Histol Histopathol (2004) 19: 951-961

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Neuropeptides, apoptosis and ion changes in prostate cancer. Methods of study and recent developments

J. Vilches[1], M. Salido[1], E. Fernández-Segura[2] and G.M. Roomans[3]

[1]Department of Cell Biology, School of Medicine, University of Cadiz, Spain, [2]Department of Histology, School of Medicine, University of Granada, Spain and [3]Department of Medical Cell Biology. Biomedical Center, Uppsala University. Sweden

Summary. It has been suggested that neuroendocrine (NE) cells provide paracrine stimuli for the propagation of local carcinoma cells and that NE differentiation is associated with the progression of prostate cancer toward an androgen-independent state. Apoptosis comprises a critical intracellular defense mechanism against tumorigenic growth and is associated with a number of changes in the elemental content of the cell. The neuropeptides bombesin and calcitonin, which inhibit etoposide-induced apoptosis, also inhibit the etoposide-induced elemental changes in prostate carcinoma cells. This important fact strengthens the link between apoptosis and changes in the intracellular elemental content. This protective effect on etoposide-induced apoptosis appears to be quite similar in androgen-dependent and androgen-independent cell lines. This confirms that neuropeptides confer antiapoptotic capabilities on non-neuroendocrine cells in close proximity to neuroendocrine cells. It can therefore be speculated that certain neuroendocrine peptides can increase the survival and further growth of neighboring cells and may thereby contribute to the aggressive clinical course of prostate tumors containing neuroendocrine elements. In addition, this correlation provides an objective basis for the study of neuropeptide target points and may be helpful for alternative therapeutic protocols using neuropeptide inhibitors in the treatment of patients with advanced prostatic carcinoma. The culture techniques described were, thus, designed in order to achieve two important goals. First, the development of an in vitro model that allows an approach to neuroendocrine differentiation in prostate cancer and its role in apoptosis blockage. Second, the method has been designed in order to permit rapid cryofixation of intact cell monolayers for subsequent x-ray microanalysis.

Offprint requests to: José Vilches, Department of Cell Biology, School of Medicine. University of Cadiz, Plaza Falla 9, 11003 Cádiz, Spain. e-mail: jose.vilches@uca.es

Key words: Prostate cancer, X-ray microanalysis, Ions, Apoptosis, Neuropeptides

Introduction

Prostate cancer is a frequently diagnosed condition in the aging population and the incidence of this disease is expected to increase in the years to come. More than 80% of prostate carcinomas initially respond to androgen ablation, but most relapse, due to the heterogeneous presence of androgen-dependent and androgen-independent clones. The pathways of cellular proliferation and apoptosis are inexorably linked to minimize the occurrence of neoplasia, and dysfunction of apoptosis is proposed as a pathogenic process in malignant tumors. Androgen-dependent prostatic cancer cells undergo apoptosis after androgen deprivation, but androgen-independent tumors do not due to a defect in the initiation step. However, they retain the basic cellular machinery for undergoing apoptosis (Salido et al., 1996, 1999) A possible role for neuroendocrine differentiation in the onset and regulation of apoptosis in prostatic neoplasia is suggested.

Neuroendocrine differentiation in prostatic malignancy has been associated with poor prognosis, tumor progression and androgen-independence of the tumor (di Sant'Agnese,1998; Abrahamsson, 1999; Hansson and Abrahamson, 2001). It has been shown that bombesin and calcitonin can stimulate growth of human prostate cancer cell lines (Bologna et al., 1989; Shah et al., 1994; Ritchie et al., 1997). It has been suggested that bombesin antagonists could be important in the treatment of advanced prostate cancer, especially after relapse when both clonal selection and adaptive mechanisms appear in heterogeneous tumors composed of cells that respond differently to androgen ablation, acquiring androgen independence (Pinski et al., 1993a,b, 1994; Jungwirth et al., 1997).

The mechanism by which bombesin stimulates growth of prostate cancer cells is still a matter of debate,

and is probably complex. Bombesin appears to affect various signaling pathways, involving Ca^{2+} ions (Han et al., 1997; Wasilenko et al., 1997), cAMP (Jongsma et al., 2000), and tyrosine kinases such as Src (Allard et al., 2000). It has also been proposed that bombesin can activate extracellular proteolytic activity and in that way contribute to metastatic spread (Festuccia et al., 1998) . Recently, we have shown that bombesin and calcitonin inhibit etoposide-induced apoptosis in human androgen-independent prostatic cancer cell lines (Salido et al., 2000), and these neuropeptides could thus disrupt the balance between cell death and cell growth in the tumor. This is an important point for the knowledge of the relationship between neuroendocrine differentiation and prognosis, because according to the kinetics of tumor growth, an increase in a neoplastic cell population is the result of imbalance between the two processes controlling tissue homeostasis: cell proliferation and cell death. Apoptosis, therefore, comprises a critical intrinsic cellular defense mechanism against tumorigenic growth which, when suppressed, may contribute to malignant development.

Apoptosis is associated with specific changes in cell morphology and cellular macromolecules, but is also accompanied by changes in ion distribution and membrane potential. In particular, apoptosis is associated with a loss of K^+ ions from the cell (Bortner et al., 1997; Dallaporta et al., 1998, 1999). The loss of K^+ accounts for the changes in cell volume that are universally associated with apoptosis and, interestingly, may actually activate enzymes in the apoptotic cascade and promote DNA degradation (Hughes and Cidlowski, 1999; Montague et al., 1999). In addition, there are indications that K^+ loss is not just a consequence of apoptosis, but that this in itself may promote apoptosis, since caspases and nucleases are inhibited by high intracellular K^+, and inhibition of K^+ efflux results in inhibition of apoptosis. (Hughes et al., 1997). Indeed, recent studies have coined the term apoptotic volume decrease (AVD) for the early-phase cell shrinkage associated with activation of K^+ and Cl^- channels (Barros et al., 2001), and have shown that AVD induction precedes and is a prerequisite for cytochrome-c release, caspase-3 activation, DNA laddering and ultrastructural alterations (Maeno et al., 2000)

Neuroendocrine secretory products and their interactions with epithelial prostate cells are currently under investigation in order to understand their significance in the pathogenesis of the prostate gland, prognosis and therapy (Zelivianski et al., 2001) Calcitonin (CT), one of these neuropeptides, is reported to be associated with the growth of prostate cancer (Craft et al., 1999; Isaacs, 1999; Chien et al., 2001) Bombesin acts as a survival and migratory factor for androgen-independent prostate cancers, may influence growth, invasiveness, metastatic processes and angiogenesis in prostatic carcinoma (Pilat et al., 1999) and can induce LNCaP growth in the absence of androgens.

In an effort to understand the role that neuroendocrine differentiation plays in the progression and metastatic spread of prostate carcinoma, the electron probe X-ray microanalysis is unique because it combines the ability to undertake chemical analysis with the high resolution of the electron microscope, and thus is capable of correlating chemical information with known ultrastructural details (Salido et al., 2001). The development of in vitro models for an adequate approach to neuroendocrine differentiation of prostatic carcinoma and its implications on this disease is imperative. The Transmission and Scanning Electron Microscopy and X-ray microanalysis study of ionic changes that occur during etoposide-induced apoptosis in prostate cancer cell lines described in this review shows how a combined treatment with etoposide and the neuropeptides bombesin and calcitonin inhibits etoposide-induced apoptosis in these cells, and also inhibits the major changes in intracellular ion concentrations in androgen-independent and in androgen-dependent prostate cancer cell lines. Much work is required to clarify the interactions between epithelial and neuroendocrine cells in hormone-dependent microenvironments, such as prostate cancer, and a better comprehension of the mechanisms of tumor progression are, therefore, of paramount importance.

