Epidermal growth factor-induced neuroendocrine differentiation and apoptotic resistance of androgen-independent human prostate cancer cells

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Abstract

Neuroendocrine differentiation (NED) has been implicated in prostate cancer progression and hormone-therapy failure. Neuroendocrine cells are non-proliferating and escape apoptotic cell death, although their origin and the causes of their apoptotic resistance have as yet been poorly elucidated. This study demonstrates a new mechanism involved in controlling NED. We report that epidermal growth factor (5–50 ng/ml) promotes neuroendocrine-like differentiation of androgen-independent DU145 prostate cancer cells. This differentiation is associated with an increase in the expression of Neuron Specific Enolase (NSE) and a reduction in cell proliferation and is blocked by inhibiting tyrosine kinase activity with genistein and with compound 56 (C56). An increase in the cAMP level, using dibutyl cAMP (db-cAMP) (1 mM) and isobutylmethylxanthine (100 µM), does not promote NED by itself, but does increase the effect of EGF on NED. In addition, EGF-induced NED protects cells from apoptosis induced with thapsigargin (1 µM) by reducing the thapsigargin-induced cytosolic calcium overload. In order to describe how EGF-induced NED protects cells against thapsigargin-induced calcium overload we investigated the spatiotemporal calcium signalling linked to apoptosis. By using thapsigargin in various conditions on DU145 cells and using micro-fluorimetric calcium measurements, we show that depletion of intracellular calcium store induces apoptosis and that the amplitude and duration of the capacitive calcium entry are two apoptosis-modulating parameters. We show that protection against thapsigargin-induced apoptosis conferred by NED is achieved by reducing the amount and the speed of calcium that can be released from calcium pools, as well as modulating the amplitude of the subsequent calcium entry.

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Introduction

Prostate cancer (PC) is the second greatest cause of cancer-related death in many countries and in northern Europe it has already taken the number one position as the main cause of male cancer-related death (Landis *et al.* 1998). Androgen ablation is initially beneficial to nearly all men with PC, since androgen-dependent PC undergoes apoptotic death by such treatment (Kyprianou *et al.*

1990). Unfortunately, androgen ablation is only palliative and patients relapse into androgen-independent PC, with dramatic consequences (Crawford 1989). Indeed, androgen-independent PC cells do not enter the programmed cell death pathway following androgen ablation (Denmeade *et al.* 1996). Neuroendocrine cells are one of the phenotypes of apoptosis-resistant cells. The frequency of neuroendocrine cells correlates with tumour malignancy, loss of androgen sensitivity and poor

prognosis (Cussenot *et al.* 1998, Jongsma *et al.* 1999). In addition, significant and clustered neuroendocrine differentiation (NED) in PC can lead to an increase in proliferation and earlier tumour progression (Grobholz *et al.* 2005). Tumours with an increased neuroendocrine cell population are often more aggressive and eventually become androgen-independent (Abrahamsson 1999). However the molecular and cellular mechanisms controlling NED and the apoptosis resistance of neuroendocrine cells are only partially understood and improvement in our understanding of the biological nature of neuroendocrine cells is urgently required (Vashchenko & Abrahamsson 2005).

According to the existing hypotheses, neuroendocrine cells are either derived from undifferentiated basal cells of the prostatic epithelium or have a neurogenic origin (Aumuller *et al.* 1999, Bonkhoff & Remberger 1996). It is also described that PC cells themselves have the ability to transdifferentiate into cells with a neuroendocrine-like phenotype. This transdifferentiation generally occurs from androgen-dependent PC cells and is induced when cells are cultured with pharmacological agents that increase the intracellular level of cAMP, in the presence of interleukin or in a steroid-free medium (Bang *et al.* 1994, Burchardt *et al.* 1999, Cox *et al.* 2000, Mori *et al.* 1999, Zelivianski *et al.* 2001). Neuroendocrine cells represent an androgen-insensitive cell phenotype in the prostate (Krijnen *et al.* 1993). So, one may postulate that neuroendocrine cells could originate from a population of androgen-independent cancer cells, but this hypothesis remains to be proved.

Over the last 20 years, the ever-expanding pool of knowledge of growth factor-regulated pathways and their role in the pathogenesis of PC has been translated into rationally designed drug therapy (Barton *et al.* 2001). Epidermal growth factor (EGF) is known to have anti-apoptotic properties in numerous cell types including prostate cells (Mimeault *et al.* 2003). In addition to the well-described effect of growth factors in PC cells, it has been recently shown that heparin-binding EGF-like growth factor was able to induce transdifferentiation in LNCaP cells (Kim *et al.* 2002).

In an effort to understand how neuroendocrine cells progressively emerge in prostate malignancies and their relationship to hormone-refractory prostate carcinoma, we studied the EGF effect on transdifferentiation of androgen-independent DU145 cells.

In this study we show that EGF mediates NED in androgen-independent PC cells. This differentiation is linked to an increase in Neuron Specific Enolase (NSE) expression, reduction in cell growth and to a resistance to apoptosis induced by thapsigargin (TG).

