is a nuclear protein and that its dephosphorylation does not affect its localization.

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