cAMP-dependent protein kinase activation inhibits proliferation and enhances apoptotic effect of tumor necrosis factor- α in NCI-H295R adrenocortical cells

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Abstract

Adrenocorticotropin is the major regulator of adrenocortical development and function. It acts mainly through the cAMP-dependent protein kinase A (PKA) pathway. Our aim was to study the interaction of tumor necrosis factor- α (TNF α) and the PKA pathway in adrenocortical cell proliferation and apoptosis. The PKA activator Dibutyryl cAMP ((Bu)₂cAMP) strongly induced differentiation and inhibited proliferation in the human adrenocortical cell line NCI-H295R (H295R). TNF α induced apoptosis of H295R cells. Interestingly, (Bu)₂cAMP treatment clearly enhanced TNF α -induced apoptosis in H295R cells, but not in another human adrenocortical cell line SW-13, the mouse adrenocortical Y-1 cell line or the human HeLa cell line. This synergistic effect was not due to the (Bu)₂cAMP-induced glucocorticoid secretion since dexamethasone had no significant effect on the TNF α -induced apoptosis. (Bu)₂cAMP treatment rapidly increased the expression of the proto-oncogene *c-myc* in H295R cells, but not in SW-13, Y-1 or HeLa cells. In transient *c-myc* transfection assay, *c-myc* expression associated with decreased expression of the proliferation marker Ki-67 in H295R cells. In conclusion, cAMP-dependent protein kinase activation reduced proliferation and augmented TNF α -induced apoptosis in adrenocortical H295R cells, and these effects were associated with increased *c-myc* expression.

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Introduction

The adrenal glands are organs that undergo dynamic changes in cell growth and death in physiological and pathophysiological conditions (Keegan & Hammer 2002, Kirschner 2002). The essential regulator adrenocorticotropin (ACTH) affects adrenocortical cell differentiation, proliferation and cell death mainly by increasing the intracellular concentration of cyclic adenosine monophosphate (cAMP), which serves as a second messenger. The ubiquitous regulatory molecule cAMP binds to protein kinase A (PKA), leading to bi-directional effects on the proliferation of mammalian cells: inhibition in several cell types but stimulation in others (Keegan & Hammer 2002, Kirschner 2002, Langlois *et al.* 2002).

Meticulously regulated differentiation, proliferation and apoptosis play fundamental roles in the development of multicellular organisms and the maintenance of tissue homeostasis (Abrams 2002, Corn & El-Deiry 2002). We previously found that ACTH is able to induce differentiation and apoptosis, but it inhibits proliferation of rat fetal adrenocortical cells in primary cultures (Arola *et al.* 1993). Expression of the proto-oncogene *c-myc* gene is lost in human adrenocortical carcinomas (Liu *et al.* 1997) and induction of *c-myc* expression by PKA activation is observed during ACTH treatment in primary cultures of human adrenocortical cells (Liu *et al.* 1996b). In many cell types, induction of *c-myc* expression puts the cells on the crossroads of coupled pathways: it promotes cell cycle progression, inhibits cell differentiation and

sensitizes the cells to apoptosis (Pelengaris *et al.* 2002). The situation is different in adrenocortical cells, where *c-myc* expression is associated with ACTH-induced differentiation and inhibited proliferation (Arola *et al.* 1993, Heikkilä *et al.* 1995, Liu *et al.* 1996b), leaving the mechanisms underlying the physiological actions of ACTH and PKA unclear.

A number of stimuli, including tumor necrosis factor- α (TNF α), Fas, TNF-related apoptosis-inducing ligand and radiation, have been demonstrated to induce apoptosis in different tissues (Zimmermann & Green 2001, Karin & Lin 2002). The intensively studied potent cytokine TNF α is produced by many cell types, and functions through its two receptors (TNF-R1 and TNF-R2). Although TNF-R2 can have an important contribution in a number of responses in specific cell types, in most cells TNF α -induced effects are mediated by TNF-R1 (Heyninck & Beyaert 2001). TNF α binding to its receptors results in recruitment of signal transducers that can simultaneously activate at least two signaling pathways. One leads to apoptosis through the activation of the caspase cascade, whereas the other leads to cell survival by inhibition of the death signal. For the latter, evidence for an important role of nuclear factor- κ B-mediated anti-apoptotic gene expression has already been obtained. When both pathways are activated by TNF α , the final decision to survive or die is mostly dependent on the activity ratio of these two pathways in individual cells (Baud & Karin 2001).

There is increasing evidence that TNF α is involved in the regulation of adrenocortical function. TNF α and its receptors are present in human adrenal steroidogenic cells, as well as in resident macrophages. The release of the cytokine from adrenal cells is regulated by factors that alter adrenal function (Jäätelä *et al.* 1990, González-Hernández *et al.* 1996, Judd *et al.* 2000). TNF α decreases steroidogenic P450 enzyme gene expression and cortisol release from human fetal adrenal cells, but increases cortisol release from adult human adrenal cells (Darling *et al.* 1989, Jäätelä *et al.* 1991, Ilvesmäki *et al.* 1993). Regardless of these studies on the effects of TNF α on adrenal steroid production, the apoptosis-inducing effect of TNF α in adrenals and its mechanisms are still largely unknown. Our goal was therefore to

study the interaction of TNF α and the PKA pathway in adrenocortical cell proliferation and apoptosis.

