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The International Journal of Biochemistry & Cell Biology 38 (2006) 2219–2228
IJBCB
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Thrombospondin-1 C-terminal-derived peptide protects thyroid cells from ceramide-induced apoptosis through the adenylyl cyclase pathway

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Received 2 June 2006; received in revised form 17 July 2006; accepted 18 July 2006
Available online 12 August 2006

Abstract

Thrombospondin-1, a multi-modular matrix protein is able to interact with a variety of matrix proteins and cell-surface receptors. Thus it is multifunctional. In this work, we examined the role of thrombospondin-1 in ceramide-induced thyroid apoptosis. We focused on the VVM containing sequence localized in the C-terminal domain of the molecule. Primary cultured thyroid cells synthesize thrombospondin-1 depending on their morphological organization. As it leads thyrocytes to organize into monolayers before inducing apoptosis ceramide can modulate this organization. Here, we established that C_2-ceramide treatment decreased thrombospondin-1 expression by interfering with the adenylyl cyclase pathway, thus leading to apoptosis. Furthermore, we demonstrated that the thrombospondin-1-derived peptide 4N1 (RFYVVMWK) abolished ceramide-induced thyroid cell death by preventing intracellular cAMP levels from dropping. Finally, we reported that 4N1-mediated inhibition of ceramide-induced apoptosis was consistently associated with a down-regulation of the caspase-3 processing. Integrin-associated protein receptor (IAP or CD47) was identified as a molecular relay mediating the observed 4N1 effects. Taken together, our results shed light for the first time on anti-apoptotic activities of the thrombospondin-1-derived peptide 4N1 and provide new information on how thrombospondin-1 may control apoptosis of non-tumoral cells.

Keywords: 4N1; Ceramide; Adenylyl cyclase; Apoptosis; Thyroid cells

1. Introduction

The involvement of the extracellular matrix in a wide number of physiological and pathological functions has been a long-standing topic of investigations. The extracellular matrix dynamic role in cell migration, adhesion, differentiation and survival has widely been documented (Bornstein, 1995; Davis & Senger, 2005; Raines, 2000). These biological functions exist in multifunctional modular domain-composed proteins at large. Among them, the thrombospondins (TSPs) are

Abbreviations: TSP-1, thrombospondin-1; IAP, integrin-associated protein; TSH, thyrothropin; 8-Br-cAMP, 8-bromo-cAMP; PKA, protein kinase A

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doi:10.1016/j.biocel.2006.07.004

a family of five members. Thrombospondin-1 (TSP-1) was the first identified (Lawler, Slayter, & Coligan, 1978). Synthesis and secretion of TSP-1 were observed in a variety of cell types pointing to a general function of TSP-1 (Adams, 2001). The pro- or anti-apoptotic effect of TSP-1 remains controversial and depends on cell lines. For example, TSP-1 inhibited angiogenesis by inducing apoptosis in activated endothelial mouse cells while it stimulated the proliferation of human colon carcinoma cells (Jimenez et al., 2000). The modular structure of TSP-1 allows its interaction with numerous other matrix molecules and cell surface molecules (Adams, 2004; Chen, Herndon, & Lawler, 2000; Sid et al., 2004). Thus, a VVM containing sequence located in the C-terminal globular domain of TSP-1 is able to bind to integrin-associated protein (IAP) also named CD47 (Gao et al., 1996). Some results assigned CD47 an essential role as receptor in apoptosis (Freyberg, Kaiser, Graf, Vischer, & Friedl, 2000). CD47-binding peptide 4N1 (RFYVVMWK), derived from the TSP-1 C-terminal domain, was described as inducing cell death (Johansson, Higginbottom, & Londei, 2004) and exhibiting antiangiogenic activity (Miyata, Koga, Takehara, Kanetake, & Kanda, 2003).

Thyroid cells in primary cultures have been shown to synthesize TSP-1 according to their organization (Bellon et al., 1994; Claisse et al., 1999). When thyroid epithelial cells are organized into follicle-like structures in presence of thyrothropin (TSH) they express little TSP-1. With transforming growth factor beta one the follicle-like structures are disrupted and cells express more TSP-1. In porcine thyroid cells, we also previously reported that ceramide modulate the differentiated states of thyroid cells and contribute to monolayer cell organization (Schneider et al., 2000). Ceramide, the central molecule in sphingomyelin pathway, serves as a second messenger for cellular functions ranging from proliferation and differentiation to growth arrest and apoptosis (Perry & Hannun, 1998). Thus ceramide has long been reported to induce apoptosis in many cell types (Mathias, Pena, & Kolesnick, 1998). In primary cultured thyrocytes, ceramide treatments also reduce cAMP production and are able to induce apoptosis via activation of atypical protein kinase C zeta (Schneider et al., 2000, 2001) and inhibition of ERK-1/2 phosphorylation (El Btaouri et al., 2006). Recently, we suggested a crosstalk between cAMP and ceramide signalling pathways in thyroid cells (Schneider et al., 2005). This could represent an important process in the TSH-controlled differentiation state of thyrocytes.