We also investigated the mechanisms involved in calcium-induced apoptosis in non-differentiated DU145 cells and we elucidated the mechanism by which EGF-induced NED renders neuroendocrine cells resistant to apoptosis induced by calcium overload. We show that in non-differentiated DU145 cells apoptosis may be induced by Ca^{2+} -pool depletion alone and is amplified by extracellular Ca^{2+} entry. Our observations also reveal that apoptosis resistance of EGF-differentiated PC cells following TG exposure occurs via a reduction of both the amount of Ca^{2+} that can be released from Ca^{2+} pools and the amplitude of the subsequent Ca^{2+} entry. Moreover, we show for the first time that EGF-induced differentiation of DU145 cells is a mechanism that can increase the number of apoptosis-resistant cells in PC. This latter result also strengthens the evidence for the existence of a common mechanism centred on a depression of the calcium signal linked to apoptotic resistance in PC.

Experimental procedures

Cell culture

The androgen-independent human PC cell line DU145, obtained from the American Type Culture Collection, was maintained in culture as previously described (Humez *et al.* 2004a). Prior to fluorescence measurements, the cells were trypsinized and transferred to glass coverslips. Cells were used 1–4 days after trypsinization.

Measurement of *in vitro* cell growth

Cells were seeded at an initial density of 500 cells/well in 96-well plates (Poly Labo, Strasbourg, France). After 48 h, cells were cultured in treatment media (day 0). From day 0, the treatment medium was changed daily for each condition. Cells were harvested on day 2. The CellTiter 96 Aqueous Non radioactive Cell Proliferation Assay (Promega Corp., Madison, WI, USA) was used to determine the number of viable cells (Humez *et al.* 2004a).

Apoptosis assays with Hoechst staining

The level of apoptosis was estimated from the number of apoptotic bodies visualized by Hoechst staining. The percentage of apoptotic cells was determined by counting at least 500 cells in random fields. The detailed procedure has been described previously (Humez *et al.* 2004b). The percentages of apoptotic cells presented in Figs 4 and 5 (see below) are representative of three independent experiments.

Analysis of cell cycle and apoptosis

Flow cytometry was used to analyse the cell cycle and apoptosis, as previously described (Zamzami *et al.* 1995). Cells were fixed overnight at 4 °C with 70% ice-cold ethanol/PBS followed by propidium iodide (50 µg/ml) staining and analysed in a Coulter xL cytofluorometer. Cell-cycle compartments were deconvoluted from a single-parameter DNA histogram of 30 000 events using the WINCYCLE 32 program (Phoenix Flow Systems, San Diego, CA, USA) after the exclusion of cell doublets. The results were expressed as percentages of the cell population. A significant proportion of apoptotic cells were lost due to the repeated washing procedures, so the apoptosis measured with flow cytometry was therefore underestimated.

Ca²⁺ measurements

For cytosolic calcium measurement, fluorescence measurements were conducted on fura 2 acetoxymethyl ester (fura 2/AM; 5 µM)-loaded (45 min) DU145 cells using a photomultiplier-based system (Photon Technologies, Princeton, NJ, USA) and a double-wavelength (340 and 380 nm) excitation protocol to quantify the absolute value of calcium concentration (Grynkiewicz *et al.* 1985). Direct quantification of Ca²⁺ within the endoplasmic reticulum (ER) [Ca²⁺]_{ER}, was conducted in DU145 cells loaded (45 min) with the AM-ester derivative of Mag-fura 2 (2 µM) as previously described (Humez *et al.* 2004b).

Western blotting

Western-blot analysis of protein expression was carried out as previously described (Humez *et al.* 2004b). Anti-NSE antibody was from Dako, Trappes, France. Anti-actin antibody was from Sigma, France.

Data analysis

Results were expressed as means ± S.E.M. except when indicated in the figure legend. Plots were produced using Origin 5.0 (Microcal Software Inc., Northampton, MA, USA). Each experiment was repeated at least three times. The Tukey–Kramer test was used for statistical comparison among means and differences and $P < 0.05$ was considered significant.

Results

EGF induces NED of DU145 cells

The effect of EGF on DU145 cell morphology was examined. When DU145 cells were cultured in a

medium containing 10 or 2% foetal calf serum (FCS), their growth plate, by forming colonies in which cells were rounded, was in close association and did not exhibit any processes (Fig. 1a and b). From time to time, isolated cells showing short processes and oval cell bodies, which look like differentiated cells, were observed and represented about 5% of the cells. Following treatment with EGF for 3 days (5 ng/ml), morphological changes appeared. Figure 1c shows that treatment of DU145 with EGF resulted in a significant level of morphological differentiation as shown by the appearance of neuritic-like extensions that often possessed growth cone-like structures. In addition, EGF-treated DU145 cells are individualized and did not form clusters, as is usually observed (Fig. 1a and b). These morphological changes were similar to descriptions of PC cells undergoing differentiation towards neuroendocrine phenotypes (Bang *et al.* 1994). The proportion of neuroendocrine-like cells observed after a 3-day EGF treatment was $35 \pm 4.7\%$ ($n = 3$).

To evaluate NED more objectively, expression of NSE was examined by western blotting. This marker has been used previously to assess the extent of differentiation in LNCaP cells (Mori *et al.* 1999, Vanoverberghe *et al.* 2004). As shown by Fig. 1d, NSE expression dramatically increased when cells were treated with EGF as compared with cells cultured with 2% FCS. In addition Fig. 1d also shows that NSE expression was higher when cells were cultured with 10% FCS rather than when they were cultured with 2% FCS. As depicted in Fig. 1f, morphological differentiation was time- and dose-dependent. The morphological differentiation induced by EGF (5 ng/ml), estimated by counting morphologically differentiated cells, increased with time and reached a plateau after 3 days of culture. Increasing the dose of EGF significantly increased the level of morphological differentiation in a dose-dependent manner that reached 63% of the cell population when 50 ng/ml EGF was applied. In addition, increasing doses of EGF also augmented NSE expression (Fig. 1g).

