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Ammonium-chloride-induced prostatic hypertrophy in vitro: urinary ammonia as a potential risk factor for benign prostatic hyperplasia

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Abstract To test the possibility that urinary ammonia could be a risk factor for benign prostatic hyperplasia (BPH), we explored the cellular effects of ammonium chloride (NH_4Cl) on prostatic cancer cells used as an experimental model. Following treatment of human prostatic cancer DU-145 cells with the varying concentrations of NH_4Cl for 3 days, cell growth was inhibited by approximately 50% at 5 mM NH_4Cl and almost completely inhibited at 10 mM NH_4Cl. However, the individual cell size in these treated cells became approximately 2-fold larger and cellular protein content was also up to 2.5-fold greater than in untreated cells. This protein increase appeared to result from the reduced protein degradation, verified by metabolic labeling with [^{14}C]valine. Western blot analysis further suggested that such reduced protein turnover could in part be due to the inactivation of a lysosomal acid protease, cathepsin D. Taken together, these studies demonstrate NH_4Cl-induced hypertrophy in prostatic cancer cells, as evidenced by the growth inhibition, cell enlargement, and cellular protein increase. Therefore, ammonia is not an inert metabolic product; instead, its chronic effects on the prostate may ultimately lead to significant cellular and biochemical alterations of the prostate such as BPH.

Key words Ammonium chloride · Prostatic hypertrophy
Cathepsin D · DU-145 cells · Urinary ammonia ·
Benign prostatic hyperplasia

Introduction

Benign prostatic hyperplasia (BPH) and prostate cancer (CAP) are the most common prostatic diseases that significantly affect the aging male population in the United States. Approximately 70% of 70-year-old men are found to develop BPH [1], while CAP is ranked as the second leading cause of male cancer death [15]. Although the incidence of BPH/CAP may correlate somewhat with risk factors such as race, diet, lifestyle, environment, specific diseases, etc., only two factors – aging and 5α-dihydrotestosterone (DHT) – are common to the majority of cases of actual disease occurrence [8]. DHT is currently believed to play a pivotal role in the development and progression of BPH/CAP [1], but whether DHT is the primary factor for "triggering" these diseases is still uncertain. Particularly in the case of BPH, the exclusive occurrence of BPH in two specific regions of the prostate – the transition and the peri-urethral zone [1, 8] – prompted us to assume that some component of the urine besides DHT could be involved in the initiation step of BPH development. Such a potential factor that we are interested in is urinary ammonia.

Ammonia (NH_3/NH_4^+) is a common metabolic product and one of the major components of urine [10], but its biological and physiological significance is not fully understood. Clinically, the serum ammonia level has been a great concern because an increase (e.g., hyperammonemia) is well known to be highly toxic to the brain, resulting in brain damage, coma, and death [11]. The cellular effects of ammonia have also been documented. Chronic ammonia exposure was shown to induce renal cell alterations, such as renal enlargement or hypertrophy [4]. It has been suggested to play a regulatory role in protein synthesis and degradation [2, 6], RNA/DNA synthesis [2, 18], and activity of growth factor [5]. However, most of these studies were mainly focused on the kidneys and only a few were performed in other urological organs. We are thus interested in the effects of ammonia on the *prostate*, hypothesizing that it could be one such unidentified extracellular factor exerting its adverse effects on the prostate, possibly leading to the development of BPH.

To test this working hypothesis, we investigated the cellular and biochemical effects of ammonium chloride

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(NH_4Cl) on human prostatic cancer cells, which were used as an experimental model due to the difficulty and unavailability of normal prostate cells in vitro. These studies may provide valid information regarding the substantial effects of ammonia on prostate (cancer) cells, which have not yet been thoroughly explored. More details are described and the possibility of (urinary) ammonia as a risk factor for BPH will be also discussed.