In the present study, we examined the role of TSP-1 in ceramide-induced apoptosis in primary cells. First, we demonstrated that C_2-ceramide decreased TSP-1 expression through interference with the adenylyl cyclase pathway. Second, we evidenced that the TSP-1 C-terminus-derived peptide 4N1 protects thyroid cells from ceramide-induced apoptosis in a caspase-3-dependent manner. The set of our results provide new information on how TSP-1-derived peptides may interfere in the control of cell apoptosis.

2. Materials and methods

2.1. Reagents

Eagle's medium and trypsin were purchased from Invitrogen (Cergy Pontoise, France). TSH, PKI and H89 were from Calbiochem (VWR International, Fontenay-sous-bois, France) and bovine foetal serum from Dutscher (Brumath, France). The BCA protein assay was from Uptima (Interchim, Montluçon, France). Monoclonal caspase-3 antibodies (#9664) were from Cell Signalling (Beverly, USA). Monoclonal TSP-1 Ab-11 antibodies (#MS-1066) were from Labvision (Interchim, Montluçon, France). B6H12 antibody (556044) came from BD Pharmingen (Le Pont de Claix, France). Secondary antibodies, β-actin antibodies (A-5441), 8-bromo-cAMP and C_2-ceramide were purchased from Sigma (Lyon, France). ECL Western blotting detection reagents were from Amersham (Freibourg, Germany). Products used for molecular biology were purchased from Promega (Charbonnieres, France) and QIAGEN (Courtaboeuf, France). The 4N1 peptide (RFYVVMWK) came from Bachem (Weil am Rhein, Germany) and the 4NGG peptide (RFYGGMWK) that serves as a control peptide, was synthesized by Dr. Patigny IFR 53 (Gao et al., 1996; Manna & Frazier, 2004). All other chemicals were from Sigma. C_2-ceramide, PKI, H89 and 8-Br-cAMP were dissolved in dimethyl sulfoxide and used at concentrations for which no obvious cytotoxicity was observed and an equal volume of dimethyl sulfoxide was added to cells in corresponding control experiments.

2.2. Isolation and primary culture of porcine thyroid cells

Fresh porcine thyroid glands were obtained from Sobevir (Rethel, France). Thyroid cells were dissociated by a discontinuous trypsin-EGTA treatment according to the method of Fayet and Lissitzky (1970), modified by Mauchamp et al. (1979). Freshly isolated cells were suspended in Eagle's medium supplemented with 10%

(v/v) foetal calf serum, penicillin (200 U/mL), streptomycin sulfate (0.05 mg/mL) and TSH (1 mU/mL). Cells were then seeded into 6-well poly (L-lysine)-treated culture dishes at the density of 4×10^6 cells per well and incubated at 37 °C under 5% CO_2 water-saturated atmosphere. In presence of TSH, cells organize into follicle-like structures adhering to the plastic-treated surface (Claisse et al., 1999). After 2 days incubation with TSH, the medium was withdrawn and replaced with serum-free Eagle's medium containing different agonists and incubation was performed for various periods as indicated.

2.3. Western blot analyzes

Following incubation, supernatants were collected and cells were lysed in ice-cold lysis buffer pH 7.4 (50 mM glycerol phosphate, 1.5 mM EGTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1% NP-40, v/v). After quantification (BCA assay), equal amounts of proteins were resolved by SDS-polyacrylamide (10%) gel electrophoresis, blotted on nitrocellulose membrane (Schleicher & Schuell GmbH) and probed with anti-TSP-1 antibodies (1/400), anti-caspase-3 (1/1000) or anti-β-actin antibodies (1/8000). Horseradish peroxidase-conjugated goat anti-rabbit (1/4000) and anti-mouse IgG (1/10,000) were used as secondary antibodies. β-Actin was used as internal control and proteins were detected by chemiluminescence. Each membrane was stained for total protein to ensure equal loading of each well. Time exposure was adjusted to ensure that the ECL signals were not saturated.

2.4. Reverse transcription-polymerase chain reaction

After incubation, total RNAs were extracted using the RNeasy Mini Kit. 1 μg total RNA was reverse transcripted and PCR amplification was carried out using specific primers for TSP-1 (CTCAGGACCAAAGGCTGCTC and ACTCCTGAATGTGGCAGGTC) and S26 (GTGCGTGCCCAAGGATAAGG and ATGGGCTTTGGTGGAGGTCG). The resulting PCR products were visualized by ultraviolet after staining with ethidium bromide. Number of cycles was adjusted to ensure that amplification was in a linear range.

2.5. cAMP assay

Cells were cultured as indicated above. Four days after the onset of cultures, they were rinsed with 20 mM Earle's Hepes buffer (pH 7.2) and incubated for 5 min at 37 °C in the same buffer containing 1 mM isobutyl methyl xanthine and TSH (10 mU/mL) in order to acutely stimulate cAMP release (Schneider et al., 2000). Reactions were stopped by $HClO_4$ (1 M) addition followed by immersion into an ice bath. Cells were homogenized and their cAMP content quantified by a radioimmunological method as described by Cailla, Racine-Weisbuch, and Delaage (1973), except bound and free ligand that were separated by precipitation of bound ligand with a mixture of γ-globulin (2.5 mg/mL) with citrate buffer (pH 6.2) and polyethyleneglycol 6000 (20%, w/v).