Materials and methods

Cell cultures

Human adrenocortical carcinoma cell line NCI-H295R (H295R) from the American Type Culture Collection (ATCC; Rockville, MD, USA) was grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM-F-12; Sigma Chemical Company, St Louis, MO, USA) supplemented with 1% ITS+1 liquid media supplement (Sigma), 2% Ultrosor SF (Bioprepa, Marlborough, MA, USA), 2 mM L-glutamine (Gibco-BRL, Paisley, Strathclyde, UK) and antibiotics (125 μ g/ml streptomycin and 125 IU/ml penicillin; Orion Pharmaceutical Co., Espoo, Finland) at 37 °C in a 5% CO₂ atmosphere. Human adrenocortical carcinoma SW-13, mouse adrenal carcinoma Y-1 and human cervical adenocarcinoma HeLa cells (ATCC) were cultured in DMEM-F-12 medium supplemented with 10% fetal calf serum, glutamine and antibiotics. The medium was refreshed every other day, and the cells were split at a ratio of 1:3 to 1:6 with trypsin after reaching confluence. Treatment or transfection was initiated on the second day after reseeding of the cells. Triplicate dishes were used for the experiments which were repeated at least three times. Cortisol was routinely measured from the conditioned cell culture media with the Technicon Immuno 1 System (Bayer Corporation, Tarrytown, NY, USA).

RNA analysis

Cytoplasmic RNA was extracted from the cultured cells (Voutilainen *et al.* 1986). Northern blotting, probe sequences, labeling and hybridizations were the same as previously described (Liu *et al.* 1996a,b). The relative intensities of autoradiographic signals were quantified by densitometric scanning. All Northern blotting data shown here were normalized with the respective 28S ribosomal RNA values. For detecting TNF α mRNA expression, RT-PCR was performed. Briefly, 1 μ g RNA was used for cDNA synthesis with the First Strand

cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). The PCR reaction was run as denaturing at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; and then final extension at 72 °C for 10 min. The reaction volume was 50 μ l containing 0.2 mM each dNTP, 0.5 μ M each primer, 1 $\times$ reaction buffer, 2.5 mM MgCl₂, 1.5 U *Taq* DNA polymerase (Fermentas Tamro Corporation, Vantaa, Finland) and template cDNA with respect to 50 ng cytoplasmic RNA. The primer set was 5'-TCA GAT CAT CTT CTC GAA CC-3' (sense) and 5'-CCA GAC TCG GCA AAG TCG AG-3' (antisense), corresponding to the human TNF α mRNA (GenBank Accession Number XM 041847). Ten microliters of the PCR products were resolved on 1.8% agarose gels and Southern blotted onto nylon membrane. The membrane was hybridized with a ³²P-labeled internal probe. The sequence of this 30-mer oligonucleotide probe was 5'-GGC AAG GGC TCT TGA TGG CAG AGA GGA GGC-3'.

Western blot

Cells grown on 10 cm plates were collected and washed with ice-cold phosphate-buffered saline (PBS) twice. Then, 160 μ l protein sample buffer (63.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) without β -mercaptoethanol was added to the cell pellets. The mixture was sonicated briefly on ice and centrifuged at 4 °C for 10 min. The supernatant was collected and the total protein concentration was determined by the standard BCA protein assay kit (Pierce Boston Technology, Woburn, MA, USA). Subsequently, 4 μ l β -mercaptoethanol was added to each sample and mixed. Samples were incubated at 95 °C for 5 min. Total protein (25 μ g) was loaded onto each lane and separated in 10% SDS-PAGE, and then transferred onto nitrocellulose membrane. Non-specific antibody binding was minimized by blocking with 5% non-fat milk in PBS-0.05% Tween 20. The membrane was incubated with mouse monoclonal antibody against *c-myc* (Nordic Biosite AB, Täby, Sweden). The secondary antibody was a horseradish peroxidase-conjugated polyclonal antibody against mouse (DAKO, Glostrup, Denmark) and the immunoblots were developed with ECL film (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Monoclonal anti- β -tubulin antibody (Sigma) was used for the loading control.

Immunocytochemical staining

The staining was performed on H295R cells cultured on two-well chamber plastic slides (Nalge Nunc International, Naperville, IL, USA). The cells were permeabilized by microwaving at 600 W for 3 $\times$ 5 min in citrate buffer (10 mM Na-citrate, pH 6.0). The slides were then washed in PBS and non-specific staining was blocked with normal goat serum for at least 30 min at room temperature. Endogenous peroxidase activities were blocked by 1% H₂O₂ for 30 min. The TNF-R1 protein was detected with two affinity-purified polyclonal antibodies (sc-7895 from Santa Cruz Biotechnology, Santa Cruz, CA, USA and CSA-810 from StressGen Biotechnologies, Victoria, BC, Canada). Expression of the TNF-R2 was also studied using two polyclonal antibodies (sc-7862 and sc-1072 from Santa Cruz Biotechnology). The proliferation marker Ki-67 antigen was detected with a monoclonal antibody MIB-1 (Immunotech, Marseille, France). The primary antibodies diluted at 1:100 in PBS were added onto the adrenal cells and incubation was performed overnight at 4 °C. The primary antibodies were detected using biotin-conjugated goat anti-rabbit IgG from the corresponding ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA), followed by incubation with ABC solution according to the manufacturer's instructions. Finally, light counterstaining was performed with hematoxylin, and the slides were dehydrated and mounted. For the negative controls, the primary antibodies were replaced with normal rabbit serum or PBS alone. To exclude the effect of possible endogenous biotin, biotin blocking (avidin-biotin blocking kit; Vector Laboratories) was performed for each antigen detection before the addition of the primary antibodies. The immunoreactivity was quantified by calculating the ratio of positively stained cells to the total number of cells.