Materials and methods

Cell culture

Human prostatic cancer DU-145 cells [19] were obtained from the American Type Culture Collection (Rockville, Md.). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). For experiments, cells were seeded in culture flasks or 6-well plates at the initial cell density of 1.3×10^4 cells/cm^2. NH_4Cl was added to cell cultures at specified concentrations, and cell number and viability at harvest were determined by the trypan blue exclusion method. RPMI-1640, FBS, antibiotics, and NH_4Cl were purchased from Sigma (St Louis, Mo.).

Cell staining

Control and NH_4Cl-treated cells were stained with hematoxylin-eosin. Cells were grown on a Lab-Tex chamber-slide (Nalge Nunc, Naperville, Ill.), fixed in acetone-methanol (1:1) solution for 5 min, and washed twice with phosphate-buffered saline (PBS). The slide was placed in a hematoxylin jar for 1 min, washed with water three times, and counterstained with eosin solution for 1 min. The slide was air-dried and photographed.

Preparation of cell lysates

Cell pellets from control and NH_4Cl-treated cells were resuspended in cell lysis buffer (10 mM HEPES-KOH, pH 7.4, 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 5% glycerol, 0.5% NP-40) supplemented freshly with 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Cell lysis was performed by three freezing-thawing cycles in liquid nitrogen. Cell lysates were then obtained by centrifugation at 12 000 rpm for 15 min at 4°C and stored at −80°C. Protein concentration was determined using the protein assay reagent, following the vender's protocol (Pierce, Rockford, Ill.).

Protein synthesis assay

The rate of protein synthesis was evaluated by the [^{14}C]valine incorporation method. After cells were cultured with or without 10 mM NH_4Cl for 3 days, spent medium was discarded and cells were incubated with 0.5 μCi of L-[U-^{14}C]Val (ICN, Irvine, Calif.) in valine-free minimum essential medium (MEM) for 4 h. Cells were precipitated with 10% trichloroacetic acid (TCA) on ice for 15 min, and the radioactivity incorporated into TCA-insoluble material was determined using a scintillation counter.

Protein degradation assay

The procedure for the protein degradation assay was previously described by Rabkin et al. [16]. Following cell culture with 10 mM NH_4Cl for 48 h, medium was discarded and cells were cultured with 0.5 μCi of [^{14}C]Val in MEM for 24 h. Cells were washed and preincubated in fresh medium for 3 h to remove valine from short-lived protein degradation. Medium was refreshed again and incubation was continued for a further 4 h. Both medium and cells were obtained and precipitated with 10% TCA. After centrifugation, 2 ml aliquots of medium were counted for the released [^{14}C]Val (TCA-soluble counts) by scintillation counting. TCA-insoluble proteins from cells were also counted (TCA-insoluble counts). The percentage (%) of protein degradation was then calculated as: "TCA-soluble counts" divided by "Total radioactivity present" (TCA-soluble + TCA-insoluble counts).

DNA synthesis assay

The rate of DNA synthesis was determined by the [^{3}H]thymidine incorporation. Control and 10 mM NH_4Cl-treated cells for 3 days were incubated with 0.8 μCi of [^{3}H]thymidine (NEN Life Science, Boston, Mass.) for 4 h. Following the removal of medium, cells were washed twice with PBS, solubilized in 0.3 N NaOH-ethanol (4:1), and the [^{3}H]thymidine incorporation measured by scintillation counting.

Western immunoblot analysis

The detailed protocol for the Western immunoblot analysis was previously described elsewhere [14]. Briefly, 7 μg of cell lysates was first subjected to 10% slab gel, followed by protein transfer to a nitrocellulose membrane. After a blocking of the blot with 3% non-fat milk overnight, it was incubated for 90 min with the primary cathepsin D antibody (Calbiochem, La Jolla, Calif.), followed by incubation with the secondary rabbit antibody conjugate for 30 min. Following the detection of the specific immunoreactive proteins by chemiluminescence, those protein bands were quantitated using densitometric scanning.