A direct correlation has previously been demonstrated between NED of LNCaP cells and inhibition of mitotic activity (Burchardt *et al.* 1999). We therefore used a cell-proliferation assay to determine the number of viable cells and to measure mitotic activity of DU145 cells treated with EGF. Relative to untreated cells, the mitotic activity of EGF-treated cells was significantly reduced with maximum mitotic activity reduction of about 20%, when cells were treated with EGF from 1.6 to 6.2 ng/ml (Fig. 1e). When EGF was used at higher concentrations (up to 12.5 ng/ml) it had no significant effect on cell growth. In addition,

Figure 1 EGF effects on cell morphology and on expression of NSE in human PC DU145 cells. Cells were cultured in media supplemented with 10% FCS. On the following day, the medium was replaced by a medium containing 2% serum supplemented with EGF. Cells were treated for 3 days in these conditions. The medium was renewed daily. (a–d) EGF induces morphological differentiation of DU145 cells. DU145 cells were maintained for 72 h in a medium containing (a) 10% FCS, (b) 2% FCS, (c) 2% FCS and 5 ng/ml EGF. (d) EGF increases the expression of the neuroendocrine marker, NSE. Semi-quantitative Western blot after 30 μ g per well of total protein extraction for the conditions presented in (a–c). Actin expression was used as a control to compare NSE expression between different experimental conditions. (e) EGF reduces cell growth. Cell growth measured after 3 days of treatment with EGF from 0.1 to 50 ng/ml compared with non-treated cells (control). Morphological differentiation: 10–30% (+); 30–40% (++); $\leq$ 40% (+++). Results are expressed as means $\pm$ s.d. (f) EGF induces morphological differentiation of DU145 cells in a time- and dose-dependent manner. Cells were cultured from 1 to 4 days with 5, 10, 20 or 50 ng/ml EGF. Morphological differentiation was determined after counting morphologically differentiated cells. (g) Semi-quantitative western blot after 30 μ g per well of total protein extraction for the conditions presented after 72 h incubation with increasing doses of EGF.

Figure 2 Morphological differentiation induced by EGF was reduced following inhibition of tyrosine kinase activity with genistein (gen) and following inhibition of EGF receptors with C56. (A) Cells were cultured in media supplemented with 10% FCS. On the following day, the medium was replaced by a medium containing (a) 2% serum supplemented with (b) 5 ng/ml, (c) 10 μ M genistein or (d) both. Cells were treated with for 3 days in these conditions. The medium was renewed daily. (B) Semi-quantitative Western blot after 30 μ g per well of total protein extraction for the conditions in (A). (C) Cells were cultured in media supplemented with 10% FCS. On the following day, the medium was replaced by a medium containing (a) 2% serum supplemented with (b) 5 ng/ml EGF, (c) 15 pM C56 or (d) both. Cells were treated with for 3 days in these conditions. The medium was renewed daily. (D) Semi-quantitative Western blot after 30 μ g per well of total protein extraction for the conditions presented from (C). Actin expression was assessed to compare NSE expression to that of actin between different experimental conditions.

treatment of DU145 cells with EGF from 0.8 to 50 ng/ml lead to morphological differentiation in a dose-dependent manner.

As depicted by Fig. 2A and B, the differentiation induced by EGF was blocked when cells were treated with genistein, a specific inhibitor of tyrosine autophosphorylation of the EGF receptor. When cells were cultured with EGF (5 ng/ml) and genistein (10 μ M), the rate of differentiation was 2-fold less than the rate of differentiation observed when cells were treated with EGF alone. In effect, the rate of differentiation was $31.4 \pm 4\%$ ($n = 6$) when cells were treated with EGF whereas it was 15.8 ± 5.3 ($n = 6$) when EGF was added with genistein. In addition, the NSE expression was reduced. The differentiation induced by EGF was also

blocked when cells were treated with C56, a specific inhibitor of the EGF receptor (Fig. 2C and D). When cells were cultured with EGF (5 ng/ml) and C56 (15 pM) the rate of differentiation was 50% less than that observed when cells were treated with EGF alone and NSE expression was inhibited.

Increase in cAMP level enhances EGF-induced NED of DU145 cells

It has been well described that cAMP-dependent protein kinase (PKA) plays a central role in the acquisition of NED observed in LNCaP cells (Burchardt *et al.* 1999, Cox *et al.* 2000, Mariot *et al.* 2002). It has also been clearly described that activation of cAMP-dependent

Figure 3 Increase in intracellular cAMP does not cause morphological differentiation but enhances morphological differentiation induced by EGF. Cells were cultured in media supplemented with 10% FCS. On the following day, the medium was replaced by a medium containing (a) 2% serum supplemented with (b) 5 ng/ml EGF, (c) 1 mM db-cAMP and 100 μ M IBMX or (d) db-cAMP, IBMX and EGF. Cells were treated for 3 days in these conditions. The medium was renewed daily. (e) Semi-quantitative western blot after 30 μ g per well of total protein extraction for the conditions in (a–e). Actin expression was assessed as a control to compare NSE expression between different experimental conditions.