Ionic changes related to apoptosis

Seven of the most relevant elements involved in cell viability and cell death can be simultaneously quantified under the same analytical conditions as described below. Healthy cells have a characteristic elemental pattern of viable cells, with low Na and high K, Mg and P and we will thus try to identify the elemental alterations that appear in neoplastic cells and the role of neuropeptides in cultured neoplastic prostate cancer cells. Our research interest will focus mainly on those parameters that indicate cell viability (Na/K ratio) and those that reflect alterations in the nucleus to cytoplasm ratio, as a result of changes in cell volume and nuclear changes which are described as morphological hallmarks of apoptosis (P/S ratio, Cl, Na and K concentrations).

The difference in concentrations of Na^+ and K^+ over the cell membrane is crucial for the maintenance of physiological intracellular gradients. The process requires adequate energy supply, an intact cell membrane and specific enzymatic activities. When the Na^+/K^+ pump in the cell membrane cannot maintain the ionic gradients, in the absence of a membrane potential, calcium ions flow through the voltage-dependent ion channels. Thus, the increase in Na/K ratio reflects the physiopathological conditions of the cell, and can be used as a very sensitive measure of various kinds of cell injury, and can be detected by X-ray microanalysis in cells and tissues undergoing programmed cell death. (Bowen et al., 1988; Sandstrom et al., 1994; Warley, 1997; Berger and Garnier, 1999; Ingram et al., 1999; Skepper et al., 1999). Voltage-dependent K^+ channels

have been described in most mammalian cells and their activation results in efflux of K^+ with a net decrease of intracellular K^+, as well as an imbalance in other ions such as Na^+ and Mg^{++} (Fernandez-Segura et al., 1999; Fujii et al., 1999; Skepper et al., 1999). Some studies have also pointed to the fact that K^+ channel function may be also required for tumor cell surface vesicle formation and shedding, changes in membrane permeability and nuclear fragmentation, i.e., the events leading to what we know by the term "programmed cell death" (Smith et al, 1998).

Recent studies have coined the term apoptotic volume decrease, AVD, for the early-phase cell shrinkage associated with activation of K^+ and Cl^- channels in apoptosis (Barros et al., 2001), and have shown that AVD induction precedes and is a prerequisite for cytochrome-c release, caspase-3 activation, DNA laddering and ultrastructural alterations (Maeno et al., 2000). Cells maintain and regulate their volume by controlling intracellular solute concentrations and water flux across the cell membrane. The channel activation leads to a significant decrease in K content, and a significant increase in Na. The variation in cellular volume is evaluated using the sum of the Na and K content. In addition, a tendency to higher P can be noted. Hence, the increase in P/S ratio may be explained as a relative increase in P-containing macromolecules (mainly nucleotides) relative to S-containing macromolecules (mainly proteins).

One of the most frequently studied events in apoptosis is the activation of the endonucleases responsible for DNA fragmentation that occurs as part of the apoptotic process. Apoptosis is characterized by DNA digestion mediated by either a Ca^{2+}/Mg^{2+}-dependent endonuclease or the acid- activated deoxyribonuclease II (DNase II), pH dependent, or both X-ray microanalysis detects total Ca (in the millimolar range) whereas in the initial stages of apoptosis changes in free Ca^{2+} (in the nanomolar to micromolar range) are relevant. These cannot be measured by X-ray microanalysis. However, later in the apoptotic process also significant changes in total Ca also occur, as described by us and others (Sandstrom et al., 1994; Warley, 1997; Gutierrez et al., 1999).

Approaches for the assessment of ionic changes

Cell culture

Culture protocols and treatment administration: Androgen-independent cells PC-3, Du 145 (ATCC, Manassas USA) grown in Dulbecco Modified Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 4% penicillin-streptomycin and 0.4% gentamycin were kept under standard conditions, in a water saturated atmosphere of 5% CO_2 until the experiment started. LNCaP androgen-dependent cells (ATCC, Manassas USA) were grown in RPMI 1640 under the same conditions. All experiments started with unsynchronized exponentially growing cells, and the culture medium was changed to 5% FBS supplemented medium.

Androgen-independent cells

Cells were seeded in microplates at a density of 200.0000 cells/well under the conditions described above and 48 hours later exposed to different treatment protocols.

Androgen-dependent cells

A second protocol including hormonal deprivation was used. 200,000 cells/well were seeded in microplates in RPMI-1640 under the conditions described above and 72 hours later changed to RPMI containing 5% Steroid Free Serum (SFS) and 48 hours later exposed to the different treatment protocols.

Treatment protocols

Cells were exposed to etoposide-added from a 2mM stock solution in DMSO-doses 80 μM, (Du 145) and 150 μM (PC-3, LNCaP) for 48 h. Inhibition of etoposide-induced apoptosis: neuropeptides and androgen exposure. Cells were exposed to combined treatments with etoposide (as described above) and bombesin (1 nM) or calcitonin (500 pg/ml). In LNCaP a combined group with dihydrotestosterone (DHT) (1 nM) was added. Control groups: A control group cultured in the standard medium during the experiment was used in all experiments. Positive controls were treated with bombesin (1 nM), calcitonin (500 pg/ml) and DHT (1 nM) in LNCaP for 48 h .

Characterization of apoptosis with ligh and fluorescence microscopy

Direct examination by phase contrast microscopy was performed once the experiment started. For microscopical quantification of apoptotic cells, cytospin preparations obtained from in vitro cell cultures were used (Fig. 1). The sample was taken by collecting the supernatant, which contained the floating apoptotic cells, followed by trypsinization of the rest of the monolayer, which contained healthy cells. Both fractions were added together to reconstitute the total population and then centrifuged at 1000 rpm for 5 minutes to get the pellet. Cells were then washed twice in PBS and cytospun by means of cytobuckets at 1500 rpm for 5 minutes. Air-dried samples were stained for light (haematoxylin-eosin) and fluorescence microscopy (fluorescent Dapi)[1].

[1]*Haematoxylin-eosin staining:* Air dried slides were fixed in 10% formaldehyde and stained in haematoxylin and counterstaiend with eosin. *Fluorescent DAPI:* Air dried slides were fixed in metanol at -20 °C for 20 min, air dried and stained with DAPI at room temperature and in the dark for 20 minutes, and mountd with an antifading medium, O-phenylendiamine in glycerol, and preserved in the dark at -20° C until examination, at a fluorescence range between 300 and 400 nm.

The percentage of apoptotic cells was defined as (number of apoptotic cells/ total cell number) x 100. At least 200 cells should be counted for each experiment.

TEM, SEM and morphology of etoposide-induced apoptosis

Scanning electron microscopy

Those cells that are to be examined by scanning electron microscopy can be directly grown on sterile glass coverslips. Once the experiment finished, cells cultured on glass coverslips were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO_4 in the same buffer, dehydrated in a graded acetone series and critical-point dried. After critical point drying, the cells were coated with gold prior to examination.

Transmission electron microscopy

Cells that are to be examined by transmission electron microscopy are directly grown on small Petri dishes. Once the experiment finished cells were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO_4 in the same buffer, dehydrated in a graded acetone series and resin-embedded.