Prostate specimen collection and protein extraction

Prostate tissue specimens (n = 30) were obtained either from patients with BPH (n = 10) at transurethral resection of the prostate (TURP) or from patients with CAP (n = 20) at radical prostatectomy. A small amount of excised tissue (~50 mg) was first homogenized in cell lysis buffer using a tissue grinder, followed by cell lysis in liquid nitrogen as described earlier. Cell extracts (proteins) from both BPH and CAP specimens were then subjected to Western blots for cathepsin D analysis as described above.

Results

Effect of NH_4Cl on cell growth

DU-145 cells were treated with varying concentrations of NH_4Cl (1–10 mM) for 3 days and cell numbers were determined at harvest. Figure 1a shows that cell growth was significantly suppressed with increasing NH_4Cl concentrations, resulting in complete growth retardation at 10 mM. The 50% inhibitory concentration (IC_{50}) of NH_4Cl was estimated to be ~5 mM, but cell viability remained >90% in all conditions. Hematoxylin-eosin staining revealed that morphologically the cell size of NH_4Cl-treated cells appeared to be almost 2-fold larger than that of controls (Fig. 1b). These results demonstrate that NH_4Cl is capable of inhibiting the growth of DU-145 cells, accompanied by the increase in the cell size.

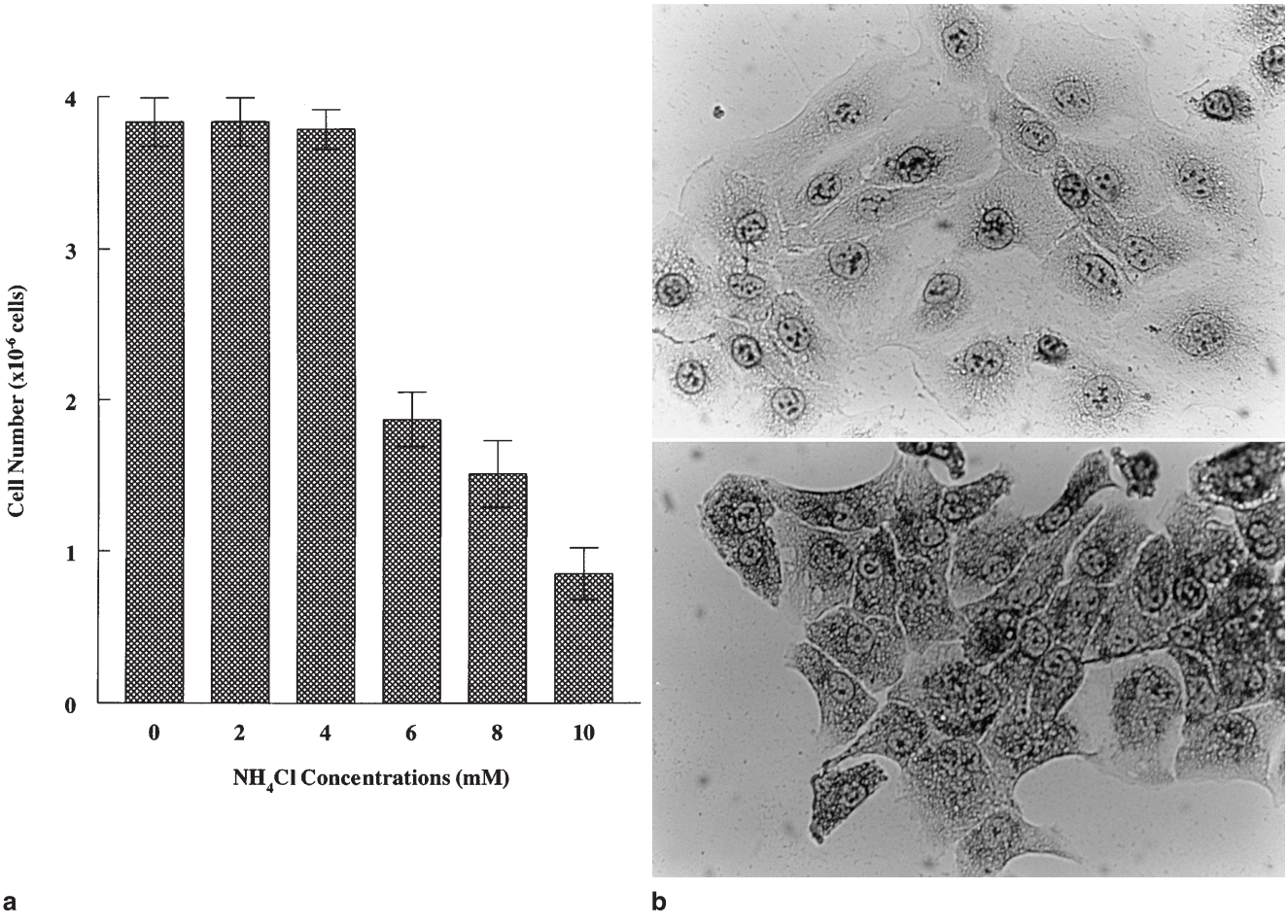

Fig. 1 a Effect of NH_4Cl on cell growth. DU-145 cells were treated with varying concentrations of NH_4Cl ranging from 0 to 10 mM for 3 days. Cell numbers were determined at harvest and those at specified concentrations are shown. **b** Effect of NH_4Cl on cell morphology. Cells were cultured with or without 10 mM NH_4Cl for 3 days. Hematoxylin-eosin stained control (*top*) and NH_4Cl-treated cells (*bottom*) are shown (×400)

Effect of NH_4Cl on protein content