protein kinase is both necessary and sufficient for stimulation of certain neuroendocrine characteristics by adenylate cyclase activators (Cox *et al.* 2000). Thus, we first tested the effect of increasing cAMP level on DU145 cell morphology (Fig. 3a–d). When DU145 cells were treated with dibutyl cAMP (db-cAMP; 1 mM) and isobutylmethylxanthine (IBMX; 100 μ M), only a few cells acquired a neuroendocrine morphology. In effect, the amount of cells that had a neuroendocrine phenotype was $5.0 \pm 3.0\%$ ($n = 6$) in the presence of agents that increase the cAMP level and was $2.0 \pm 3.2\%$

($n = 6$) in control conditions (Fig. 3a and c). However, treatment with db-cAMP and IBMX significantly increased the rate of morphologically observed NED induced by EGF (5 ng/ml). The rate of NED observed when cells were treated with EGF (5 ng/ml) was $31.0 \pm 3.1\%$ ($n = 6$) compared with $44.2 \pm 3.2\%$ ($n = 6$) when db-cAMP and IBMX were added to EGF (Fig. 3b and d). Interestingly, when DU145 cells were treated with db-cAMP (1 mM) and IBMX (100 μ M), NSE expression was significantly raised compared with untreated cells. Furthermore, the

addition of EGF on cells treated with db-AMP and IBMX was not able to increase NSE expression compared with that observed with db-cAMP and IBMX alone. These latter results indicate (i) that db-cAMP and IBMX promote NSE expression without achieving morphological differentiation, (ii) that EGF is able to enhance NED following db-cAMP and IBMX exposure and (iii) that EGF is able to promote NSE expression and morphological differentiation.

EGF-induced NED protects DU145 cells from the TG-induced apoptosis

As it has been established that NED protects LNCaP cells from apoptosis (Vanoverberghe *et al.* 2004), we went on to investigate the effect of EGF on apoptosis induced by calcium overload. For this we used TG, which causes a strong calcium increase, and induces apoptosis in DU145 cells (He *et al.* 2002). Our experiments show that 72-h pretreatment with 5 ng/ml EGF before a 48-h treatment with 1 μ M TG reduced the level of TG-induced apoptosis (assessed by Hoechst staining) by 50%. Indeed, in these conditions, the apoptosis level of neuroendocrine-differentiated cells was 6% whereas it reached 12% when cells were kept in a non-differentiated state (Fig. 4A). The resistance of apoptosis conferred by NED was also detected by flow cytometry (Fig. 4B).

We then investigated whether EGF-induced NED was able to affect the TG-induced calcium overload. Our experiments demonstrate that NED stably reduced the basal intracellular calcium concentration ($[Ca^{2+}]_i$) measured after a 48 h TG exposure (Fig. 4C). Indeed, when cells were exposed to TG for 48 h, $[Ca^{2+}]_i$ reached 206 ± 12 nM ($n = 113$). However, in neuroendocrine-differentiated cells $[Ca^{2+}]_i$ rose to 88 ± 3 nM ($n = 73$; Fig. 4C). In addition, as depicted in Fig. 4D the protection from the calcium overload induced by NED was abolished when the EGF receptors were blocked with C56 (15 pM).

Mechanisms of Ca^{2+} homeostasis involved in apoptosis induction in androgen-independent PC DU145 cells

To understand the mechanisms that lead EGF to induce a protective effect from calcium-induced apoptosis we studied the spatiotemporal calcium signalling leading to apoptosis in DU145 cells. With this aim, we treated cells with 1 μ M TG in various extracellular conditions (Fig. 5). Sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase inhibition by TG generally results in a biphasic increase in $[Ca^{2+}]_i$, reflecting a passive Ca^{2+} release through endoplasmic reticulum leak channels,

followed by a capacitative Ca^{2+} entry through plasma membrane Ca^{2+} channels activated by store depletion. In a Ca^{2+} -free medium, a 48-h treatment by TG (1 μ M) induced only a short, transient increase in $[Ca^{2+}]_i$ (at the time of TG application) due to Ca^{2+} store mobilization (see trace in Fig. 5a). Such a treatment induces apoptosis in 5.2% of cells in 48 h. In cells treated with the Ca^{2+} chelator 50 μ M BAPTA-AM (bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)) in a Ca^{2+} -free medium (Fig. 5b), TG induced the emptying of Ca^{2+} stores, but without any increase in free $[Ca^{2+}]_i$. In these conditions, TG also induced apoptosis in 5% of cells. Note that a 48-h treatment in a Ca^{2+} -free medium supplemented with 50 μ M BAPTA-AM alone (without TG) did not induce apoptosis (data not shown). Thus, independently of the cytosolic Ca^{2+} level, the mobilization of Ca^{2+} stores induced apoptosis in $5 \pm 1\%$ ($n = 3$) of DU145 cells.

In order to evaluate the role of cytosolic increase in Ca^{2+} in the induction of apoptosis in these cells, apoptosis was measured after treating DU145 cells in a medium which contained 2 mM Ca^{2+} . Indeed, readmitting 2 mM Ca^{2+} into the extracellular medium after the transient increase in Ca^{2+} -free medium caused by TG (1 μ M), induced capacitative calcium entry (see trace in Fig. 5c), causing in sustained increase in $[Ca^{2+}]_i$ (see the table in Fig. 5c, after 24 and 48 h of treatment). In these conditions, when Ca^{2+} is stably raised for 48 h, apoptosis is induced in 10.5% of cells. Thus, even if by emptying the Ca^{2+} stores TG induces apoptosis in 5% of cells, our results clearly show that when TG induces a sustained increase in $[Ca^{2+}]_i$ it doubles the apoptosis level, pointing to capacitative Ca^{2+} entry as one of the Ca^{2+} events in apoptosis induction by TG in DU145 cells.