X-ray microanalysis (Stem mode)

The study of cellular and subcellular events that take place during pharmacologically-induced apoptosis by using electron probe X-ray microanalysis is unique because it gives us the possibility to combine the ability to undertake chemical analysis with the high resolution of the electron microscope. This allowed us to correlate chemical information with all ultrastructural details observed in treated and non-treated cells. Microprobe analytical techniques take advantage of the physical interactions that occur when an electron beam impinges on a specimen. One such interaction is the production of x-rays with energy and wavelength characteristics indicative of the elemental composition of the specimen interacting with the electron beam. (Warley, 1997; Ingram et al., 1999; Salido et al., 2001). Cell and tissue culture offer an interesting perspective to increase the number of problems to which x-ray microanalysis can be applied. Among the numerous applications of x-ray microanalysis in cell biology and cell pathology, we will focus our interest on the role of ions in programmed cell death in cancer cells (Smith et al., 1998; Fernandez-Segura et al., 1999; Gutierrez et al., 1999; Masson, 1999; Skeeper et al., 1999; Salido et al., 2001, 2002), as cancer growth is the result of the imbalance between cell death and cell proliferation of the neoplastic population.

Whole mounts

For analysis in the scanning transmission electron microscope (STEM) the cells were cultured on titanium grids covered with a Formvar film and sterilized by ultra-violet light overnight. Cells were previously grown under standard conditions in a water saturated atmosphere of 5% CO_2 until a density of 50,000 cells/ml was achieved. Grids were distributed in small Petri dishes, 3 or 4 in each, and then a drop of about 10 μl of the cell suspension was carefully seeded on each of the grids. Petri dishes were kept in the incubator for at least 30 min. to allow cells to attach to substrate, then filled with 2-3 ml of complete medium and kept in the

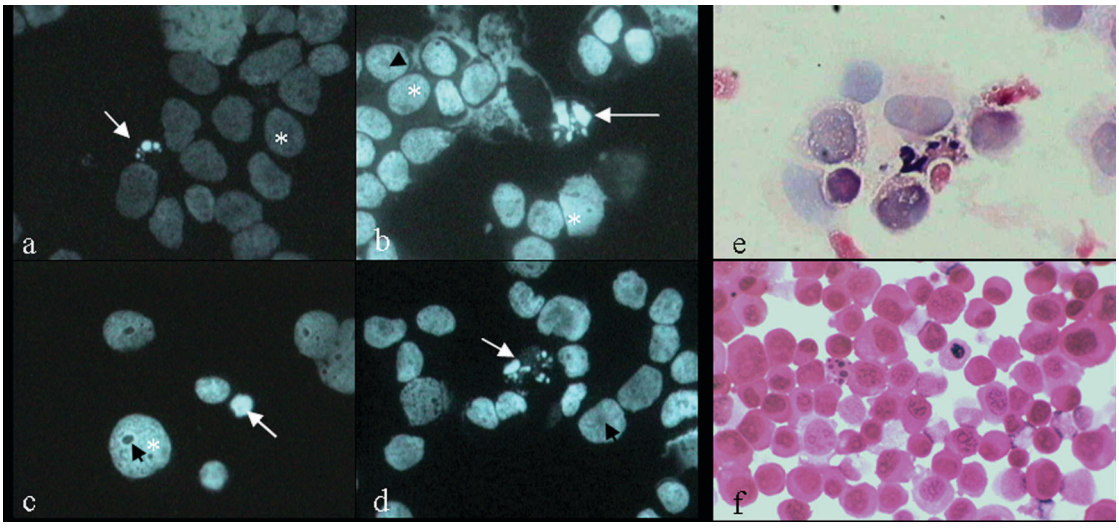

Fig. 1. Prostate cancer cell line PC3 after etoposide treatment. Apoptotic cells pass through different stages with progressive condensation of the nuclei. Control cells (white star) appear bigger, with round nuclei and well preserved nucleoli (black arrow). Apoptotic cells (arrow) show progressive hypercondensation of nuclear content and surface changes such as membrane blebs (black star) until the whole cell breaks into apoptotic bodies with or without nuclear fragments that are then phagocyted by neighboring cells. In **a, b, c, d:** nuclei stained with DAPI as described below, x 60. In **e, f:** haematoxylin-eosin-stained cells, x 60, x 40

incubator.

The cells were cultured for 48 h in the culture medium as described above, and then exposed to etoposide (150 μM), etoposide (150 μM)$^+$ bombesin (1 nM), etoposide (150 μM)$^+$ calcitonin (500 pg/ml), bombesin (1 nM), or calcitonin (500 pg/ml) for 48 h. Control cells were cultured in the standard medium for an additional 48 h. After the exposure period, the cells on the grids were rinsed briefly in cold distilled water (4 °C), frozen in liquid nitrogen-cooled liquid propane (-180 °C), freeze-dried in vacuum overnight at -130 °C and slowly brought to room temperature under vacuum (Roomans, 2002). Finally, the freeze-dried specimens were coated with a conductive carbon layer. X-ray microanalysis wasperformed at 100 kV in the STEM mode of an electron microscope with an energy dispersive spectrometer system. Quantitative analysis is carried out based on the peak-to-continuum ratio after correction for extraneous background (Roomans, 1988, 2002) and by comparing the spectra from the cells with those of a standard, which consists of known concentrations of mineral salts in a 20% gelatin and 5% glycerol matrix, frozen, cryosectioned and freeze-dried to resemble the specimen in its physical and chemical properties (Roomans, 1988). Spectra are acquired for 100 seconds and only one spectrum is obtained from each cell.

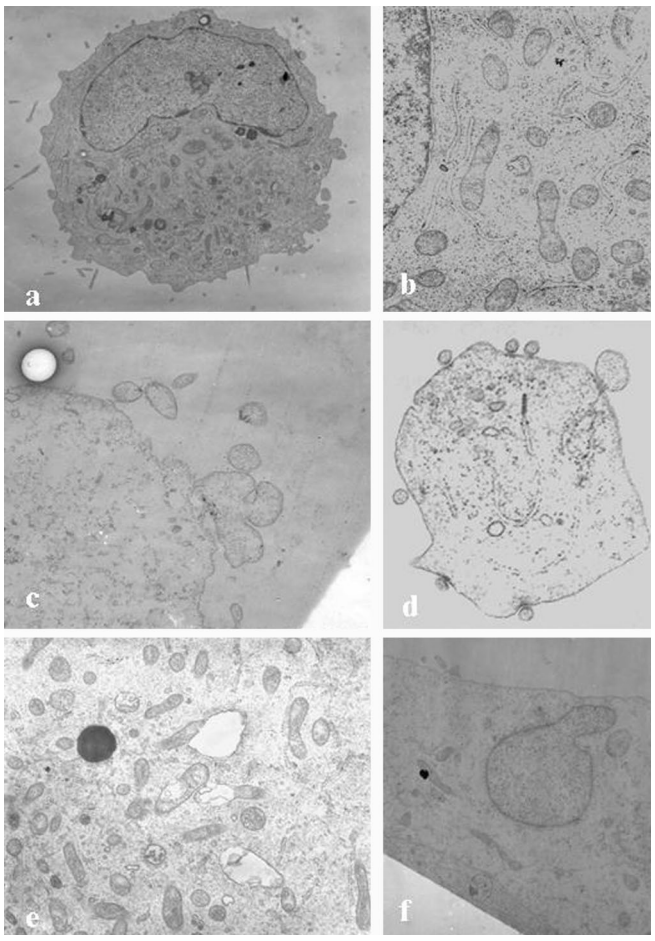

Fig. 2. Transmission electron microscopy of prostate cancer cells. **a.** Control PC3 cells in without chromatin condensation and preserved nucleolus. The cytoplasm shows a number of functional organelles. **b.** When the neuropeptide bombesin is added, the cytoplasm is full of granular endoplasmic reticulum with ribosomes and healthy mitochondriae. After etoposide treatment, membrane starts blebbing as cells enter into apoptosis for Du 145 cells **(c)**, for PC3 cells **(d)**, as cytoplasmic organelles degenerate. Mitochondriae are relatively well preserved with continuous membranes until the last stages of apoptosis in PC3 cells **(e)** after etoposide treatment, and in Du 145 cells after etoposide treatment **(f)**. a, d, x 4,000; b, e, f, x 8,000; c, x 7,000