Since such cell enlargement without cell proliferation in NH_4Cl-treated cells was indicative of hypertrophy, the effect of NH_4Cl on cellular protein content (amount) was examined next. Figure 2a shows that the cellular protein concentrations increased almost proportionally as the NH_4Cl concentrations were increased. In cells treated with 10 mM NH_4Cl, protein concentration was over 2.5-fold greater than that of controls. There was also an *inverse* correlation between cell numbers and protein concentrations (Fig. 2b): the NH_4Cl-dependent reduction in cell numbers coincided with the increase in protein concentrations. Thus, these studies confirm that NH_4Cl-induced growth inhibition was indeed associated with the increased cellular protein content, supporting induction of cellular hypertrophy.

Effects of NH_4Cl on protein synthesis and degradation and on DNA synthesis

To better understand how the increased cellular proteins were attained with NH_4Cl, protein synthesis and degradation were examined by metabolic labeling using [^{14}C]valine. It is generally known that an increase in cellular protein content is most likely due either to excessive protein synthesis over protein degradation or to normal protein synthesis with reduced protein degradation. The results of such studies are summarized in Table 1. No significant difference in protein synthesis was observed between controls and 10 mM NH_4Cl-treated cells, whereas protein degradation was significantly depressed by 42% ($P < 0.01$) in treated cells compared with controls. Thus, the increased protein content in NH_4Cl-treated cells appears to be the result of the reduced cellular protein breakdown or slow protein turnover. In addition, DNA synthesis, measured by [^{3}H]thymidine incorporation, was dramatically reduced by 80% ($P < 0.001$) with NH_4Cl, consistent with the significant reduction in cell number with 10 mM NH_4Cl (see Fig. 1a). Taken together, these results further support the idea of NH_4Cl-induced cellular hypertrophy of DU-145 cells.