2.6. Hoechst reagent staining

Cell monolayers cultured as described above were incubated with the indicated treatments for 24 h. Cells were then stained with propidium iodide (10 μg/mL) and Hoechst 33258 reagent (20 μg/mL) 15 min at room temperature and photographed under fluorescence microscopy (λ excitation: 351.1–363.8 nm; λ emission: 390–480 nm). Apoptotic cells, which contained condensed chromatin fragments, were scored in three replicate wells for each treatment and each assay was repeated at least four times. Results indicated in the text are expressed as percentage of total cell number.

2.7. Caspase-3 activity

After incubation, cell pellets were washed twice with PBS and scrapped in ice-cold lysis buffer provided in the caspACE assay kit from Promega. The caspase-3 activity was measured according to the manufacturer's conditions: 50 μg of cytosolic fraction were incubated with caspase-3 colorimetric substrate that absorbs at 405 nm following its cleavage. Absorbance was measured with a multichannel fluorimetric plate reader (Perkin-Elmer). Z-VAD, a general caspase inhibitor, was used at 50 μM and serves as a control.

2.8. Statistics

All experiments using primary cultured pig thyroid cells were performed on three separate cell preparations and results are expressed as the mean ± S.D. ANOVA was used to compare the mean responses among experimental and control groups for all experiments. Dunett's test was used to determine between which groups significant differences existed. A p-value of <0.05 was considered significant.

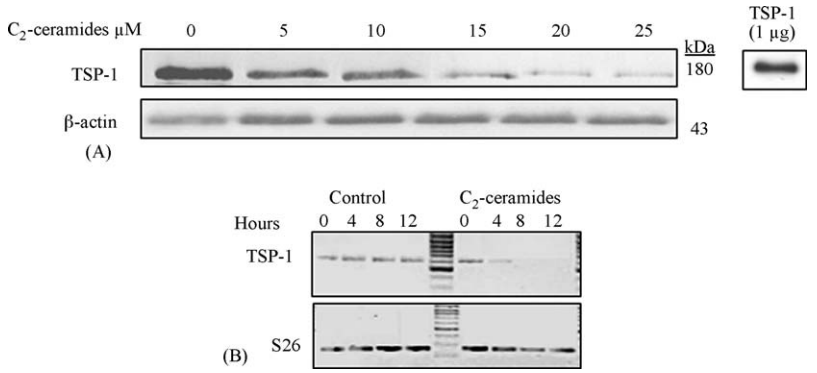

Fig. 1. Effect of C_2-ceramide on TSP-1 expression. After TSH treatment (1 mU/mL) for 2 days, thyroid cells were incubated for 24 h in the presence or absence of increasing doses of C_2-ceramide (0–25 μM). TSP-1 was detected in the culture medium by Western blot (A). 1 μg purified TSP-1 was used to ensure the specificity of the anti-TSP-1 antibody and cell extracted β-actin was used as loading control. For TSP-1 mRNA analyzes, thyrocytes were treated for the indicated periods with 20 μM C_2-ceramide (B). RNAs were isolated and used for detection of TSP-1. S26 amplification served as internal control. The gels shown are representative of three separate experiments.

3. Results

3.1. Inhibition of TSP-1 expression under C_2-ceramide treatment

Since ceramide was shown to induce apoptosis in thyroid primary cells (El Btaouri et al., 2006) and TSP-1 was recently reported to be a molecular relay during cell apoptosis induction (Bruel, Touhami-Carrier, Thomaidis, & Legrand, 2005; Friedl, Vischer, & Freyberg, 2002), we tested whether TSP-1 expression could be modulated in ceramide-treated thyrocytes (Fig. 1A). Controls were done to ensure that at least 80% of exogenous C_2-ceramide penetrated into living cells (data not shown). After 24 h incubation with increasing doses of C_2-ceramide (from 0 to 25 μM), inhibition of TSP-1 secretion in thyrocytes was observed in a dose-dependent manner. The extracellular amount of TSP-1 was reduced by about 20% with 5 μM C_2-ceramide treatment and nearly abolished under 20 or 25 μM C_2-ceramide (inhibition by about 85%). Then, TSP-1 mRNA analyses were performed using the most efficient 20 μM C_2-ceramide treatment for 0, 4, 8 and 12 h (Fig. 1B). Ceramide decreased TSP-1 transcription by about 80% from 4 h and TSP-1 mRNA became undetectable from 8 h.