X-ray microanalysis (SEM mode)

For analysis in the scanning electron microscope (SEM), the cells were grown on Millipore Millicell® filters (Fernández-Segura et al., 1999; Salido et al., 2001, 2002). Polypropylene filters were placed in each of the wells of a 24-well plate, and 10 μl of a 50,000 cells/ml suspension were pipetted onto the filter. Plates are then stored in the incubator for at least 30 min. to allow cells to attach to the substrate, and then the wells were filled with complete medium. Culture conditions and exposure to the various substances were as described above for analysis in the STEM. The experiment was terminated by rinsing the filters with the cells in distilled water at 4 °C. After blotting excess fluid with a filter paper, the cells were frozen immediately in liquid propane cooled by liquid nitrogen and freeze-dried overnight at -30 °C. The dried filters were coated with a conductive carbon layer to avoid charging in the electron microscope. The cells on the filter were analyzed in a scanning electron microscope (SEM) with an energy-dispersive X-ray microanalysis system at 20 kV, spot mode. Quantitative analysis was performed by determining the ratio (P/B) of the characteristic intensity (peak, P) to the background intensity (B) in the same energy range as the peak and comparing this P/B ratio with that obtained by analysis of a standard[2] (Roomans, 1988; Salido et al., 2001). Each spectrum was acquired for 200 seconds. Only one spectrum was acquired from each cell.

[2]The concentration of element x in the specimen (C_{xso}) was obtained with the peak-to-local-background (P/B) ratio method, and was calculated acorrding to the formula

$$C_{xsp} = \frac{(Px/Bx)_{sp}}{(Px/Bx_{std}} \cdot \frac{G_{sp}}{G_{sttd}} \cdot C_{xstd}$$

where Cx is the concentration of element x in millimoles per kilogram (dry weight), (Px/Bx) is the peak to background ratio for the element x, the subscriptis sp and std refer to the specimen and standard, respectively, and The G value is the meabn value of the atomic number squared (Z^2) and divided by the atomic weight (A) in the sample. Cellular elemental concentrations were obtained with reference to 20% dextran standards containing known amounts of inorganic salts (Warley, 1990) Roomans and Sevéus, 1976. J. Cell Sci. 21, 119-127.

Data obtained after examination

Scanning and transmission electron microscopy Figs. 2-4 of the cultured prostatic cells (LNCaP, Du 145, PC3) confirmed the results obtained with light and fluorescence microscopy (Salido et al.,1999, 2000). Control cells were round with well-preserved cytoplasm and plasma membrane, and treated cells passed through

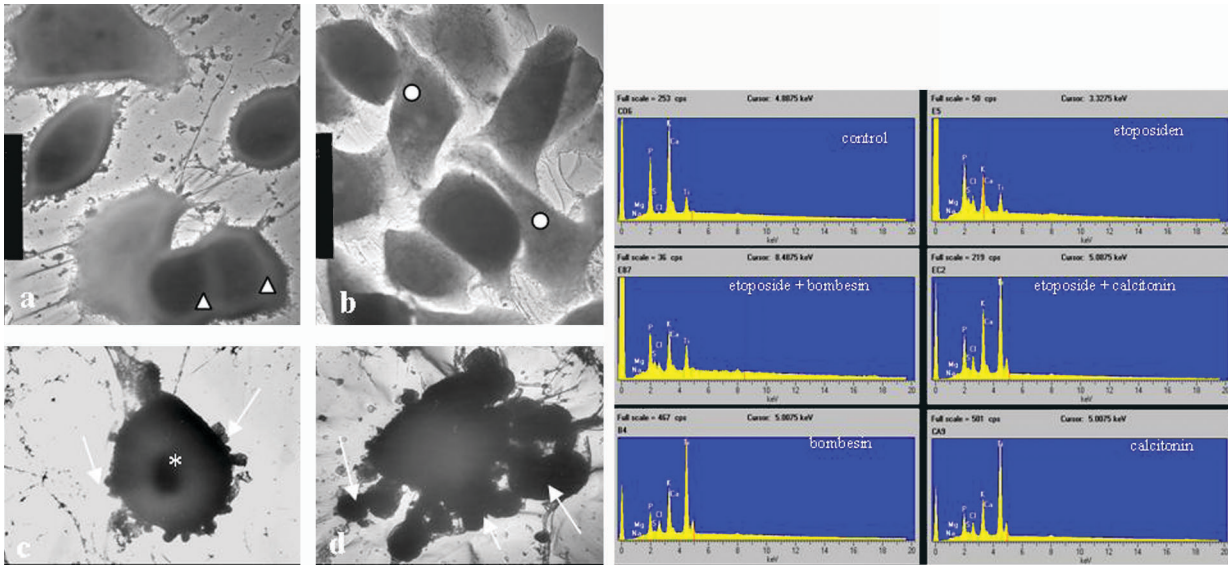

Fig. 3. Du 145 cells as examined in the Transmission Electron Microscope, in STEM mode together with the spectra from quantitative analysis of the cells. Cells are grown on titanium grids covered with a formvar film. **a.** Etoposide-treated cells (triangle) show a ruffled membrane and condensed nuclei and become round prior to detachment from the substrate and neighbouring cells. As shown in **b** for control cells, viable cells remain together and with well preserved morphology (circles). As cells pass to later stages **c**, nuclei appear more and more condensed (*) and blebs are more evident as shown in **d**. X-ray microanalysis reveals that etoposide treatment causes changes in the most relevant ions implicated in cell viability and cell death, with an increase in Na^+ and a decrease in K^+ and changes in Cl-, P, and S that are blocked by neuropeptides, as described in the text.