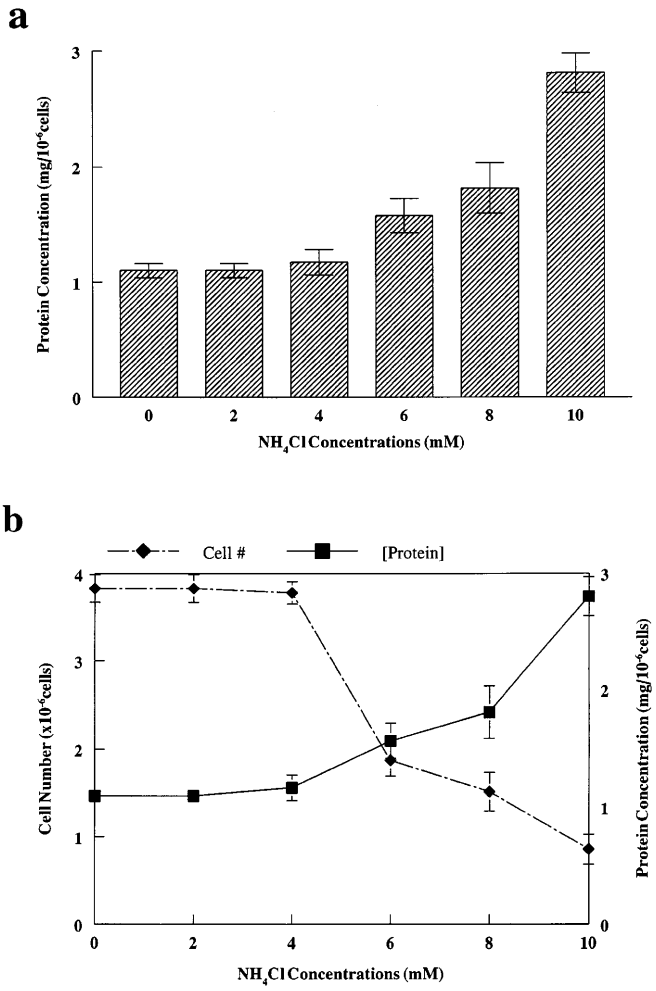

Fig. 2 a Effect of NH_4Cl on protein content. Following treatment of cells with various NH_4Cl concentrations for 3 days, protein concentrations of cell lysates were determined as shown. **b** Correlation between cell number and protein content. Changes in cell number (*diamonds*) and protein content (*squares*) were plotted against NH_4Cl concentrations as shown

Table 1 Effects of NH_4Cl on protein synthesis and degradation and on DNA synthesis

	Protein synthesis (cpm/10^6 cells)	Protein degradation (%)	DNA synthesis (cpm/10^6 cells)
Control	5400 ± 760[a]	8.5 ± 0.17	15 700 ± 2100
$+NH_4Cl$[b]	5600 ± 910	4.9 ± 0.08	3200 ± 290

[a] Mean ± standard deviation (SD) of three separate experiments
[b] Concentration at 10 mM

Effect of NH_4Cl on cathepsin D

It was of interest to explore further how such protein turnover could be modulated by NH_4Cl. Intracellular protein turnover is well known to be regulated by various proteases, which are also shown to play a key role in cell remodeling, proliferation, and differentiation [12]. The most plausible cause of slow protein turnover by NH_4Cl could be linked to the inactivation of those proteases that are active only in an acidic pH, because of intracellular alkalization (raising a pH) caused by NH_4Cl [9]. Accordingly, we examined the effect of NH_4Cl on cathepsin D (Cat.D) [3], a lysosomal acid protease, using Western blots. The results revealed that processing of Cat.D, i.e. the conversion of procathepsin D (Pro.Cat.D) to mature Cat.D, was severely interrupted by NH_4Cl in a dose-dependent manner, as evidenced by a gradual increase in Pro.Cat.D expression with a concomitant decrease in mature Cat.D (Fig. 3a). Since Pro.Cat.D is an inactive precursor form while mature Cat.D is an active form [3], this accumulation of Pro.Cat.D with NH_4Cl is indicative of the catalytic inactivation of Cat.D. Thus, the reduced protein degradation induced by NH_4Cl could be due at least in part to the inactivation of Cat.D.

To establish whether Cat.D may also be linked to cell growth, the relative expression of Pro.Cat.D (quantitated by densitometer) and cell numbers were plotted against NH_4Cl concentrations (Fig. 3b). The results show that the NH_4Cl-dependent growth reduction was associated with the increase in the Pro.Cat.D levels or the inactivation of Cat.D. This suggests that Cat.D may also play a pivotal role in the growth of DU-145 cells.