3.2. Involvement of the cAMP/PKA pathway in ceramide-mediated regulation of TSP-1 expression

As a crosstalk between both ceramide and cAMP signalling pathway was recently suggested (El Btaouri et al., 2006; Schneider et al., 2005), we tested whether adenylyl cyclase pathway could regulate the C_2-ceramide-mediated inhibition of TSP-1. PKI and H89 treatments, respectively, ATP non-competitive and competitive PKA inhibitors (Constantinescu, Diamond, & Gordon, 1999; Kimura, Uchiyama, Takahashi, & Shibuki, 1998), were used to investigate the secretion of TSP-1. Doses from 50 nM to 10 μM were tested for H89 and from 10 nM to 1 μM for PKI. The first significant effects were obtained with 130 nM H89 and 36 nM PKI (Fig. 2A). Both treatments down-regulated the extracellular TSP-1 amount

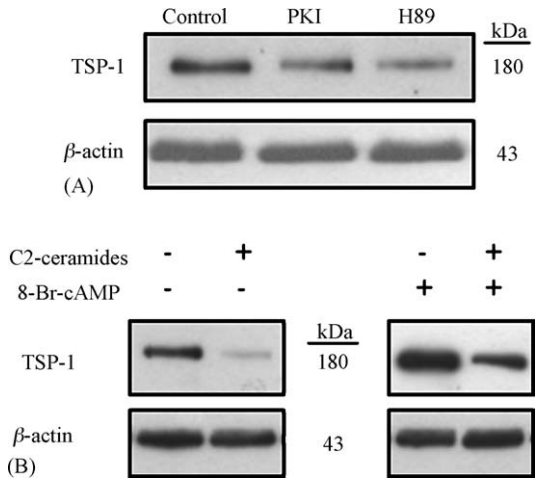

Fig. 2. Involvement of adenylyl cyclase pathway in ceramide-inhibited TSP-1 expression. Two days TSH-treated cells were incubated for 24 h in the presence or absence of PKI (36 nM) or H89 (130 nM), both PKA inhibitors (A). TSP-1 was detected in the culture medium by Western blot. Cell extracted β-actin was used as loading control. After TSH treatment for 2 days, cells were incubated for 24 h in the presence or absence of 20 μM C_2-ceramide and/or 10^{-3} M 8-Br-cAMP (B). TSP-1 was detected in the culture medium by Western blot. Cell extracted β-actin was used as loading control. The gels shown are representative of three separate experiments.

by about 40%. Furthermore, the 8-bromo-cAMP (8-Br-cAMP), a non-metabolic cAMP analog, was used to avoid the drop in cAMP level under ceramide treatment, as already reported (Schneider et al., 2000). Cells were incubated with 20 μM C_2-ceramide in presence or absence of 10^{-3} M 8-Br-cAMP and TSP-1 secretion was analyzed (Fig. 2B). Results evidenced that 8-Br-cAMP prevented ceramide-induced TSP-1 inhibition.

Expression of TSP-1 mRNA was also analyzed in presence of H89 and under 8-Br-cAMP with/without C_2-ceramide for 0, 4, 8 or 12 h (Fig. 3). H89 decreased TSP-1 mRNA expression from 4 h, mimicking the effect of C_2-ceramide. Additionally, we detected an elevated TSP-1 mRNA level in 8-Br-cAMP-treated cells as compared to control cells. Moreover, the data indicated that in presence of 8-Br-cAMP, C_2-ceramide-mediated inhibition of TSP-1 transcription was abolished. These data support the concept that ceramide-dependent regulation of TSP-1 expression was mediated through cAMP/PKA pathway inhibition.

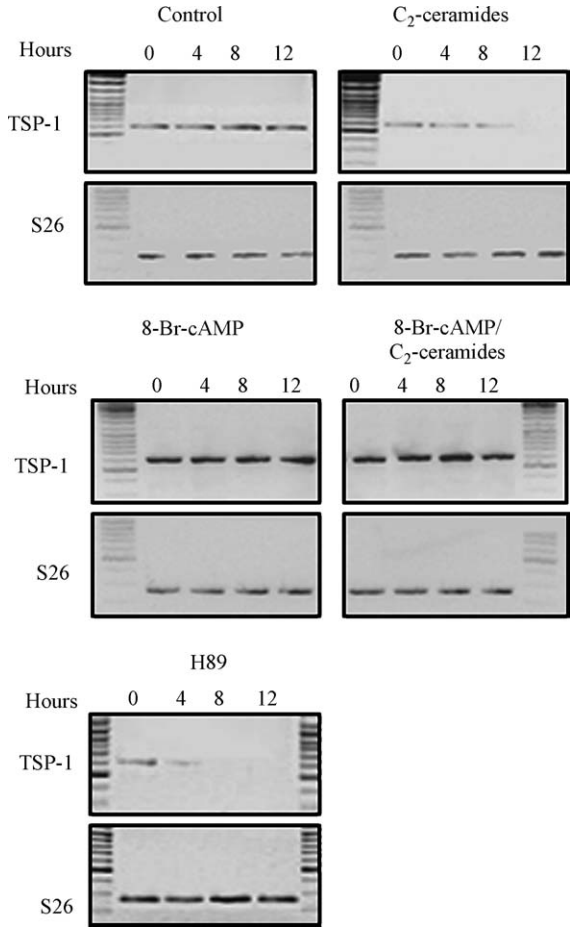

Fig. 3. TSP-1 mRNA analysis. Cells were treated for the indicated periods with C_2-ceramide (20 μM) alone or in presence of 8-bromo-cAMP (10^{-3} M), or H89 (130 nM). RNAs were isolated and used for detection of TSP-1 and S26 that served as internal control. The gels shown are representative of three separate experiments.