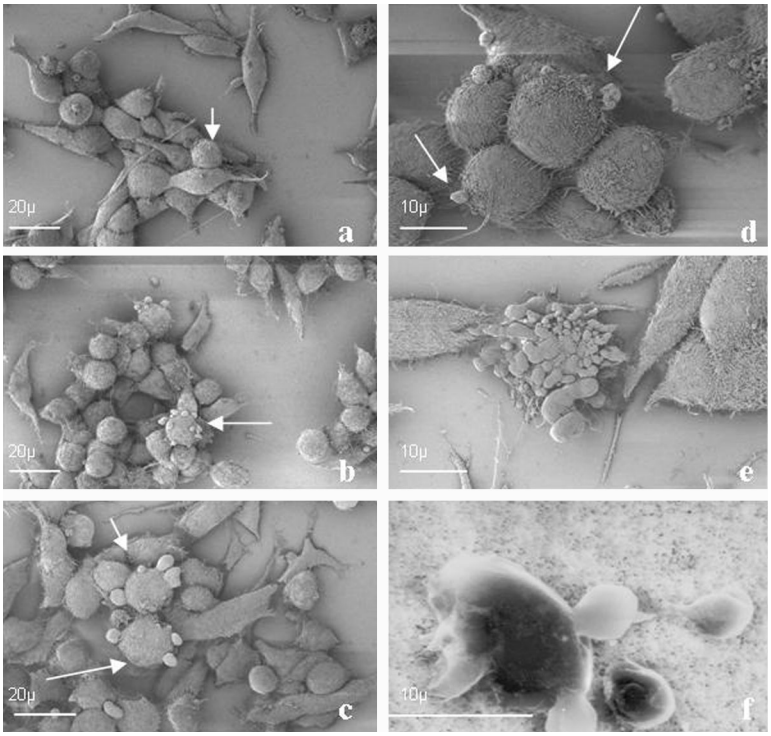

Fig. 4. Scanning electron microscopy of LNCaP cells that shows different stages of apoptosis after treatment with etoposide. In non-treated cells only **(a)** some androgen-dependent cells become apoptotic under the deprivation protocol (arrow) and start rounding. After treatment, cells are progressively more altered as shown in **(b), (c), (d**), and finally blow into apoptotic bodies covered with plasma membrane, **(e)** and **(f).**

a series of morphologically identifiable stages in their pathway to death. In the initial phase of the apoptotic process, cells shrank due to a loss of cytoplasmic volume, became detached from their neighbors and from culture substrata and adopted a smooth contour. In a following phase, the plasma membrane showed ruffles and blebs. In the third phase, progressive degeneration of residual nuclear and cytoplasmic structures was observed.

When X-ray microanalysis was performed on the cells after etoposide treatment, a progressive decrease in intracellular K^+, as cells passed from the first to second stage of induced apoptosis, and a dramatic lowering in the third stage was observed (Salido et al., 2001) The morphology of the cells changed in parallel: cells in the second stage become ruffled and blebbed, as intracellular Na and Mg increase and K decreases (Figs. 3, 5). Nevertheless, the cell membrane permeability at this stage remained unaltered as demonstrated by dye exclusion assays. The ionic ratios were also indicative of an adequate function of the Na^+/K^+ pump. A progressive increase in Na/K ratio compatible with cell injury as cells progressed through the stages of apoptosis finally showed values which could be considered as a sign of severe cell damage (Sandstrom et al., 1994; Warley, 1997; Kampf et al., 1999; Orlov et al., 1999). At the time that cells have reached the third phase of the process, the cell membrane was permeable to vital dyes.

In the third phase of the process, a positive correlation between the levels of calcium and phosphorus indicated the possibility that high local calcium concentrations could be due to deposition of calcium phosphate. Control cells appeared to have the characteristic elemental pattern of viable cells, with low Na and high K, Mg and P during the assay (Hongpaisan and Roomans, 1995; Grängsjö et al., 1997; Fernandez-Segura et al., 1999; Roomans et al., 2001).

In androgen-independent cell lines PC3 and Du 145, etoposide treatment consistently induced a decrease in K and an increase in Na. The Na/K ratio increased markedly after exposure to etoposide, and both bombesin and calcitonin fully inhibited the etoposide-induced changes in the Na/K ratio. The data also indicate that apoptosis is associated with a relative increase in P, in particular, with an increase in the P/S ratio. Calcitonin, but not bombesin, also reversed the etoposide-induced changes in the P/S ratio. Because the change in the P/S ratio probably is multifactorial (Grundin et al., 1985; Smith and Cameron, 1999; Gränsjö et al., 2000) an explanation for this difference cannot be provided currently but requires further study.

In the LNCaP androgen-sensitive cell line after androgen withdrawal, etoposide treatment also induced a decrease in K and an increase in Na and, as a consequence, the Na/K ratio increased, also markedly, and both bombesin and calcitonin blocked this increase. After etoposide treatment the intracellular concentrations of P, S and Cl increased, thus lowering the P/S ratio and increasing the ratio of Cl to K. The addition of the neuropeptides inhibited the increase in the Cl/K ratio and also increased the P/S ratio. Bombesin, but not calcitonin blocked changes in chloride concentrations and both neuropeptides inhibited the changes observed in the rest of elements analyzed. Calcitonin was more effective in blocking changes in K, S, and P, whereas bombesin was more effective in blocking changes in Mg, Na and Cl.

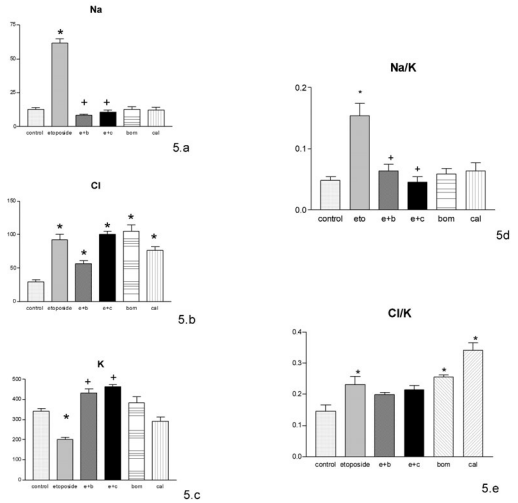

Fig. 5. Potassium, sodium and chloride concentrations as assessed by X-ray microanalysis (in STEM mode) in LNCaP cells. The Na/K ratio in LNCaP cells increases after treatment with etoposide, but this increase is inhibited by bombesin **(e^+b)** or calcitonin **(e^+c)**. Treatment with bombesin (bom) or calcitonin (cal) without etoposide does not affect the Na/K ratio of the cells. Bombesin blocks the increase in chloride concentration, and both bombesin and calcitonin added to etoposide treatment block the increase in the ratio of chloride to potassium.

Concluding remarks

The culture techniques described were designed in order to achieve two important goals. First, the development of an in vitro model that allows an approach to neuroendocrine differentiation in prostate cancer and its role in apoptosis blockage. Second, the method has been designed in order to permit rapid cryofixation of intact cell monolayers for subsequent x-ray microanalysis.

There appears to be a direct relationship between the density of NE cells and enhancement of prostate cancer characteristics, such as increased Gleason grade and lose of androgen sensitivity. It has been suggested that NE cells provide paracrine stimuli for the propagation of local carcinoma cells and that NE differentiation is associated with the progression of prostate cancer toward an androgen-independent state (Cox et al., 1999; Hansson and Abrahamsson, 2001; Ito et al., 2001; Segawa et al., 2001; Goodin and Rutherford, 2002).

The approach taken mimics the presence of neuroendocrine cells in prostatic carcinoma and makes it possible to correlate the morphological changes and

elemental patterns that appear in neuropeptide-induced resistance to apoptosis. Etoposide induces a decrease in the cellular K concentration, and an increase in the cellular Na concentration. Calculation of the Na/K ratio, a sensitive indicator of cell injury, shows that this ratio increases after etoposide treatment. We have described similar ionic changes after induction of apoptosis in androgen-independent prostate cell lines (Salido et al., 2001, 2002) and also in other systems by other groups (Yu and Choi, 2000; Tapia-Vieyra and Mas-Oliva, 2001) These changes may be due to activation of K^+ channels, or to inhibition of the Na^+/K^+-ATPase, presumably due to lack of ATP. Most likely, the changes are due to both factors. Activation of K^+ channels is one of the common features of apoptosis and prevention of etoposide-induced apoptosis in thymocytes by blocking K channels has been reported earlier (Dallaporta et al., 1998; Ouadid-Ahidouch et al., 1999). The addition of bombesin or calcitonin to etoposide-treated cells reduces the percentages of apoptotic cells, with an increase in K and a decrease in Na, and a subsequent decrease of the Na to K ratio, as an objective expression of the increase in the viability of the neoplastic cells in the presence of neuropeptides.