Analyses of apoptotic cells

Terminal transferase-mediated fluorescent-dUTP nick end-labeling (TUNEL) was performed with the *in situ* cell death detection kit (1684795; Roche Diagnostics GmbH, Penzberg, Germany)

according to the producer's instruction manual. Briefly, the cells were cultured on two-well chamber plastic slides. After treatment with different agents, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Then 50 μ l label solution was added onto each chamber and the slides were incubated in a humidified atmosphere for 60 min at 37 °C in the dark. The apoptotic cells were analyzed by Zeiss Axiophot fluorescence microscopy. For the morphological analysis of apoptotic cells, the cultured cells were fixed with methanol and stained with DAPI (or Hoechst 33258; Sigma) dye. Apoptotic cells were examined for nuclear morphology using a fluorescence microscope. End-stage apoptotic cells were also detected by trypan blue staining.

Proliferation assays

H295R cell proliferation was evaluated immunocytochemically with a cell proliferation kit (Amersham Pharmacia Biotech, Amersham, Bucks, UK) according to the manufacturer's instructions. In this assay, 5-bromo-2'-deoxyuridine (BrdU) is incorporated into replicating DNA during 15 h of incubation, localized with a specific monoclonal antibody and stained with a peroxidase-based detection system. The culture area was photographed and the nuclei were counted from each culture dish. The proliferation percentage was calculated on the basis of the positively stained nuclei in relation to all nuclei. Flow cytometric analysis was performed on cells treated with Dibutyl cAMP ((Bu)₂cAMP). The cells were trypsinized, collected into 70% ethanol, and stained with propidium iodide. DNA content was analyzed with a flow cytometer. Cell cycle analysis was performed using the Sync Wizard Model of ModFit LT software (Becton Dickinson, Franklin Lakes, NJ, USA). Ki-67 immunocytochemistry was also used to estimate the proliferation of H295R cells.

Transfection of c-myc into H295R cells

Semi-confluent cells were transfected with the pSVT c-myc construct (Mäkelä *et al.* 1992) using FuGene 6 (Roche) transfection reagents. Cells were plated (1:5) on cover slips in a 35 mm culture dish 1 day before the transfection. The ratio of transfection reagent/vector DNA was 4:1 (1 μ g DNA per

dish) in all experiments. Otherwise the transfection procedures were performed in serum-free medium according to the manufacturer's instructions. After 36 h the cells were fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. The cells were washed three times in PBS, and the cover slips were then immunostained with c-myc and Ki-67 antibodies for an hour at room temperature and followed by three washes with PBS. To visualize the localization of antibodies, we used FITC- or TRITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After incubation, the cover slips were washed with PBS three times for 5 min each. Finally, the cover slips were mounted onto Anti-FADE media (Molecular Probes, Eugene, OR, USA) and analyzed with a Zeiss inverted fluorescence microscope (Axophot 2) equipped with a cooled CCD camera (SensiCam; PCO Computer Optics GmbH, Kelheim, Germany). The data presented in this paper are from the total pool of the transfection experiments.

Statistical analyses

All experiments were repeated at least three times. Non-parametric Kruskal–Wallis test was used to detect differences among the different experimental groups. If significant differences were found, Mann–Whitney U test was subsequently used for statistical evaluation in two-group comparisons. Chi-square test was used for analyzing the correlation between Ki-67 and transfected c-myc expression. The level of significance was chosen as $P < 0.05$.

Results

Activation of PKA stimulates differentiation but inhibits proliferation of adrenocortical cells

To find out if the ACTH-induced apoptosis in primary cultures of rat fetal adrenal cells (Kahri *et al.* 1996) can be reproduced in human adrenocortical cells and to further study its mechanisms, we used the human adrenocortical carcinoma cell line H295R as a model. As reported previously in primary cultures of normal rat and human adrenocortical cells (Arola *et al.* 1993, Liu *et al.* 1996a) and in H295 cells (the parent cell line of the H295R cells; Staels *et al.* 1993), (Bu)₂cAMP

Figure 1 Effect of (Bu)₂cAMP on the differentiation of H295R cells. (A) Regulation of cortisol secretion by (Bu)₂cAMP (1 mM) treatment for different time-periods. The open bars represent cortisol secretion from control cultures and the solid ones from (Bu)₂cAMP-treated ones. Each bar represents cumulative cortisol secretion (means $\pm$ S.E. of three experiments in triplicate dishes) with the respective control values adjusted to 100. * $P < 0.01$ compared with the control. (B) A representative Northern blot analysis to show the induction of steroidogenic acute regulatory protein (StAR) mRNA expression after (Bu)₂cAMP (24 h) treatment at the concentrations indicated. 28S ribosomal RNA was used as a loading control.