3.3. Ceramide-induced cell death was abolished by the TSP-1 C-terminal peptide 4N1

As ceramide repressed TSP-1 through the adenylyl cyclase pathway in thyroid cells, we investigated whether 4N1, a TSP-1 C-terminal derived peptide previously reported to modulate apoptosis (Johansson et al., 2004; Manna & Frazier, 2004), was capable of regulating the ceramide-induced cell death. Thyrocytes were incubated with C_2-ceramide alone or in combination with the 4N1 peptide, the control peptide 4NGG (Gao et al., 1996; Manna & Frazier, 2004), in presence or absence of CD47 blocking antibody B6H12 (McDonald, Dimitry, & Frazier, 2003; Saumet, Jesus, Legrand, & Dubernard, 2002) or with 8-Br-cAMP, then double stained with Hoechst reagent and propidium iodide to visualize apoptotic cells (Fig. 4). Results showed that 40 ± 3% of apoptotic cells were detected under 20 μM C_2-ceramide and an equal apoptosis percent was obtained with ceramide when associated with the scramble peptide 4NGG (Fig. 4B, F and L), thus validating this control peptide. On the contrary, we did not observe any apoptotic cells under simultaneous ceramide and 4N1 treatment (Fig. 4D and L) while B6H12 totally thwarted this effect (42 ± 2%, Fig. 4G and L) unlike rabbit IgG (Fig. 4H and L). Identical results were obtained by using the mature form of TSP-1 instead of the TSP-1-derived peptide 4N1 (data not shown). Additionally, 8-Br-cAMP, reported to induce TSP-1 expression (Fig. 2B), was able to wholly abolish the ceramide-induced cell death while H89 treatment was capable of inducing apoptosis in thyroid cells to 48 ± 4% (Fig. 4J–L).

3.4. The TSP-1-derived 4N1 peptide prevented ceramide-dependent drop of cAMP

Our data suggested that 4N1 could protect thyroid cells from ceramide-induced apoptosis in a cAMP/PKA-dependent manner. To validate this hypothesis, the intracellular cAMP levels were measured with or without C_2-ceramide and in presence or absence of the 4N1 or the 4NGG peptide (Fig. 5). B6H12 was also tested. The results confirmed that C_2-ceramide provoked a dramatic decrease in cAMP production, as already described (Schneider et al., 2000). Moreover, the intracellular cAMP levels were induced by about 2.8 times in 4N1-