The data also show an increase in the cellular Cl concentration, which can also be expressed as an increased Cl/K ratio. This may appear to contradict the notion that chloride channel activation occurs at the onset of apoptosis (Nilius, 2001). However, if the increase in the cellular Na/K ratio is to be interpreted as an effect of energy deficiency, the cell would be unable to maintain its low intracellular chloride concentration and chloride ions would flow into the cell along the electrochemical gradient. Further studies are required to clarify the role of chloride ions in the onset and development of apoptosis.

The data further indicate that apoptosis is associated with an increase in both P and S, but more in S, which results in a decreased P/S ratio. The P signal mainly represents nucleotides and phosphorylated proteins, whereas the S signal mainly represents protein-bound sulfur. This correlates to the morphological changes associated with apoptosis, The fact that these changes are very marked, and can be inhibited to a large extent by neuropeptides indicates that they are important in the process of apoptosis.

This protective effect on etoposide-induced apoptosis cells appears to be quite similar in androgen-dependent and androgen-independent cell lines (Salido et al., 2001, 2002). This confirms that neuropeptides confer antiapoptotic capabilities on non-neuroendocrine cells in close proximity to neuroendocrine cells. It can therefore be speculated that certain neuroendocrine peptides can increase the survival and further growth of neighboring cells (Yu et al., 2001) and may thereby contribute to the aggressive clinical course of prostate tumors containing neuroendocrine elements (Hoosein, 1998). According to our data, neuroendocrine cells, thus, have a value as an indicator of poor prognosis in patients with prostate carcinoma, independently of the hormonal status of the epithelial cell tumors.

With respect to the culture techniques employed, the method allows the rapid cryofixation and adequate analysis by means of electron-probe x-ray microanalysis. When cells are grown on a solid substrate as we have described for polypropylene filters, and are analyzed in SEM, there is a theoretical possibility that the electron beam penetrates the cell entirely and excites the substrate even at low accelerating voltage.. The likelihood for this increases when the cell shrinks, as is the case during apoptosis. This implies that a measured decrease in elemental concentration under such conditions can be (in part) due to a decrease in cell size. This possible artefact has to be taken into account in the interpretation of data obtained by analysis of cells on a solid substrate (Fernandez-Segura et al., 1999; Roomans et al., 2001). To normalize the intensity counts of the different elements with respect to the mass of the cell analyzed, the phosphorus intensity signal is often taken as a measure of the analyzed mass and as a unit of reference for evaluating the peak intensity of the other elements (Abraham et al., 1985). However, this requires that the P content is constant during the experiment, and this is not always true during apoptosis, a process that affects cellular macromolecules, and progresses over a considerable period of time. Analysis of cells grown on thin plastic films on grids in the STEM avoids these problems, although the method as such is technically more difficult (Von Euler et al., 1993; Roomans et al., 1996). A methodological comparison of the two methods[3] showed that systematic errors in the absolute

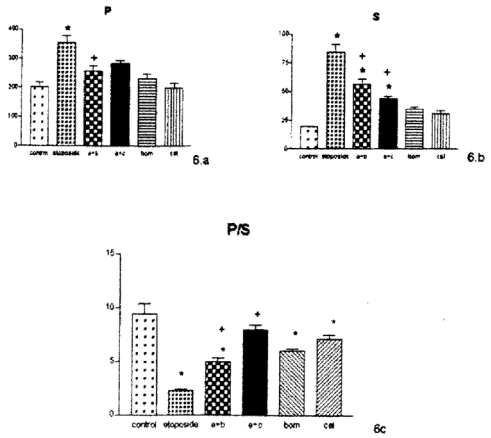

Fig. 6. Phosphorus and sulfur concentrations in LNCaP cells. Control cells are only treated with androgen deprivation. Etoposide cells show a relative increase with respect to control cells in absolute values but a net decrease when phosphorus to sulfur ratio was analyzed. Bombesin and calcitonin block the ionic changes.

[3]Roomans GM. X-ray microanalysis of cultured cells in the scanning electron microscope and the scanning transmisison electron microscope: a comparison. Scanning Microsc. 1999, 13, 159-165.

concentrations could be easily introduced in both methods, but that elemental ratios would be relatively free of such artefacts. We have, therefore, also expressed our data as elemental ratios and the direction of the changes is the same in both types of measurement (cf. Figs. 5 and 6). Hence, the increase in P/S ratio may be explained as a relative increase in P-containing macromolecules (mainly nucleotides) relative to S-containing macromolecules (mainly proteins). The relative increase in P content matches with the etoposide-induced arrest in G2/M which would result in an increased nucleotide content (Barbiero et al., 1995; Salido et al., 2001)

The use of electron probe x-ray microanalysis made it possible to evaluate alterations in total element composition in individual cells during apoptosis. The three morphologically identifiable stages of apoptosis are associated with alterations of intracellular ions, mainly sodium, potassium and chlorine, as well as with changes in the phosphorus/sulfur ratio. The use of x-ray microanalysis, thus, can be a helpful tool for further studies on cellular mechanisms involved in the control of programmed cell death of prostatic cancer cells. Induction of apoptosis is a process of high significance for the treatment of cancer. As we have shown, there is a strong correlation between apoptosis and elemental changes in the cell, and this could be used to control the apoptotic process in a better way. In particular, insight may be gained into the way in which neuropeptides such as calcitonin and bombesin, which can be secreted by neuroendocrine cells in close proximity to prostate carcinoma cells, inhibit etoposide-induced apoptosis in prostate cancer cells, and this knowledge may be used to overcome the stimulatory effect of these peptides on prostate tumor growth. The putative function of neuroendocrine cells in stimulating proliferation and/or inhibiting the apoptotic process, worsening the prostate cancer outcome, through paracrine hormonal mechanisms, provides a rationale for the experimental use of drugs which are able to inhibit the secretion of neuroendocrine products, with the aim to counteract tumor progression.

Acknowledgements. The authors thank Marianne Ljungkvist and Leif Ljung for excellent technical assistance.This work was partially supported by an Instituto de Salud Carlos III Grant (FIS 01/0727)

References

Abraham E.H., Breslow J.L., Epstein J., Chang-Sing P. and Lechene C. (1985). Preparation of individual human diploid fibroblasts and study of ion transport. Am. J. Physiol. 248, C154-164.

Abrahamsson P.A. (1999). Neuroendocrine differentiation in prostatic carcinoma. Prostate 39, 135-148.

Allard P., Beaulieu P., Aprikian A. and Chevalier S. (2000). Bombesin modulates the association of Src with a nuclear 110-kd protein expressed in dividing prostate cells. J. Androl. 21, 367-375.

Barbiero G., Duranti F., Bonelli G., Amenta J.S. and Baccino F.M. (1995). Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. Exp.Cell Res. 217, 410-418.

Barros L.F., Hermosilla T. and Castro J. (2001). Necrotic volume increase and the early physiology of necrosis. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 30, 401-409.