induced dose- and time-dependent differentiation of H295R adrenocortical cells (measured by morphological change, steroidogenic gene expression and cortisol secretion) (Fig. 1; other data not shown). Using the BrdU incorporation assay, inhibition of proliferation was found at the 72 h time-point and maintained at least for 1 week in (Bu)₂cAMP (1 mM)-treated H295R cell cultures. The proliferation percentage of the (Bu)₂cAMP (1 mM for 7 days)-treated cells was approximately 60% that of the control group (means $\pm$ S.E. proliferation percentage $20.6 \pm 8\%$ for the treated cells and $32.7 \pm 16\%$ for the controls, $n = 5$, $P < 0.01$). Direct counting of the cells also showed a decrease in cell number in (Bu)₂cAMP-treated cultures down to approximately 65% of the control (Fig. 2A). Immunostaining of Ki-67 showed similar results to the BrdU staining (data not shown). We further analyzed at what stage in the cell cycle the cells had been arrested by measuring their DNA content with propidium iodide staining and flow cytometric analysis (Fig. 2B). Under normal growth

conditions, more than 60% of the H295R cells were in the G₀/G₁ phase and only 18% were in the G₂/M phase. In contrast, over 30% of the cells were in the G₂/M phase after 3 days of sustained treatment with (Bu)₂cAMP (1 mM) and the fraction of the G₀/G₁ cells decreased down to 45%. The average DNA content of the cells increased during (Bu)₂cAMP treatment in the G₀/G₁, G₂/M and S phases. (Bu)₂cAMP treatment for 7 days showed a similar result (data not shown). Immunocytochemical staining of BrdU and Ki-67 also showed enlarged nuclei after (Bu)₂cAMP treatment. Another analog of cAMP, 8-bromo-cAMP, also induced differentiation and reduced proliferation of H295R cells at the same concentration range as did (Bu)₂cAMP (data not shown). The effect of (Bu)₂cAMP was significantly blocked by the PKA inhibitor H-89 (data not shown). ACTH had no significant effect on either the differentiation markers or proliferation rate, probably due to the lack of functional ACTH receptors in this cell line (data not shown).

Figure 2 Decreased proliferation of H295R cells during (Bu)₂cAMP treatment. (A) Cell number after treatment with (Bu)₂cAMP (1 mM) for the times indicated. Each bar shows the cell number in (Bu)₂cAMP-treated cultures (means $\pm$ s.e. of three experiments in triplicate dishes) with the cell number in the respective control cultures adjusted to 100%. * P <0.01 compared with the control. (B) Histogram to display DNA content with or without (Bu)₂cAMP stimulation (1 mM for 3 days) as measured by propidium iodide staining and flow cytometric analysis. The hatched area between the left (G₀/G₁ phase) and right (G₂/M phase) darker grey areas represent the S phase. Apoptotic pattern is observed as sub-G₁ hypodiploid fraction (the light gray area).

TNF α induces apoptosis of H295R adrenocortical cells and the effect is enhanced by (Bu)₂cAMP

In contrast to the situation in primary rat adrenocortical cultures (Kahri *et al.* 1996), ACTH did not induce apoptosis in H295R cells. Trypan blue exclusion test and TUNEL analysis showed about 2% of the cells to be apoptotic. The apoptotic cells were usually localized in the peripheral areas of the cell colonies. (Bu)₂cAMP increased apoptosis slightly after long-term (more than 24 h) treatment as analyzed by flow cytometry (the sub-G₁ hypodiploid fraction in the histogram of Fig. 2B), trypan blue exclusion and TUNEL (Fig. 3). Since the dramatic effect of ACTH on apoptosis was observed in the primary rat adrenal cell cultures with heterogeneous population of cells (Kahri *et al.* 1996), we hypothesized that the apoptotic effect might involve some factors secreted by macrophages present in those cultures. We focused on the effect of the well-known apoptotic factor TNF α , and found as expected that TNF α induced apoptosis five- to tenfold in H295R cells

after 24 h treatment, as analyzed by both trypan blue exclusion test and TUNEL analysis (Fig. 3). Interestingly, this effect was increased two- to fivefold by co-treatment with (Bu)₂cAMP (1 mM) (Fig. 3). This synergistic effect of TNF α and (Bu)₂cAMP on apoptosis was specific for H295R cells, since it was not detectable in HeLa, mouse adrenocortical Y-1 or in another human adrenocortical cell line, SW-13 cells, although TNF α alone increased apoptosis in these cells (data not shown). Since glucocorticoids are able to regulate apoptosis in a tissue- and cell-specific manner (Amsterdam *et al.* 2002), and cortisol concentration was very high in the conditioned media of the (Bu)₂cAMP-treated H295R cells, but not in HeLa and SW-13 cells (data not shown), we used the well-known apoptosis inducer dexamethasone and steroidogenesis-inhibitor genistein treatment to clarify whether the secreted glucocorticoids affect the TNF α -induced apoptosis in H295R cells. Dexamethasone at the concentration of 500 μ g/l for 24 h had no effect on the TNF α -induced apoptosis in H295R cells. Treatment with genistein

(at the concentration of 10 μ g/ml) for 24 h reduced cortisol secretion by more than 80%, flattened the cells and prevented the contracting effect of (Bu) $_2$ cAMP on the cells. Trypan blue assay showed that genistein alone slightly increased cell death (about 5–20%) compared with the control, and this effect was not affected by co-incubation with

(Bu) $_2$ cAMP. Genistein treatment did not significantly augment the apoptotic effect of TNF α . In addition, the absence of the synergistic effect of (Bu) $_2$ cAMP and TNF α in Y-1 cells also excluded the effect of glucocorticoids, since (Bu) $_2$ cAMP strongly stimulated steroidogenesis in this cell line (data not shown).