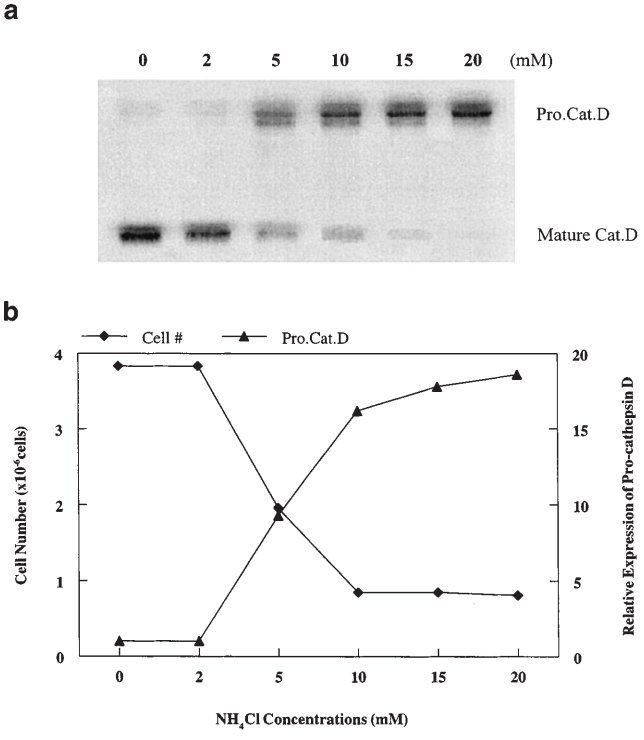

Fig. 3 a Effect of NH_4Cl on cathepsin D. Cells were treated with 0, 2, 5, 10, 15, and 20 mM NH_4Cl for 3 days and cell lysates were analyzed for cathepsin D using Western blots. Expression of procathepsin D (*Pro.Cat.D*) and mature cathepsin D (*Mature Cat.D*) is shown on the blot. **b** Correlation between cell number and procathepsin D expression. Cell numbers (*diamonds*) and procathepsin D expression (*triangles*) were plotted against NH_4Cl concentrations as shown

Discussion

The reason why BPH arises almost exclusively in the transition and the peri-urethral regions of the prostate is not fully understood. We postulated that ammonia (in the urine) might play a critical role in such disease development, because these two regions could have a greater risk for being more frequently and continuously exposed to urinary ammonia.

The concentration of urinary ammonia has been reported to be almost 1000-fold higher than that of serum ammonia, remarkably ranging from 8 to 80 mM in normal people [7], giving the estimated average of approximately 30 mM. In urine, ammonia exists in two forms: the nonelectrolyte NH_3, which is fairly diffusible, and the relatively less diffusible monovalent NH_4^+ [10]. Ammonia is also known to play a role as a cellular pH modifier in the regulation of systemic acid-base balance [10], which could affect or disturb various intracellular enzymes for maintaining normal cellular activity. It is thus tempting to assume that such highly concentrated ammonia could diffuse out of the urethra and induce serious cellular alterations in these specific regions over the years. Although all the above hypotheses are conceivable, no study of the actual effects of ammonia on normal or prostatic cancer cells has yet been documented.

In the present study, we thus investigated the potential cellular effects of ammonia on prostatic cancer cells. Our study showed that cell growth was significantly inhibited by NH_4Cl in a dose-dependent manner and that almost complete growth inhibition was attained at 10 mM NH_4Cl, which appeared to be within its physiological range. This growth inhibition was also accompanied by an increase in cell size with >90% cell viability. We further demonstrated that such cell enlargement was well associated with a concomitant increase in cellular protein concentrations, consistent with the previous report on renal tubular cells [17]. In fact, our pilot study also confirmed that two renal epithelial cell lines, namely OK and LLC-PK_1, had exhibited similar cell enlargement following NH_4Cl treatment (data not shown). Moreover, it should be noted that NH_4Cl-induced growth inhibition and cell enlargement in this study was reversible. Following the removal of NH_4Cl on day 3, normal cell growth and cell size were restored within the next 3–4 days in NH_4Cl-free medium (data not shown). However, whether *prolonged* NH_4Cl exposure may induce an irreversible effect on the cells has not been explored.