Berger R. and Garnier Y. (1999). Pathophysiology of perinatal brain damage. Brain Res. Rev. 30, 107-134.

Bologna M., Festuccia C., Muzi P., Biordi L. and Ciomei M. (1989). Bombesin stimulates growth of human prostatic cancer cells in vitro. Cancer 63, 1714-1720.

Bortner C.D., Hughes F.M. Jr and Cidlowski J.A. (1997). A primary role for K^+ and Na^+ efflux in the activation of apoptosis. J. Biol. Chem. 272, 32436-32442.

Bowen I.D., Worrill N.A., Winters C.A. and Mullarkey K. (1988). The use of backscattered electron imaging, X ray microanalysis and X-ray microscopy in demonstrating physiological cell death. Scan. Microsc. 2, 1453-1462.

Chien J., Ren, Y., Quin, W.Y., Bordelon, W., Thompson, E., Davis, R., Rayford W. and Shah G. (2001). Calcitonin is a prostate-epithelium-derived growth stimulator peptide. Mol. Cell. Endocrinol. 181, 69-79.

Cox M.E., Deeble P.D., Lakhnai S. and Parsons S.J. (1999). Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. Cancer Res. 59, 3821-3838

Craft N., Chhor C., Tran C., Belldegrun A., De Kernion J., Witte O.N., Said J., Reiter R.E. and Sawyers C.L. (1999). Evidence for clonal outgrowth of androgen independent prostate cancer cells from androgen-dependent tumors trough a two-step process. Cancer Res. 59, 5030-5036.

Dallaporta B., Hirsch T., Susin SA., Zamzami N., Larochette N., Brenner C., Marzo I. and Kroemer G. (1998). Potassium leakage during the apoptotic degradation phase. J. Immunol. 160, 5605-5615.

Dallaporta B., Marchetti P., de Pablo M.A., Maisse C., Duc H.T., Metivier D., Zamzami N., Geuskens M. and Kroemer G. (1999). Plasma membrane potential in thymocyte apoptosis. J. Immunol. 162, 6534-6542.

di Sant'Agnese P.A. (1998). Neuroendocrine differentiation in prostatic carcinoma: an update. Prostate suppl 8, 74-79.

Fernandez-Segura E., Cañizares F.J., Cubero M.A., Warley A. and Campos A. (1999). Changes in elemental content during apoptotic death studied by electron probe X-ray microanalysis. Exp. Cell Res. 253, 454-462.

Festuccia C., Guerra F., D'Ascenzo S., Giunciuglio D., Albini A. and Bologna M. (1998). In vitro regulation of pericellular proteolysis in prostatic tumor cells treated with bombesin. Int. J. Cancer 75, 418-431.

Fujii F., Kimura J. and Tase C. (1999). Ca^{2+} activated K^+ current induced by external ATP in PC12 cells. Clin. Exp. Pharmacol. Physiol. 26, 39-47.

Goodin J.L. and Rutherford C.L. (2002). Identification of differentially expressed genes during cyclic adenosine monophosphate-induced neuroendocrine differentiation in the human prostatic adenocarcinoma cell line LNCaP. Mol. Carcinogen. 33, 88-98.

Grängsjö A., Lindberg M. and Roomans G.M. (1997). Methods for x-ray microanalysis of epidermis: the effect of local anaesthesia. J. Microsc. 186, 23-27.

Grängsjö A., Ybo A., Roomans G.M. and Lindberg M. (2000). Irritant-induced keratinocyte proliferation evaluated with two different methods: immunohistochemistry and X-ray microanalysis. J

Submicrosc. Cytol. Pathol. 32, 11-16.

Grundin T.G., Roomans G.M., Forslind B., Lindberg M. and Werner Y. (1985). X-ray microanalysis of psoriatic skin. J. Invest. Dermatol. 85, 378-380.

Gutierrez A.A., Arias J.M., Garcia L., Mas-Oliva J. and Guerrero-Hernandez A. (1999). Activation of Ca^{2+} permeable cation channel by two different inducers of apoptosis in a human prostatic cancer cell line. J. Physiol. (London) 15, 95-107.

Han K., Viallet J., Chevalier S., Zheng W., Bazinet M. and Aprikian A.G. (1997). Characterization of intracellular calcium mobilization by bombesin-related neuropeptides in PC-3 human prostate cancer cells. Prostate 31, 53-60.

Hansson J. and Abrahamsson P.A. (2001). Neuroendocrine pathogenesis in adenocarcinoma of the prostate. Ann. Oncol.12 suppl 2, 145-152.

Hongpaisan J. and Roomans G.M. (1995). Use of postmortem and in vitro tissue specimens for x-ray microanalysis. J. Microsc. 180, 93-105.

Hoosein N.M. (1998). Neuroendocrine and immune mediators in prostate cancer progression. Front. Biosci. 3, 1274-1279.

Hughes F.M. Jr., Bortner C.D., Purdy G.D. and Cidlowski J.A. (1997). Intracellular K^+ suppresses the activation of apoptosis in lymphocytes. J. Biol. Chem. 272, 30567-30576.

Hughes F.M. Jr and Cidlowski J.A. (1999). Potassium is a critical regulator of apoptotic enzymes in vitro and in vivo. Adv. Enzyme Regul. 39, 157-71.

Ito T., Yamamoto S., Ohno Y., Namiki K., Aizawa T., Akiyama A. and Tachibana M. (2001). Up-regulation of neuroendocrine differentiation in prostate cancer after androgen deprivation therapy, degree and androgen independence. Oncol. Rep. 8, 1221-1224.

Ingram P., Shelburne J.D. and Le Furgey A. (1999). Principles and instrumentation. In: Biomedical applications of microprobe analysis. Ingram P., Shelburne J., Roggli V. and Le Furgey A. (eds). Academic Press. London. pp 1-58.

Isaacs J.T. (1999). The biology of hormone refractory prostate cancer. Why does it develop? Urol. Clin. North. Am. 26, 263-273.

Jongsma J., Oomen M.H., Noordzij M.A., Romijn J.C., van der Kwast T.H., Schröder F.H. and van Steenbrugge G.J. (2000). Androgen-independent growth is induced by neuropeptides in human prostate cancer cell lines. Prostate 42, 34-44.

Jungwirth A., Pinski J., Galvan G., Halmos G., Szepeshazi K., Cai R.Z, Groot K., Vadillo-Buenfil M. and Schally A.V. (1997). Inhibition of growth of androgen-independent Du-145 prostate cancer in vivo by luteinising hormone-releasing hormone antagonist Cetrorelix and bombesin antagonists RC-3940-II and RC-3950-II. Eur. J. Cancer 33, 1141-1148.

Kampf C., Relova A.J., Sandler S. and Roomans G.M. (1999). Effects of TNF-alpha, IFN-gamma and IL-beta on normal human bronchial epithelial cells. Eur. Resp. J. 14, 84-91.

Maeno E., Ishizaki Y., Kanaseki T., Hazama A. and Okada Y. (2000). Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. Proc. Natl. Acad. Sci. USA 97, 9487-9492.

Mason R.P. (1999). Calcium channel blockers, apoptosis and cancer: is there a biologic relationship?. J. Am. Coll. Cardiol. 34, 1857-1866.

Montague J.W., Bortner C.D., Hughes F.M. Jr. and Cidlowski J.A. (1999). A necessary role for reduced intracellular potassium during the DNA degradation phase of apoptosis. Steroids 64, 563-569.