Expression of the *c-myc* gene does not correlate with proliferation of H295R cells

For clarifying the potential mechanisms underlying the synergistic effects of TNF α and (Bu) $_2$ cAMP on apoptosis, we compared *c-myc* gene expression in H295R, SW-13, Y-1 and HeLa cells, since our previous studies showed that *c-myc* expression was upregulated by (Bu) $_2$ cAMP in primary cultures of normal human adrenal cells and was lost in adrenocortical carcinomas (Liu *et al.* 1996b, 1997). Preceding the decreased cell proliferation, *c-myc* expression was increased by (Bu) $_2$ cAMP in a dose- and time-dependent manner in H295R cells. Interestingly, this effect was not detected in SW-13, Y-1 or HeLa cells (Fig. 4). For analyzing further *c-myc* functions, we transfected *c-myc* cDNA into H295R cells in five different experiments (Fig. 5). Interestingly, overexpression of *c-myc* gene was not associated with stronger Ki-67 staining in the transfected than in the untransfected cells (Chi square=0.229, Phi=0.071, $P=0.632$). Approximately 7% of the Ki-67-positive cells expressed the *c-myc* gene simultaneously and only 35% of the *c-myc*-overexpressing cells were Ki-67 positive, suggesting that induction of *c-myc* expression alone in this cell line may not be coupled to cell proliferation.

Figure 3 Synergistic effect of (Bu) $_2$ cAMP (1 mM) and TNF α (100 ng/ml) treatments (24 h) on apoptosis in H295R cells. The cell culture conditions and apoptosis measurements were as described in the Materials and methods. (A) Trypan blue exclusion analysis. Each bar represents the mean percentage of positively staining cells ($\pm$ s.e. of three experiments in triplicate dishes). * $P<0.01$. (B) TUNEL analysis. (a and b) Control cells without stimulation; (c and d) (Bu) $_2$ cAMP alone; (e and f) TNF α alone; (g and h) combined TNF α and (Bu) $_2$ cAMP treatment. (a, c, e and g) Phase contrast and (b, d, f and h) fluorescence microscopic images.

Figure 5 A representative immunostained area in transfection analysis to show dissociation of *c-myc* and Ki-67 expression in H295R cells. The *c-myc* vector was transiently transfected into H295R cells, and the expression of *c-myc* and Ki-67 was analyzed by immunocytochemistry with a fluorescence microscope. Original magnification $\times 200$.

H295R cells express TNF α receptors

Although TNF α mRNA was not detectable in H295R cells with RT-PCR analysis (data not shown), weak staining of both TNF-R1 and TNF-R2 (Fig. 6) was detected in these cells, with occasional strong expression for TNF-R2 in some cells (Fig. 6B). The positive staining was mainly in the cytoplasm, with occasional weak staining in the nuclei. When the primary antibodies were replaced with non-specific rabbit IgG or PBS, there was no specific staining. These results indicated that the components for TNF α signal transduction are available in H295R cells. Treatment with (Bu) $_2$ cAMP had no significant effect on the expression of TNF α receptors (data not shown).

Discussion

This study was undertaken to characterize the interaction of PKA activation and TNF α on the apoptotic process in cultured H295R adrenocortical cells. (Bu) $_2$ cAMP induced H295R cell differentiation, reduced proliferation and increased the sensitivity of the cells to TNF α -induced apoptosis. This finding is similar to the previous report that the cAMP inducers, such as forskolin and

Figure 6 Immunostaining of TNF α receptors (A) TNF-R1 and (B) TNF-R2 in H295R cells. The cells were cultured for 1 day after splitting, and immunocytochemistry was performed as described in the Materials and methods. Original magnification $\times 200$.

8-bromo-cAMP, inhibited cell growth but also induced cell death in some other cell types (Chen *et al.* 1998). Nevertheless, the H295R cell line originated from a human adrenocortical carcinoma (Rainey *et al.* 1994), giving the cells particular characteristics. Previous reports have described that adrenocortical cell apoptosis is affected by the *in vitro* culture system, species and differentiation status (Negoescu *et al.* 1995, Carsia *et al.* 1998, Spencer *et al.* 1999).

Although this cell line was recently reported to respond to ACTH (Suzuki *et al.* 2004), previous studies showed no response in either the H295R cell line or in its parental H295 cells, probably because of low ACTH receptor expression (Staels *et al.* 1993, Rainey *et al.* 1994, Denner *et al.* 1996). Similarly, our H295R cells did not show significant response to ACTH treatment. We therefore used (Bu) $_2$ cAMP to mimic the ACTH signal pathway. In the present study, (Bu) $_2$ cAMP reduced H295R cell proliferation by arresting the cell cycle mainly to the G $_2$ /M phase. (Bu) $_2$ cAMP treatment seems to override the cell-cycle inhibition in the G $_1$ checkpoint and promote cells into the cell cycle,

Figure 4 Effect of (Bu) $_2$ cAMP on *c-myc* gene expression in different adrenocortical cell lines. The cell culture conditions, RNA analysis and Western blotting were as described in Materials and methods. (A) Time-dependent effect of (Bu) $_2$ cAMP (1 mM) treatment on *c-myc* mRNA accumulation in H295R cells. Each bar represents the relative *c-myc* mRNA level (means $\pm$ S.E. of three experiments in triplicate dishes) with the control values adjusted to 100. The open bars represent values for the control cultures and the solid bars for the treated ones. $*P < 0.01$ compared with the control. (B) A representative Northern blot showing dose-dependent effect of (Bu) $_2$ cAMP (2 h) on *c-myc* mRNA levels in H295R cells. 28S ribosomal RNA was used as a loading control. (C) A representative Western blot of *c-myc* expression in H295R cells treated with (Bu) $_2$ cAMP (1 mM) for 2 h. β -Tubulin was used as a loading control. (D, E and F) Northern blots showing the effect of (Bu) $_2$ cAMP (1 mM for 2 h) and ACTH (30 nM for 2 h in (E)) in SW-13, Y-1 and HeLa cells respectively.