To further investigate the role of capacitative Ca^{2+} entry in apoptosis induction in DU145 cells, we studied the role of the amplitude of the capacitative Ca^{2+} influx in this phenomenon. For this, we lowered the capacitative calcium entry by readmission of 200 μ M Ca^{2+} into the extracellular medium (instead of 2 mM) after TG treatment made in a calcium-free medium (see Fig. 5d with 200 μ M Ca^{2+} , as compared with Fig. 5c with 2 mM Ca^{2+}). A decrease in the amplitude of the sustained (24 and 48 h) Ca^{2+} increase was also observed (see the table in Fig. 5d as compared with the table in Fig. 5c). This treatment for 48 h induced apoptosis in 6.8% of cells. As reducing the extracellular calcium concentration reduces TG-induced apoptosis by about 30%, it is clear that in androgen-independent DU145 PC cells the level of apoptosis induced by TG depends on the amplitude of the Ca^{2+} increase due to the capacitative Ca^{2+} influx.

Figure 4 EGF protects DU145 cells from the TG-induced apoptosis and from the long-term cytotoxic Ca^{2+} overload induced by TG. Cells were pretreated with 5 ng/ml EGF for 72 h prior the 48 h 1 μM TG treatment (combined with EGF). (a) Apoptosis was labelled using the Hoechst staining method, as indicated in the Experimental procedures section. (b) Apoptosis was measured using flow cytometry. Results represent the relative level of apoptosis. Apoptosis observed in control (CTL) conditions is labelled as 1. (c) $[\text{Ca}^{2+}]_i$ was measured after a 48-h TG (1 μM) treatment in a culture medium containing 2 mM Ca^{2+} . Experiments were performed on control cells cultured with 2% FCS and on cells treated by 5 ng/ml EGF 48 h before the TG treatment. Note that EGF was maintained during the TG treatment. (d) $[\text{Ca}^{2+}]_i$ was measured after a 48-h TG (1 μM) treatment in a culture medium containing 2 mM Ca^{2+} . Experiments were performed with C56 (15 pM) on control cells cultured with 2% FCS, and on cells treated with 5 ng/ml EGF 48 h before TG treatment. Note that EGF was maintained during TG treatment.

Finally, we studied the role of the duration of the capacitative Ca^{2+} entry in apoptosis induction in DU145 cells. As shown in Fig. 5c, readmission of 2 mM Ca^{2+} into extracellular media after the transient increase caused by TG in a Ca^{2+} -free medium induced a sustained (see Fig. 5c) capacitive Ca^{2+} entry and apoptosis in 10% of cells within 48 h. By removing extracellular Ca^{2+} at a specific time after the beginning of the capacitative Ca^{2+} influx (represented by T on

Fig. 5e), Ca^{2+} returned to its basal level (see Fig. 5e). In these conditions, by labelling the apoptosis level 48 h after the return to basal conditions, the effect of the duration of capacitative Ca^{2+} influx was studied. The table on Fig. 5e shows that whereas a 5- or 30-min capacitative Ca^{2+} influx is insufficient to increase apoptosis, as compared with calcium store mobilization alone after 48 h (see Fig. 5a and b), a 2-h Ca^{2+} influx induces apoptosis in 9.7% of cells after 48 h.

Figure 5 Characterization of the role of Ca^{2+} homeostasis perturbation in apoptosis induction in DU145 cells. Cells were cultured in media supplemented with 10% FCS. On the following day the medium was replaced by medium containing 2% serum supplemented with the indicated doses of Ca^{2+} and BAPTA-AM. Cells were treated with $1 \mu\text{M}$ TG for 48 h in these conditions. Traces show the short-lived modifications in $[\text{Ca}^{2+}]_i$ induced by TG treatment, whereas the tables illustrate the long-lasting (24 and 48 h) effects of these treatments on $[\text{Ca}^{2+}]_i$, as well as apoptosis level at 48 h. (a, b) Role of Ca^{2+} -store mobilization in TG-induced apoptosis: (a) Effects of TG application in a Ca^{2+} -free medium, on $[\text{Ca}^{2+}]_i$ and apoptosis: in a Ca^{2+} -free medium a 48-h TG treatment induced only a short-lived increase in $[\text{Ca}^{2+}]_i$ due to Ca^{2+} -store mobilization without any sustained modification of $[\text{Ca}^{2+}]_i$ (see table). (b) Role of transient $[\text{Ca}^{2+}]_i$ increase due to Ca^{2+} -store mobilization in TG-induced apoptosis: the trace shows the effect of TG application in a Ca^{2+} -free medium supplemented with BAPTA-AM ($50 \mu\text{M}$). In these conditions the emptying of the stores induced by TG did not modify $[\text{Ca}^{2+}]_i$. However, after 48 h apoptosis was induced at a similar level to that in (a). (c–e) Role of capacitative calcium entry in TG-induced apoptosis. (c) Effect of capacitative calcium entry induced by TG on $[\text{Ca}^{2+}]_i$ and apoptosis: the emptying of the store induced by TG activated a sustained capacitative calcium influx measurable by readmitting 2 mM extracellular calcium. Apoptosis was measured after a 48-h treatment in these conditions, and the apoptosis level increased as compared with cells exposed to calcium-store mobilization alone (a and b). (d) Role of the amplitude of capacitative calcium entry in TG-induced apoptosis: by reducing the extracellular calcium concentration to $200 \mu\text{M}$, both the amplitude of the sustained capacitative calcium influx and apoptosis level induced by TG were reduced (as compared with c, in 2 mM extracellular Ca^{2+}). (e) Role of the duration of the capacitative calcium influx in apoptosis induction: by removing extracellular calcium at a determined time after the beginning of the capacitative calcium influx (represented by T), calcium stably returned to the basal level. The role of the duration of capacitative calcium influx was studied by labelling apoptosis level in these conditions, 48 h after the return in basal cytosolic calcium concentrations.