Nilius B. (2001). Chloride channels go cell cycling J. Physiol. 532, 581.

Orlov S.N., Thorin-Trescases N., Kotelevtsev SV., Tremblay J. and Hamet P. (1999). Inversion of the intracellular Na^+/K^+ ratio blocks apoptosis in vascular smooth muscle at a site upstream of caspase-3. J. Biol. Chem. 274, 16545-16552.

Ouadid-Ahidouch H., Van Coppenolle F., Le Bourhis X., Belhaj A. and Prevarskaya N. (1999). Potassium channels in rat prostate epithelial cells. FEBS Letters 459, 15-21.

Pilat M.J., Kamradt J.M. and Pienta K.J. (1999). Mechanisms of hormone resistance in prostate cancer. Cancer Metast. Rev. 17, 373-381.

Pinski J., Reile H., Halmos G., Groot K. and Schally A.V. (1994). Inhibitory effects of somatostatin analogue RC-160 and bombesin/gastrin-releasing peptide antagonist RC-3095 on the growth of the androgen-independent Dunning R-3327-AT-1 rat prostate cancer. Cancer Res. 54, 169-174.

Pinski J., Schally A.V., Halmos G. and Szepeshazi K. (1993). Effect of somatostatin analog RC-160 and bombesin/gastrin releasing peptide antagonist RC-3095 on growth of PC-3 human prostate-cancer xenografts in nude mice. Int. J. Cancer 55, 963-967.

Ritchie C.K., Thomas K.G., Andrews L.R., Tindall D.J. and Fitzpatrick L.A. (1997). Effects of the calciotrophic peptides calcitonin and parathyroid hormone on prostate cancer growth and chemotaxis. Prostate 30, 183-187.

Roomans G.M. (1988). Quantitative x-ray microanalysis of biological specimens. J. Electr. Microsc. Tech. 9, 19-44.

Roomans G.M. (2002). X-ray microanalysis of epithelial cells in culture. In: Epithelial cell culture protocols. Methods in molecular biology,. Vol. 18. Wise C. (ed). Humana Press. Totowa, NJ. pp 273-289.

Roomans G.M., Hongpaisan J., Jin Z., Mörk A.C. and Zhang A. (1996). In vitro systems and cultured cells as specimens for x-ray microanalysis. Scanning Microsc. Suppl. 10, 359-370.

Roomans G.M., Vilches J., López A. and Salido M. (2001). Neuropeptides bombesin and calcitonin inhibit apoptosis-related elemental changes in a prostate cancer cell line. In: Proceedings SCANDEM 2001. Stockholm: Scandinavian Society for Electron Microscopy, 2001: B427-B428.

Salido M., López A., Aparicio J., Larrán J. and Vilches J. (1996). Characterization of apoptosis in prostatic androgen independent cell lines. Int. J. Dev. Biol. suppl 1, 191-192.

Salido M., Larrán J., Vilches J., López A. and Aparicio J. (1999). Etoposide sensitivity of human prostatic cancer cell lines PC-3, Du 145, LNCaP. Histol. Histopathol. 14, 125-134.

Salido M., Vilches J. and Lopez A. (2000). Neuropeptides bombesin and calcitonin induce resistance to etoposide induced apoptosis in prostate cancer cell lines. Histol. Histopathol. 15, 729-738.

Salido M., Vilches J., López A. and Roomans G.M. (2001). X-ray microanalysis of etoposide induced apoptosis in the PC-3 prostatic cancer cell line. Cell Biol. Int. 25, 499-508.

Salido M., Vilches J., López A. and Roomans G.M. (2002). Neuropeptides bombesin and calcitonin inhibit apoptosis related elemental changes in prostate cancer cell lines. Cancer 94, 368-377.

Sandström P.E., Jonsson O., Grankvist K. and Henriksson R. (1994). Identification of potassium flux pathways and their role in the cytotoxicity of estramustine in human malignant glioma, prostatic carcinoma and pulmonary carcinoma cell lines. Eur. J. Cancer 30, 1822-1826.

Segawa N., Mori I., Utsunomiya H., Nakamura M., Nakamura Y., Shan, L., Katudo K. and Katsuoka Y. (2001). Prognostic significance of

neuroendocrine differentiation, proliferation activity and androgen receptor expression in prostate cancer. Pathol. Int. 51, 452-459.

Shah G.V., Rayford W., Noble M.J., Austenfeld M., Weigel J., Vamos S. and Mebust W.K. (1994). Calcitonin stimulates growth of human prostate cancer cells through receptor-mediated increase in cyclic adenosine 3',5'-monophosphates and cytoplasmic Ca^{2+} transients. Endocrinology 134, 596-602.

Skepper J.N., Karydis I., Garnett M.R., Hegyi L., Hardwick S.J., Warley A., Mitchinson M.J. and Cary N.R. (1999). Changes in elemental concentrations are associated with early stages of apoptosis in human monocyte-macrophages exposed to oxidized low-density lipoprotein: an X-ray microanalytical study. J. Pathol. 188, 100-106.

Smith N.K.R. and Cameron I.L. (1999). Ionic regulation of proliferation in normal and cancer cells. In: Biomedical applications of microprobe analysis. Ingram P., Shelburne J.D., Roggli V.L. and LeFurgey A., (eds). Academic Press. San Diego. pp 445-459.

Smith P., Rhodes N.P., Shortland A.P., Fraser S.P., Djamgoz M.B., Ke Y. and Foster C.S. (1998). Sodium channel protein expression enhances the invasiveness of rat and human prostate cancer cells. FEBS Lett. 423, 19-24.

Tapia-Vieyra J.V. and Mas-Oliva J. (2001). Apoptosis and cell death channels in prostate cancer. Arch. Med. Res. 32, 175-185.

Von Euler A., Pålsgård E., Vult von Steyern C. and Roomans G.M. (1993). X-ray microanalysis of epithelial and secretory cells in culture. Scanning Microsc. 7, 191-202.

Warley A. (1997). X-Ray microanalysis for biologists. Portland Press. Cambridge.

Warley A., Arrebola F., Zabiti S., Cañizares F., Cubero M.A., Fernandez-Segura E. and Campos A. (2000). Changes in sodium, chloride and potassium during apoptotic cell death. An X-ray microanalytical study. In: Proc 12th Eur Congress Electron Microsc. Frank L and Čiampor F. (eds). Brno: Czechoslovak Soc Electron Microsc, 2000. B427-B428.

Wasilenko W.J., Cooper J., Palad A.J., Somers K.D., Blackmore P.F., Rhim J.S., Wright G.L. Jr, Schellhammer P.F. (1997). Calcium signaling in prostate cancer cells: evidence for multiple receptors and enhanced sensitivity to bombesin/GRP. Prostate 30, 167-173.

Yu D.S., Hsieh D.S., Chen H.I. and Chang S.Y. (2001). The expression of neuropeptides in hyperplastic and malignant prostate tissue and its possible clinical implications. J. Urol. 166, 871-875.

Yu S.P. and Choi D.W. (2000). Ions, cell volume and apoptosis. Proc. Natl. Acad. Sci. USA 97, 9360-9362.

Zelivianski S., Verni M., Moore C., Kondrikov D., Taylor R. and Lin M.F. (2001). Multipathways for transdifferentiation of human prostate cancer cells into neuroendocrine-like phenotype. Biochim. Biophys. Acta 1539, 28-43.

Accepted February 26, 2004