possibly by inducing mitogenic factors, such as *c-myc*. Obviously the induction of these mitogenic factors by (Bu)₂cAMP is unable to overcome the inhibition in the G₂/M checkpoint, ultimately leading the cells to arrest in the G₂/M phase. After long-term treatment, the pool of cells in the G₀/G₁ phase and subsequent re-entering into the cell cycle could decrease, resulting in reduced DNA synthesis measured by BrdU incorporation. *c-myc* gene expression was rapidly induced by (Bu)₂cAMP treatment in H295R cells, as reported previously in primary cultures of rat fetal and normal human adult adrenocortical cells (Heikkilä *et al.* 1995, Liu *et al.* 1996b). The increased *c-myc* expression may initially promote the cells into the cell cycle through the G₁ to G₂/M phase. However, overexpression of *c-myc* gene in the transfected cells was not associated with increased Ki-67 staining, suggesting that the proliferation pathway was blocked in these *c-myc*-overexpressing cells. The previously reported putative tumor suppressor H19 and cyclin-dependent kinase inhibitor p57^{KIP2}, stimulated by (Bu)₂cAMP treatment in adrenocortical cells (Voutilainen 1998), may function as proliferation blocking factors to stop the cells within the cell cycle. During treatment with (Bu)₂cAMP for 3–7 days, the percentage of cells in the G₂/M phase and the average cellular DNA content increased, but the total cell number and the late-stage BrdU incorporation decreased. These findings also suggested an ultimate inhibition of DNA synthesis and cell division after initial entering into the cell cycle. It was previously reported that induction of *c-myc* alone is not sufficient to induce DNA synthesis in Y-1 cells (Lepique *et al.* 2000). While blocking cell proliferation, (Bu)₂cAMP-induced PKA activation may set H295R cells to a low threshold for apoptosis in the G₂/M phase, probably by increasing the expression of apoptosis-related genes (such as *c-myc*). Interestingly, PKA activation did not induce *c-myc* expression in another human adrenocortical cell line SW-13, in the mouse adrenocortical cell line Y-1 or in the human cervical adenocarcinoma cell line HeLa. In these cell lines, (Bu)₂cAMP treatment did not enhance cellular sensitivity to TNF α . In the fetal zone of the human fetal adrenals, ACTH apparently causes differentiation and hypertrophy of the cells migrating inwardly from the definitive zone. The enlarged senescent cells in the fetal zone go into apoptosis more easily than the smaller

definitive zone cells (Mesiano & Jaffe 1997). The synergistic effect of PKA activation and TNF α on apoptosis seems to be tissue specific. It was previously reported that cAMP-elevating reagents were able to suppress TNF α or Fas/APO-1 (CD95)-induced apoptosis in neutrophils and hepatocytes (Fladmark *et al.* 1997, Niwa *et al.* 1999), whereas multidrug-resistant leukemic cells with higher constitutive levels of PKA activity were more susceptible to TNF α -induced apoptosis and growth inhibition (Yin *et al.* 2000).

Glucocorticoids are tissue- and cell-specific regulators of apoptosis (Amsterdam *et al.* 2002). We therefore used dexamethasone and genistein to clarify whether the high concentration of cortisol in the conditioned media could affect the TNF α -induced apoptosis. Our results showed that treatment with dexamethasone had no effect on basal or TNF α -induced apoptosis. Inhibition of cortisol synthesis by genistein, a strong suppressor of the steroidogenic enzymes steroid 21-hydroxylase and 3 β -hydroxysteroid dehydrogenase in H295R cells (Mesiano *et al.* 1999, Ohno *et al.* 2002), could not protect the cells from apoptosis. These results suggested that the enhancing effect of (Bu)₂cAMP on TNF α -induced apoptosis in H295R cells is not due to the increased secretion of cortisol into the culture medium.

In the human adult adrenal gland, TNF α -secreting cells are predominantly localized in the zona reticularis of the cortex. ACTH reduces TNF α release from cultured rat and bovine adrenocortical cells, probably via increased intracellular cAMP (Judd *et al.* 2000). In addition, the ACTH-induced secretion of glucocorticoids may also suppress TNF α release from monocytes/macrophages surrounding the adrenal cells (Joyce *et al.* 1997). In physiological conditions, ACTH stimulation increases intracellular cAMP, which could then predispose adrenocortical cells to the TNF α -induced apoptotic effect; at the same time ACTH reduces TNF α production directly or indirectly. Thus, normal adrenocortical cells differentiate without apoptosis in the absence of TNF α ligand. Hormonally regulated TNF α levels together with cellular PKA activity may be at least one mechanism maintaining the normal dynamics of adrenocortical cells. However, in exceptional conditions, ACTH stimulated cells may meet high levels of TNF α from the circulation or from the monocytes/macrophages concentrated locally in