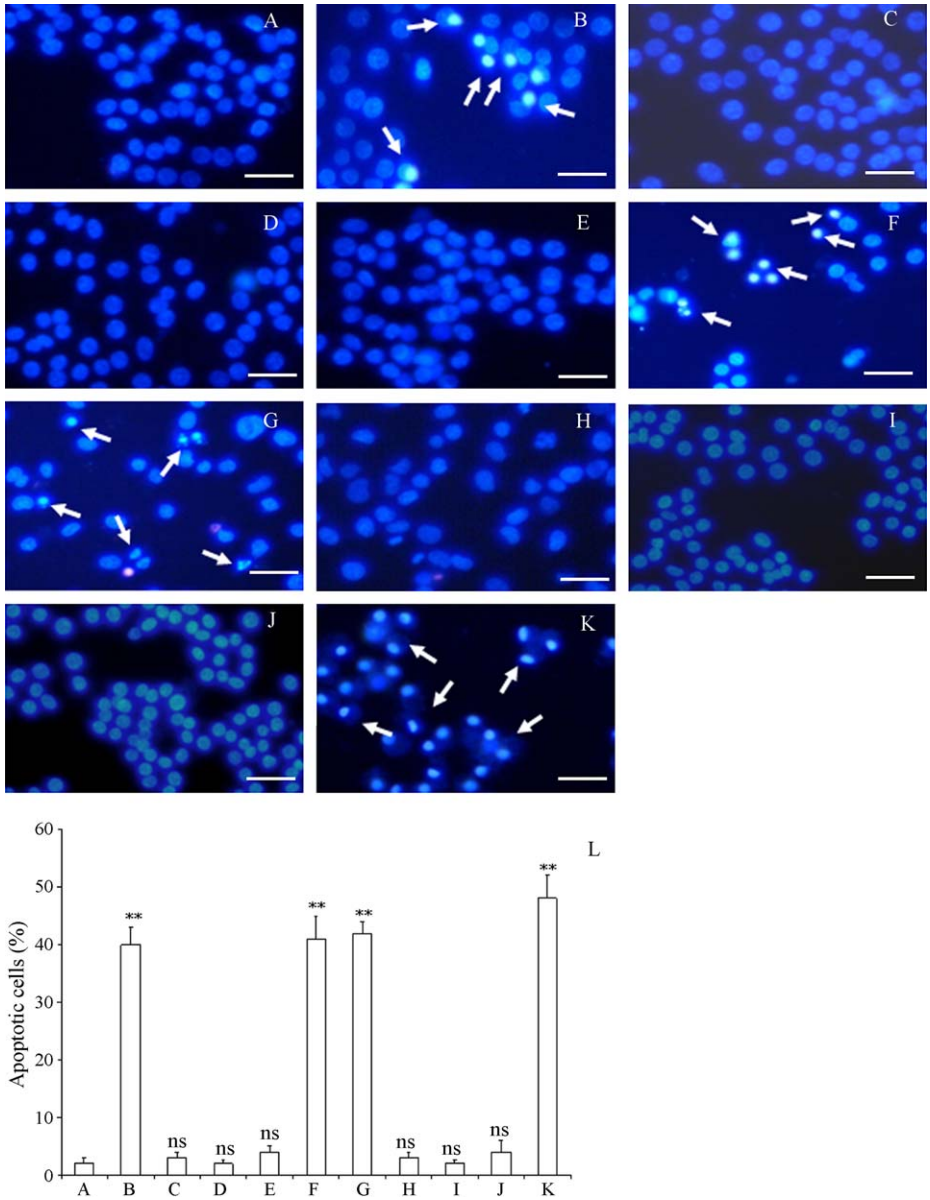

Fig. 4. Effect of the TSP-1 C-terminal-derived peptide 4N1 on ceramide-induced apoptosis. After TSH treatment for 2 days, cells were incubated for 24 h in presence of the indicated treatments. The number of Hoechst-stained cells with pycnotic nuclei were quantitated under fluorescence microscopy and photographed. Scale bars, 50 μm. Arrows indicate apoptotic cells. Results are representative of at least four independent experiments. (A) Dimethyl sulfoxide control cells. (B) C_2-ceramide, 20 μM. (C) 4N1 peptide, 100 μM. (D) C_2-ceramide + 4N1. (E) 4NGG control peptide, 100 μM. (F) C_2-ceramide + 4NGG. (G) C_2-ceramide + 4N1 + B6H12, CD47 blocking antibody, 100 μg/mL. (H) C_2-ceramide + 4N1 + anti-rabbit IgG, 100 μg/mL. (I) 8-Br-cAMP, 10^{-3} M. (J) C_2-ceramide + 8-Br-cAMP. (K) H89, 130 nM. (L) Bar graph representing quantification of the obtained results (A–K). Statistical analysis was performed on at least four independent experiments.

treated cells as compared to 4NGG-treated thyrocytes and only the 4N1 peptide is capable of preventing the C_2-ceramide-mediated drop of cAMP. The IAP dependence of the protective effect of 4N1 is demonstrated by the inhibition of the effect by the function blocking anti-IAP antibody B6H12.

3.5. 4N1 peptide abolished ceramide-induced apoptosis in a caspase-dependent manner

To test whether the 4N1 peptide abolished ceramide-induced apoptosis in a caspase-dependent manner, the 17 kDa cleaved caspase-3 pro-apoptotic form was quan-

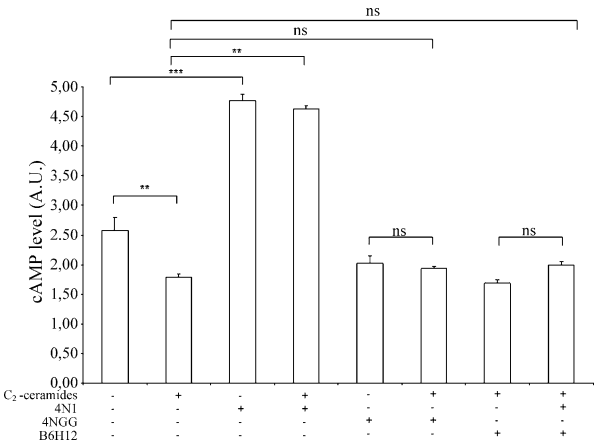

Fig. 5. Effects of C_2-ceramide, 4N1 and 4NGG peptides on cAMP intracellular levels. Cells were cultured for 4 days with TSH (1 mU/mL) and acute stimulation of cAMP production was performed as indicated in experimental procedure. Cells were incubated with C_2-ceramide (20 μM), 4N1 peptide (100 μM), 4NGG peptide (100 μM) or CD47 blocking antibody B6H12 (100 μg/mL) as indicated. The intracellular levels of cAMP were measured as described in Section 2 and expressed as arbitrary units (A.U.). Results are the means ± S.D. of three separate experiments. $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant.

tified by immunoblotting (Fig. 6A) and caspase-3 activity was measured using a fluorimetric assay (Fig. 6B). We observed that ceramide-induced cell death was accompanied by a significant activation of caspase-3 that was completely reversed under 4N1 treatment, whereas 4NGG had no effect. When IAP was blocked by B6H12, the 4N1 peptide could not prevent ceramide-induced caspase-3 activation. In addition, 8-Br-cAMP treatment artificially maintaining the intracellular cAMP levels decreased the ceramide-dependent activation of caspase-3.