Figure 6 EGF reduced the amount and the speed of the calcium that could be released from internal stores. (a) TG was used to study the amount of Ca^{2+} that is releasable from the stores, as well as the corresponding capacitative Ca^{2+} entry. Experiments were performed on control cells ({OT}), as well as on cells pretreated for 48 h with 5 ng/ml EGF ({OC}). Insert: Typical traces showing decrease in the speed of cytosolic calcium. Calcium increase was obtained in a calcium-free medium using TG. (b) Ionomycin (1 μ M) was used to study the amount of Ca^{2+} releasable from the stores in a calcium-free medium. (c) ATP (100 μ M) was used to study the amount of Ca^{2+} that is releasable from the stores in a calcium-free medium. (d) Quantification of $[Ca^{2+}]_{ER}$ measured in digitonin-permeabilized cells based on Mag-fura 2 fluorescence.

Moreover, a 6-h Ca^{2+} influx is sufficient to induce maximal apoptosis levels after 48 h (as compared with the apoptosis induced by the 48-h sustained capacitative Ca^{2+} influx shown in Fig. 5c). Finally we showed that NED by itself is not linked to a variation in the basal cytoplasmic calcium since the amount of the resting cytoplasmic calcium measured in non-treated cells was not significantly modified following NED and was 54 ± 9 nM ($n = 85$).

Taken together, these results show that in androgen-independent DU145 PC cells the depletion of intracellular Ca^{2+} store may induce apoptosis, and that apoptosis is increased in cells where capacitative Ca^{2+} entry is induced, pointing to capacitative Ca^{2+} influx as one of the events in the phenomenon of apoptosis induction. Moreover, our results provide the first evidence that in androgen-independent PC cells the

amplitude and the duration of capacitative Ca^{2+} entry are two parameters that control apoptosis.

NED of androgen-independent prostatic cells reduces the Ca^{2+} released from intracellular pools and the following capacitative Ca^{2+} entry

As our results demonstrated how the Ca^{2+} signal induces apoptosis in DU145 cells, we investigated the effect of NED on DU145 Ca^{2+} homeostasis. ER Ca^{2+} release was thus estimated by measuring the difference between basal $[Ca^{2+}]_i$ and Ca^{2+} peaks induced by TG in a Ca^{2+} -free medium. Our results show that NED is associated with a reduction in the amount of Ca^{2+} that can be released from the ER (Fig. 6). Indeed, in control conditions, the release induced by 1 μ M TG application

reached 210 ± 5 nM ($n = 54$), whereas this release reached 159 ± 10 nM ($n = 32$) in neuroendocrine-differentiated cells, corresponding to 76% of the amount released in control conditions (Fig. 6a). The speed of the calcium release induced by TG also decreased. In effect, calcium release induced by TG was 0.12 ± 0.01 nM/s ($n = 90$) when cells were differentiated with EGF while it was 0.53 ± 0.08 nM/s ($n = 90$) in control conditions.

To confirm the reduction in the amount of calcium that can be released from internal stores we measured the calcium release induced by ionomycin (1 μ M) or by activating purinergic receptors with ATP (100 μ M) as already described in DU145 cells (Fang *et al.* 1992). As depicted in Fig. 6b and c, the calcium that can be released by ionomycin or by ATP was reduced in neuroendocrine-differentiated cells. Indeed the calcium release was reduced by 49 and 41% when cells were challenged with ionomycin and ATP respectively. In comparison the level of free $[Ca^{2+}]$ in the reticulum and measured with Mag-fura 2 was not modified after NED (Fig. 6d).

Our results also show that NED affects the capacitative Ca^{2+} entry observed after readmitting extracellular Ca^{2+} . Indeed, the amplitude of capacitative Ca^{2+} entry induced by 1 μ M TG application in control conditions reached 477 ± 12 nM ($n = 54$), whereas it reached 235 ± 22 nM ($n = 32$) in neuroendocrine-differentiated cells. Thus, NED is correlated with a reduction of the amplitude of Ca^{2+} entry and the amplitude of entry corresponded to 49% of the control Ca^{2+} entry.

The results presented in this study demonstrate for the first time the ability of androgen-independent cells to undergo NED following a growth factor treatment. In addition, we demonstrate the role of the calcium-signal amplitude and duration in the induction of apoptosis in androgen-independent cell line. Furthermore, we show that a depression in calcium homeostasis confers apoptosis resistance on the neuroendocrine-differentiated DU145 cells.