the adrenals. In these conditions, enhanced apoptosis could occur, similarly to that in primary cultures of fetal rat adrenocortical tissues (Kahri *et al.* 1996). In that tissue culture system, plenty of macrophages attach on adrenocortical cells (authors' unpublished data). This phenomenon does not occur in primary cultures of human fetal or adult adrenocortical cells (Ilvesmäki *et al.* 1993, Liu *et al.* 1996b), suggesting that the enzymatic dispersion and washing process during the preparation of human adrenal cell cultures may eliminate the macrophages. The ACTH/PKA-induced enhanced sensitivity of adrenocortical cells to TNF α -induced apoptosis may have a role in fetal zone regression after birth, since human fetal adrenal cells secrete more TNF α than do adult adrenal cells (Jäättelä *et al.* 1990). H295R cells are characterized by a loss of cAMP response element (CRE)-binding protein (CREB) expression and compensatory overexpression of CRE modulator τ (CREM τ) (Groussin *et al.* 2000). The role of this shifted signal transduction pathway of PKA in the sensitizing effect of (Bu) $_2$ cAMP in H295R cells has not been ruled out, as the normal adrenal cortex expresses high levels of CREB and no or low levels of CREM τ (Groussin *et al.* 2000).

In summary, in human adrenocortical H295R cells, activation of cAMP-dependent PKA inhibited cell proliferation and increased the sensitivity of the cells to TNF α -induced apoptosis, associated with the induction of *c-myc* gene expression. The tightly regulated proliferation and apoptosis probably contribute to the dynamic changes in cell growth and death in adrenal glands.

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References

Abrams JM 2002 Competition and compensation: coupled to death in development and cancer. *Cell* **110** 403–406.

- Amsterdam A, Tajima K & Sasson R 2002 Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. *Biochemical Pharmacology* **64** 843–850.
- Arola J, Heikkilä P, Voutilainen R & Kahri AI 1993 Role of adenylate cyclase-cyclic AMP-dependent signal transduction in the ACTH-induced biphasic growth effect of rat adrenocortical cells in primary culture. *Journal of Endocrinology* **139** 451–461.
- Baud V & Karin M 2001 Signal transduction by tumor necrosis factor and its relatives. *Trends in Cell Biology* **11** 372–377.
- Carsia RV, Nagele RG, Morita Y, Tilly KI & Tilly JL 1998 Models to elucidate the regulation of adrenal cell death. *Endocrine Research* **24** 899–908.
- Chen TC, Hinton DR, Zidovetzki R & Hofman FM 1998 Up-regulation of the cAMP/PKA pathway inhibits proliferation, induces differentiation, and leads to apoptosis in malignant gliomas. *Laboratory Investigation* **78** 165–174.
- Corn PG & El-Deiry WS 2002 Derangement of growth and differentiation control in oncogenesis. *Bioessays* **24** 83–90.
- Darling G, Goldstein DS, Stull R, Gorschoth CM & Norton JA 1989 Tumor necrosis factor: immune endocrine interaction. *Surgery* **106** 1155–1160.
- Denner K, Rainey WE, Pezzi V, Bird IM, Bernhardt R & Mathis JM 1996 Differential regulation of 11 β -hydroxylase and aldosterone synthase in human adrenocortical H295R cells. *Molecular and Cellular Endocrinology* **121** 87–91.
- Fladmark KE, Gjertsen BT, Doskeland SO & Vintermyr OK 1997 Fas/APO-1(CD95)-induced apoptosis of primary hepatocytes is inhibited by cAMP. *Biochemical and Biophysical Research Communications* **232** 20–25.
- González-Hernández JA, Ehrhart-Bornstein M, Späth-Schwalbe E, Scherbaum WA & Bornstein SR 1996 Human adrenal cells express tumor necrosis factor- α messenger ribonucleic acid: evidence for paracrine control of adrenal function. *Journal of Clinical Endocrinology and Metabolism* **81** 807–813.
- Groussin L, Massias JF, Bertagna X & Bertherat J 2000 Loss of expression of the ubiquitous transcription factor cAMP response element-binding protein (CREB) and compensatory overexpression of the activator CREM τ in the human adrenocortical cancer cell line H295R. *Journal of Clinical Endocrinology and Metabolism* **85** 345–354.
- Heikkilä P, Arola J, Salmi A & Kahri AI 1995 ACTH-induced *c-myc* proto-oncogene expression precedes antimitogenic effect during differentiation of fetal rat adrenocortical cells. *Journal of Endocrinology* **145** 379–385.
- Heynink K & Beyaert R 2001 Crosstalk between NF- κ B-activating and apoptosis-inducing proteins of the TNF-receptor complex. *Molecular Cell Biology Research Communications* **4** 259–265.
- Ilvesmäki V, Jäättelä M, Saksela E & Voutilainen R 1993 Tumor necrosis factor- α and interferon- γ inhibit insulin-like growth factor II gene expression in human fetal adrenal cell cultures. *Molecular and Cellular Endocrinology* **91** 59–65.
- Jäättelä M, Carpen O, Stenman U-H & Saksela E 1990 Regulation of ACTH-induced steroidogenesis in human fetal adrenals by rTNF- α . *Molecular and Cellular Endocrinology* **68** R31–R36.
- Jäättelä M, Ilvesmäki V, Voutilainen R, Stenman U-H & Saksela E 1991 Tumor necrosis factor as a potent inhibitor of adrenocorticotropin-induced cortisol production and steroidogenic P450 enzyme gene expression in cultured human fetal adrenal cells. *Endocrinology* **128** 623–629.
- Joyce DA, Steer JH & Abraham LJ 1997 Glucocorticoid modulation of human monocyte/macrophage function: control of TNF- α secretion. *Inflammation Research* **46** 447–451.
- Judd AM, Call GB, Barney M, McIlmoil CJ, Balls AG, Adams A & Oliveira GK 2000 Possible function of IL-6 and TNF as intraadrenal factors in the regulation of adrenal steroid secretion. *Annals of the New York Academy of Sciences* **917** 628–637.