Altogether, our results demonstrated that 4N1 could protect thyroid cells from C_2-ceramide-induced apoptosis in a caspase-dependent manner, mainly by activating the cAMP/PKA pathway.

4. Discussion

The possible role of TSP-1 in the control of cell apoptosis attracts much attention from scientists interested in altering the behavior of cancer cells and regulating various pathological diseases such as neurodegenerative or autoimmune disorders. In this paper, we established for the first time that TSP-1-derived peptide 4N1 protects normal cells from ceramide-induced apoptosis.

We described a ceramide-induced apoptosis in thyroid cells, confirming what has been reported in a range of normal or tumor cells from various tissues (Haimovitz-Friedman, Kolesnick, & Fuks, 1997; Okazaki, Kondo, Kitano, & Tashima, 1998; Takai et al., 2005). Moreover, we established that ceramide down-regulated TSP-1 expression through inhibition of cAMP/PKA signalling pathway, thus leading to apoptosis induction. This is in agreement with previous results reported in our laboratory in a similar cellular context. They suggested a ceramide-mediated inhibition of the adenylyl cyclase system through activation of Gi protein. This resulted in a dramatic drop in intracellular cAMP levels associated to an increased caspase activity (El Btaouri et al., 2006; Schneider et al., 2000). Consistent with our data, Manna and Frazier (2004) demonstrated by using 8-Br-cAMP that preventing the drop in cAMP level spared the cells from apoptosis. Moreover, Morimoto, Head, MacDonald, and Casey (1998) have already described a direct relationship between adenylyl cyclase activation and the mRNA level of TSP-1, thus supporting our present observations.

Though various studies over the last decade have supported the pivotal role of TSP-1 in apoptosis induction (Bruel et al., 2005; Friedl et al., 2002; Jimenez et al., 2000; Li et al., 2003; Miyata et al., 2003; Nor et al., 2000), our results clearly shed light on anti-apoptotic activities of the TSP-1-derived peptide 4N1 in thyroid context. Among all these studies, the mainstream focused either on the mature form of TSP-1 or on synthetic peptides derived from the type I repeats (Gao et al., 1996) or the heparin-binding domain (Bruel et al., 2005) of TSP-1. Yet few were interested in the biological aspects of the 4N1 motif which could explain, at least in part, the apparent discrepancies of these results. Contrary to what takes place in thyroid environment, recent observations support the concept that TSP-1-derived C-terminal fragment is capable of inducing a rapid apoptosis-like death of human monocytes, dendritic cells or breast cancer cell lines (Johansson et al., 2004; Manna & Frazier, 2004). As a suggested mechanism, Manna and Frazier (2004) reported a dramatic decrease of intracellular cAMP levels upon 4N1 treatment. We did not observe such a phenomenon in our experimental conditions. On the contrary, we demonstrated that 4N1 peptide mediated inhibition of ceramide-induced apoptosis which was consistently associated with a down-regulation of the caspase-3 processing. Consistent with our findings, an elevated production of endogenous ceramide in primary thyroid cells was recently reported to be associated with an increased level of the cleaved caspase-3 pro-apoptotic form (El Btaouri et al., 2006). Furthermore, the TSP-1-mediated regulation of leukaemia cell apoptosis has already been reported as consistently associated with a regulation of caspase-3 processing (Li et al., 2003).