Discussion

EGF treatment induced NED in androgen-independent prostate cancerous cell line

To date, the mechanism leading to the development of the neuroendocrine cell population in advanced tumours is poorly understood. It has been described that androgen-sensitive cell lines have the ability to transdifferentiate into cells with a neuroendocrine-like phenotype. It has been also demonstrated that

neuroendocrine cells lack nuclear androgen receptor and thus represent an androgen-insensitive cell phenotype in the prostate (Krijnen *et al.* 1993). This absence of androgen receptor in neuroendocrine tumour cells suggests that these cells may represent a population that could initially be androgen-independent and therefore refractory to androgen-deprivation therapy. Here we show, for the first time, that androgen-independent DU145 cells have the ability to transdifferentiate. After this treatment, differentiated DU145 exhibit a significant level of morphological changes that were similar to descriptions in the literature of PC cells undergoing differentiation towards neuroendocrine phenotypes (Bang *et al.* 1994). Furthermore, we show that expression of NSE increases when cells are treated with EGF. Increase in NSE expression is a marker that has been used previously to assess differentiation of LNCaP cells (Mori *et al.* 1999). Taken together our results demonstrate that EGF has the ability to induce NED of DU145 cells.

It has been previously established that NED of prostate cells, including androgen-independent ones, can be induced by agents that increase cAMP (Zelivianski *et al.* 2001). Here, we show that these agents (db-cAMP and IBMX) were ineffective by themselves to promote NED of androgen-independent DU145 cells. However, db-cAMP and IBMX increased the efficiency of EGF in inducing NED.

The NED process has itself been suggested to induce cell-cycle arrest (Mori *et al.* 1999, Blutt *et al.* 1997). Here we show that NED induced by EGF (between 1.6 and 6.2 ng/ml) is correlated with a reduction in cell proliferation. This reduction of the proliferation rate was moderate (20%). This decrease in cell growth observed with EGF is surprising, since exogenous EGF has already been described as having little effect on cell growth when they are cultured at high densities, because the amount of EGF being secreted by DU145 is sufficient for optimal stimulation by EGF (Connolly & Rose 1991). In this study one may consider the fact that the additional doses of EGF considerably increase the level of EGF in our culture conditions at a doses rendering EGF able to reduce cell growth.

It has been demonstrated that the micro-environment is likely to play a critical role in growth and to control phenotypic properties of prostatic tumours (Cunha 1994, Chung 1995). Here we show that NED of androgen-independent cells is specifically induced by EGF, which is a growth factor that has been frequently been implicated in PC progression (Barton *et al.* 2001). This function of EGF in PC cells was unexpected. Such an

effect of EGF is mediated by EGF activation of a tyrosine kinase pathway since this was not observed when genistein was used. Activation of EGFR by heparin-binding EGF-like growth factor has been demonstrated to stimulate NED in LNCaP cells linked to STAT3 phosphorylation (Kim *et al.* 2002). Nevertheless, to date no effect of EGF on NED in LNCaP cells has been reported.

In this study, we identify an unrecognized function for EGF in androgen-independent DU145 cells, as a mediator of NED. We suggest that this growth factor could be a key signal linked to NED in androgen-independent prostate cell.

NED induced by EGF protects DU145 cells from the TG-induced apoptosis

Our data show that TG induces apoptosis in about 10% of androgen-independent DU145 PC cells. This result is in accordance with studies demonstrating that an increase in $[Ca^{2+}]_i$ induced by TG induced apoptosis (Orrenius *et al.* 2003). Nevertheless, levels of apoptosis observed in our study are significantly lower than the ones previously observed in the same cell type by other authors (He *et al.* 2002). However, this low apoptosis level, observed in our experimental conditions, reflects the fact that androgen-independent cells are resistant to apoptosis when compared with androgen-dependent ones (Prevarskaia *et al.* 2004). Our experiments also show that pre-treatment with EGF before a 48 h treatment by 1 μ M TG reduces the level of the TG-induced apoptosis by 48%. Indeed, we demonstrate that NED of androgen-independent cells is a mechanism by which prostate cells can escape apoptosis. Our results are in accordance with those describing how neuroendocrine tumour cells escape apoptotic cell death (Fixemer *et al.* 2002, Vanoverberghe *et al.* 2004) and that EGF protects several cell types, including prostate cells, from apoptosis (Mimeault *et al.* 2003).

Calcium signalling and induction of apoptosis in DU145 cells

To understand how the NED of androgen-independent human PC cells protects them from apoptosis due to calcium overload we first investigated the role of Ca^{2+} homeostasis in the apoptotic regulation of DU145 cell. At that time, two hypotheses were proposed about the role of Ca^{2+} in apoptosis induction. The first assumed that apoptosis may be triggered by ER Ca^{2+} -pool depletion without any requirement for cytosolic Ca^{2+} elevation due to store-operated Ca^{2+} entry (Bian *et al.* 1997, He *et al.* 1997). The second hypothesis, on the

contrary, assumed that a sustained elevation of cytosolic Ca^{2+} to a critical level is the initiator of apoptosis (Dowd *et al.* 1992, Wang *et al.* 1999). In prostate cells, these two opposing hypotheses have already been reported (Prevarskaia *et al.* 2004). Indeed, studies performed in our laboratory demonstrate that in human androgen-dependent LNCaP PC cells the depletion of intracellular Ca^{2+} stores may trigger apoptosis without any requirement for a sustained Ca^{2+} entry (Skryma *et al.* 2000). On the other hand, Ca^{2+} pool depletion appears insufficient to initiate apoptosis in rat androgen-independent PC cells. Indeed, Furuya *et al.* (1994) reported that in rat androgen-independent PC AT3 cells programmed cell death is critically dependent on an adequate (i.e. 3-4 fold) sustained (>1 h) elevation in $[Ca^{2+}]_i$ and not on depletion of the ER Ca^{2+} pools.