We then investigated the mechanism of the cellular protein increase with NH_4Cl. Such a protein increase was found to be most likely due to the reduced rate of protein degradation, not to the net increase in protein synthesis. Further studies demonstrated that Cat.D processing required for its activation was inhibited by NH_4Cl, suggesting that the reduced protein degradation is at least in part due to the inactivation of Cat.D, which also appears to play a regulatory role in DU-145 cell growth. To further explore whether such elevated Pro.-Cat.D expression due to the blockage of Cat.D processing could be associated with the different tissue status of the prostate, we analyzed expression of Cat.D in clinical prostate specimens obtained from patients with BPH or CAP. Cat.D expression was found to differ distinctly between BPH and CAP specimens: Pro.Cat.D was predominantly (>90%) expressed in BPH ($n = 10$) whereas greater expression of mature Cat.D was observed in CAP ($n = 20$) specimens (Fig. 4). These findings indicate that differences in Cat.D expression or activity appear indeed to reflect alterations in prostate tissues, further implying the significant involvement of Cat.D in such process. Therefore, it is conceivable that Cat.D could be the primary factor responsible for the increase in cellular protein content and the growth inhibition in NH_4Cl-induced prostatic hypertrophy.

Although NH_4Cl induces "hypertrophy" (defined as cell enlargement without cell proliferation) in prostatic cancer cells, BPH is actually "hyperplasia", which involves cell enlargement as well as cell proliferation. At this point, whether NH_4Cl or ammonia may induce hypertrophy or hyperplasia of the normal prostate in vivo remains uncertain, because DHT, a known key factor for the development of BPH, must be also taken into consideration. Due to the fact that DHT has a mitogenic activity stimulating cell growth [13], it is difficult to predict how two *opposing* factors could concurrently affect the prostate in vivo. Whether they work cooperatively or antagonistically probably depends on their physiological state.

Our study would certainly be more appropriate and convincing if *normal* prostate cells were available for such experiments. Unfortunately, it is generally understood that conducting in vitro studies using any normal cells, including prostate cells, is almost impossible, although BPH primary cultures can be established in vitro. However, our specific aim was to test the hypothesis that NH_4Cl (ammonia) may have the biological potency to cause substantial alterations in prostate (cancer) cells such as hypertrophy, implying its potential role as a risk factor for BPH. For this purpose, little advantage would

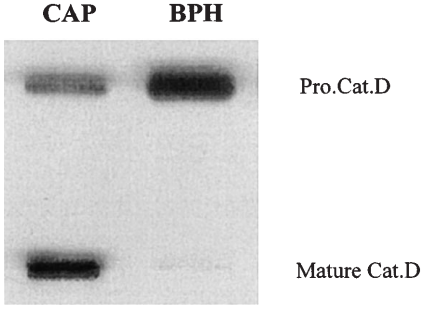

Fig. 4 Expression of cathepsin D in prostate specimens. Cell extracts from prostate cancer (*CAP*; $n = 20$) and benign prostatic hyperplasia (*BPH*; $n = 10$) specimens were subjected to Western blots. Differential expression of cathepsin D forms in these specimens is shown by two representative specimens

be gained by using established BPH cultures instead of prostatic cancer cells, which were readily available. Nevertheless, the best approach for understanding the substantial effects of ammonia and DHT on the prostate may rely on in vivo studies using an appropriate animal model. Such studies are currently under way.

In conclusion, NH_4Cl is not a simple inert metabolite but is capable of inducing hypertrophy in prostatic cancer cells, through in part modulating a lysosomal protease, cathepsin D. It is plausible that chronic exposure to ammonia (in the urine) may cause significant cellular and biochemical alterations to the prostate, probably leading to clinical manifestations such as BPH. Therefore, with aging and DHT, urinary ammonia could be considered an additional risk factor for BPH and further investigations are warranted.

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