- Kahri AI, Liu J, Arola J & Heikkilä P 1996 ACTH-induced selective death of rat adrenocortical cells in primary culture. *Endocrine Research* **22** 409–410.
- Karin M & Lin A 2002 NF- κ B at the crossroads of life and death. *Nature Immunology* **3** 221–227.
- Keegan CE & Hammer GD 2002 Recent insights into organogenesis of the adrenal cortex. *Trends in Endocrinology and Metabolism* **13** 200–208.
- Kirschner LS 2002 Signaling pathways in adrenocortical cancer. *Annals of the New York Academy of Sciences* **968** 222–239.
- Langlois D, Li JY & Saez JM 2002 Development and function of the human fetal adrenal cortex. *Journal of Pediatric Endocrinology and Metabolism* **15** (Suppl 5) 1311–1322.
- Lepique AP, Forti FL, Moraes MS & Armelin HA 2000 Signal transduction in G₀/G₁-arrested mouse Y1 adrenocortical cells stimulated by ACTH and FGF2. *Endocrine Research* **26** 825–832.
- Liu J, Heikkilä P, Kahri AI & Voutilainen R 1996a Expression of the steroidogenic acute regulatory protein mRNA in adrenal tumors and cultured adrenal cells. *Journal of Endocrinology* **150** 43–50.
- Liu J, Voutilainen R, Kahri AI & Heikkilä P 1996b Expression of the *c-myc* gene in human adrenals: regulation by adrenocorticotropin in primary cultures. *Journal of Endocrinology* **148** 523–529.
- Liu J, Voutilainen R, Kahri AI & Heikkilä P 1997 Expression patterns of the *c-myc* gene in adrenocortical tumors and pheochromocytomas. *Journal of Endocrinology* **152** 175–181.
- Mäkelä TP, Koskinen PJ, Vastrik I & Alitalo K 1992 Alternative forms of Max as enhancers or suppressors of Myc-ras cotransformation. *Science* **256** 373–377.
- Mesiano S & Jaffe RB 1997 Developmental and functional biology of the primate fetal adrenal cortex. *Endocrine Reviews* **18** 378–403.
- Mesiano S, Katz SL, Lee JY & Jaffe RB 1999 Phytoestrogens alter adrenocortical function: genistein and daidzein suppress glucocorticoid and stimulate androgen production by cultured adrenal cortical cells. *Journal of Clinical Endocrinology and Metabolism* **84** 2443–2448.
- Negoescu A, Labat-Moleur F, Defaye G, Mezin P, Drouet C, Brambilla E, Chambaz EM & Feige JJ 1995 Contribution of apoptosis to the phenotypic changes of adrenocortical cells in primary culture. *Molecular and Cellular Endocrinology* **110** 175–184.
- Niwa M, Hara A, Kanamori Y, Matsuno H, Kozawa O, Yoshimi N, Mori H & Uematsu T 1999 Inhibition of tumor necrosis factor- α induced neutrophil apoptosis by cyclic AMP: involvement of caspase cascade. *European Journal of Pharmacology* **371** 59–67.
- Ohno S, Shinoda S, Toyoshima S, Nakazawa H, Makino T & Nakajin S 2002 Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells. *Journal of Steroid Biochemistry and Molecular Biology* **80** 355–363.
- Pelengaris S, Khan M & Evan G 2002 c-MYC: more than just a matter of life and death. *Nature Reviews Cancer* **2** 764–776.
- Rainey WE, Bird IM & Mason JI 1994 The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. *Molecular and Cellular Endocrinology* **100** 45–50.
- Spencer SJ, Mesiano S, Lee JY & Jaffe RB 1999 Proliferation and apoptosis in the human adrenal cortex during the fetal and perinatal periods: implications for growth and remodeling. *Journal of Clinical Endocrinology and Metabolism* **84** 1110–1115.
- Staels B, Hum DW & Miller WL 1993 Regulation of steroidogenesis in NCI-H295 cells: a cellular model of the human fetal adrenal. *Molecular Endocrinology* **7** 423–433.
- Suzuki J, Otsuka F, Inagaki K, Takeda M, Ogura T & Makino H 2004 Novel action of actinin and bone morphogenetic protein in regulating aldosterone production by human adrenocortical cells. *Endocrinology* **145** 639–649.
- Voutilainen R 1998 Adrenocortical cells are the site of secretion and action of insulin-like growth factors and TNF α . *Hormone and Metabolic Research* **30** 432–435.
- Voutilainen R, Tapanainen J, Chung B, Matteson KJ & Miller WL 1986 Hormonal regulation of P450 scc (20,22-desmolase) and P450c17 (17 α -hydroxylase/17,20-lyase) in cultured human granulosa cell. *Journal of Clinical Endocrinology and Metabolism* **63** 202–207.
- Yin Y, Allen PD, Jia L, Macey MG, Kelsey SM & Newland AC 2000 Constitutive levels of cAMP-dependent protein kinase activity determine sensitivity of human multidrug-resistant leukaemic cell lines to growth inhibition and apoptosis by forskolin and tumour necrosis factor alpha. *British Journal of Haematology* **108** 565–573.
- Zimmermann KC & Green DR 2001 How cells die: apoptosis pathways. *Journal of Allergy and Clinical Immunology* **108** (Suppl 4) S99–S103.

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