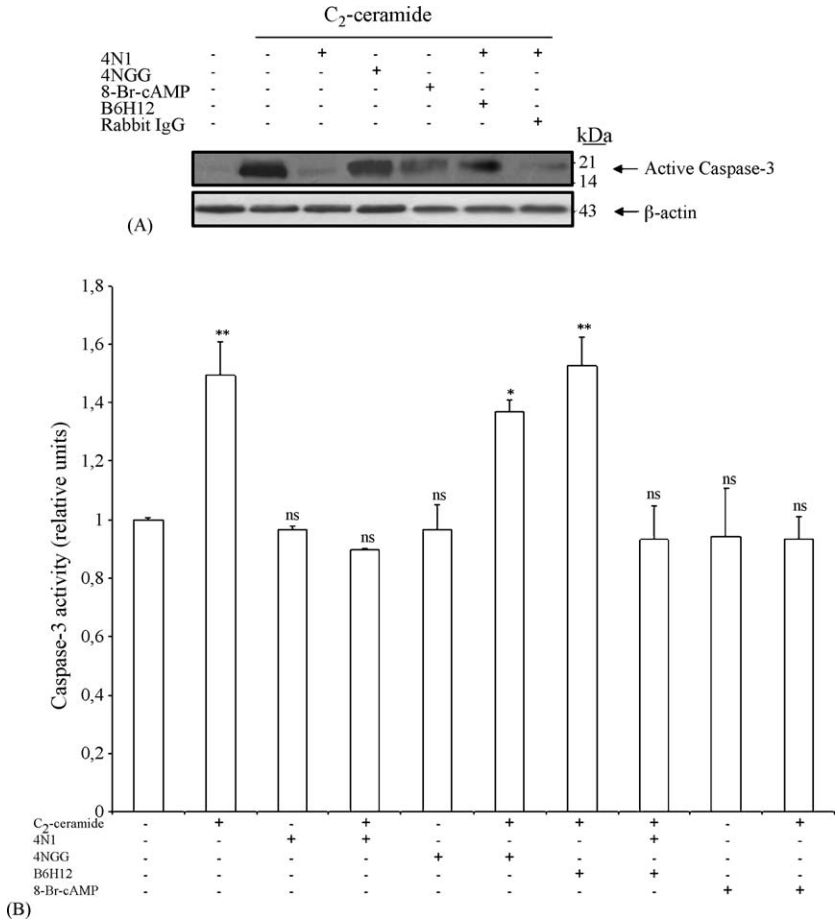

Fig. 6. Effect of the 4N1 peptide on ceramide-induced pro-caspase-3 activation. After TSH treatment for 2 days, thyroid primary cells were incubated during 4 h in absence or presence of C_2-ceramide (20 μM) and with or without the 4N1 peptide (100 μM), the 4NGG peptide (100 μM), 8-Br-cAMP (10^{-3} M), B6H12 (100 μg/mL) or rabbit IgG (100 μg/mL). The active caspase-3 was detected by Western blot by using anti-cleaved-caspase-3-fragment antibodies (A). β-Actin was used as loading control. Caspase-3 activity was measured by using a fluorimetric caspase-3 substrate (B). Results are the means ± S.D. of three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; ns, not significant.

Additionally, the caspase-mediated death pathway has been recently evoked by Nor et al. (2000) as involved in TSP-1-mediated control of endothelial cell apoptosis. However, on the contrary, others reported an absence of modulation of caspase-3 activity in 4N1-treated breast cancer cells (Manna & Frazier, 2004).

Altogether, these data indicate that regulation of apoptosis by TSP-1 must be carefully apprehended since it seems to be closely dependent on the nature of the TSP-1-derived motif. Moreover, the different cellular responses obtained are also the consequences of their cellular context, including tumor grade and cell invasiveness. In the same way and despite extensive studies over the last years, the role of TSP-1 in the regulation of other biological processes such as proliferation, adhesion or angiogenesis remains mainly uncertain and controversial (Sid et al., 2004).

The molecular basis by which the 4N1 peptide antagonized ceramide-induced cell death remains to this day largely unknown. Binding of TSP-1-derived peptides to CD36 or CD47 receptors has already been suggested to mediate the TSP-dependent regulation of cell apoptosis (Freyberg et al., 2000; Johansson et al., 2004; Li et al., 2003; Saumet, Slimane, Lanotte, Lawler, & Dubernard, 2005). However, CD36 remain undetectable under our experimental conditions (data not shown) and CD47 agonists were constantly reported to mediate cell death induction through a caspase-independent machinery (Manna & Frazier, 2004; Saumet et al., 2005). This supports the concept of a still unidentified receptor/integrin association or signalling pathway mediating the anti-apoptotic activity of the 4N1 motif in thyroid environment. On the other hand, the Ras/PI3K/Akt signalling pathway, sensitive to ceramide (Schubert,

Scheid, & Duronio, 2000; Zhou, Summers, Birnbaum, & Pittman, 1998), is well known to modulate caspase-3 response and induce anti-apoptotic signal (Franke & Cantley, 1997; Yang & Widmann, 2002). It is reported to be activated during TSP-1-mediated cellular processes such as focal adhesion disassembly (Goicoechea, Orr, Pallero, Eggleton, & Murphy-Ullrich, 2000; Orr, Pallero, & Murphy-Ullrich, 2002) or thyroid carcinoma cell invasion (Soula-Rothhut et al., 2005). This argues the idea that PI3K signalling pathway could act as a prospective pivotal molecular relay in the TSP1-dependent antagonism of ceramide-induced cell death. This is currently in progress in the laboratory.

To conclude, understanding how synthetic pharmacological agents such as TSP-1-derived peptides are capable of presenting not only well-established antitumor and antimetastasis activities but also anti-apoptotic properties against non-tumoral cells from the close environment will be of great interest in the coming years. It will allow elaboration of new systematic effective chemotherapeutic approaches against metastatic diseases.

Acknowledgements

This work was supported by MENRT, CNRS and by grants from the "Ligue Départementale Contre le Cancer" (Comité de l'Aube).

Authors thank Odette Legué and Laetitia Parent for their technical assistance and are grateful to Sobevir (Rethel, France) for the supply of thyroid glands.

We thank Sylvie Ricord for reading the paper and improving the English.

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