We show that in DU145 cells apoptosis can be induced by ER Ca^{2+} pool depletion alone, without any requirement for a cytosolic $[Ca^{2+}]$ increase. Furthermore, our data clearly demonstrate that when Ca^{2+} depletion is followed by a Ca^{2+} entry from extracellular medium apoptosis is amplified. Our results further demonstrate that the level of apoptosis is proportional to the level and the duration of the Ca^{2+} increase. Thus the amplitude and kinetics of the Ca^{2+} signal appear to be crucial regulators of apoptosis in DU145 PC cells. This result is of a great interest since it points out a fundamental difference between Ca^{2+} regulation of apoptosis in two major PC cell lines: androgen-dependent LNCaP PC cells and androgen-independent DU145 PC cells. Indeed, in our previous study, we showed that in an androgen-dependent PC cell line a decrease in the ER Ca^{2+} pool is a major factor in apoptosis induction, and that the following Ca^{2+} influx is not needed for the apoptosis phenomenon to occur (Skryma *et al.* 2000). Our results show that independently of the Ca^{2+} level the depletion of Ca^{2+} stores may induce apoptosis in DU145 cells. This result is not surprising, since the maintenance of sufficient levels of Ca^{2+} within the ER is essential not only for Ca^{2+} signal transduction, but also for protein synthesis and processing, and cell proliferation (Koch 1990, Sambrook 1990, Jiang & Julian 1998). Moreover, mechanisms by which ER depletion might contribute to apoptosis without any $[Ca^{2+}]_i$ increase have been reported by He *et al.* (2002): (i) depletion of the ER Ca^{2+} pools might destabilize the Ca^{2+} -protein gel and its associated membrane, leading to vesiculation and the formation of apoptotic blebs, (ii) disruption of protein processing and transport within the ER may contribute to TG-induced apoptosis and (iii) TG-induced ER Ca^{2+} pools

depletion releases an endonuclease into the nucleus responsible for DNA fragmentation. Moreover, alternative mechanisms include the activation of caspase 12 which can be directly stimulated by depletion of the ER Ca^{2+} pool (McConkey & Nutt 2001).

Our results are the first which describe calcium-dependent apoptosis in androgen-independent PC cells. In effect, it has been recently demonstrated the TSU-PR1 cell line originally described as human androgen-independent PC cells and requiring Ca^{2+} entry for apoptosis in fact derives from T24 bladder carcinoma cells (van Bokhoven *et al.* 2001).

Apoptosis resistance of neuroendocrine-differentiated DU145 cells is conferred by modifications in the calcium homeostasis

Since we demonstrated the involvement of Ca^{2+} homeostasis perturbation in apoptosis induction in DU145 cells, we investigated the effect of NED on Ca^{2+} homeostasis. Our results failed to demonstrate any effect of NED on basal cytosolic $[\text{Ca}^{2+}]$ or on the level of endoplasmic free calcium. By contrast, a reduction in the amount of calcium that can be released from the ER was observed following NED as well as capacitative calcium entry. This result clearly explains how neuroendocrine-differentiated cells become resistant to apoptosis, since we demonstrated that in DU145 cells the apoptosis level is proportionally dependent on the amplitude of Ca^{2+} increase induced by apoptotic signal. Thus the reduction of both the amount of Ca^{2+} that can be released from the stores and the subsequent Ca^{2+} entry into neuroendocrine-differentiated cells can account for the apoptosis resistance of neuroendocrine-differentiated cells. Such a prevention of apoptosis by a control calcium homeostasis has already been reported in mesangial cells, where a sustained increase in the $[\text{Ca}^{2+}]_i$ that triggers apoptosis was thwarted by platelet-derived growth factor (Saleh *et al.* 2000). More recently, reduction of the expression of inositol 1,4,5-trisphosphate receptor type 1 in cisplatin-resistant cells has also been demonstrated, pointing out that regulation of the calcium signalplex is important to cancer progression and resistance to treatments (Tsunoda *et al.* 2005). In addition, a reduction in the calcium signalling (i.e reduction of the calcium release and the calcium influx) has also been described as the mechanism leading to apoptosis resistance of PC cells (Prevorskaya *et al.* 2004, Vanoverberghe *et al.* 2004). In effect, apoptosis resistance conferred by NED in LNCaP cells was accompanied by a reduction in both calcium release and calcium entry (Vanoverberghe

et al. 2004). Thus, the reduction in the calcium signal caused by neuroendocrine-differentiated PC cells is a common mechanism that leads to the apoptosis resistance of PC cells besides an increase in survivin and clusterin (Xing *et al.* 2001, July *et al.* 2002).

These results highlight a new emergence path for neuroendocrine cells in prostate malignancy by demonstrating the neuroendocrine differentiating effect of EGF. Furthermore, we describe the reduction in the calcium signalling as being a common way inducing apoptosis resistance in neuroendocrine-differentiated PC cells. Our results suggest that targeting EGF and its receptor as well as calcium homeostasis in an attempt to enhance the proapoptotic potential of malignant cells may be a useful strategy in the treatment of advanced PC.

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