Role of cathepsin D in prostatic cancer cell growth and its regulation by brefeldin A

Abstract We investigated a possible relationship between brefeldin A (BFA), an antibiotic, and cathepsin D (Cat.D), a lysosomal protease, in prostate cancer proliferation. Effects of BFA (30 ng/ml) were examined on the growth of three human prostatic cancer cell lines, PC-3, DU-145, and LNCaP cells. Its effect on Cat.D in these cancer cells was assessed by Western blots and compared with Cat.D expressed in clinical prostate specimens (n = 55). BFA profoundly (> 70%) inhibited the growth of all three cancer cell lines. Western blots revealed that expression of procathepsin D (Pro.Cat.D) was markedly increased with BFA, whereas actively proliferating (control) cells greatly exhibited mature Cat.D. Analysis of prostate specimens then showed predominant Pro.Cat.D expression in non-cancerous tissues while also showing enhanced expression of mature Cat.D in all cancer specimens. Therefore, BFA-induced growth inhibition in prostatic cancer cells is associated with a blocking of Cat.D maturation (activation), suggesting a possible role of Cat.D in prostate cancer proliferation/development.

Key words Cathepsin D · Brefeldin A · Prostate cancer

Although various modalities of prostate cancer (CAP) treatment, including androgen ablation, brachytherapy, radiation, chemotherapy etc., are currently available, the outcomes of these therapies are often disappointing [10]. This led us to search for agents/drugs that could more effectively regulate CAP growth. An antibiotic brefeldin A (BFA) [17], a fungal macrocyclic lactone, was the most interesting agent among them, because BFA has been shown to induce apoptosis in several cancers [14]. We recently reported that BFA was a potent growth inhibitor as well as an apoptosis inducer in prostatic cancer cells in vitro, regardless of their androgen dependency. The primary growth regulatory mechanisms of BFA were described in such studies [2, 8, 9]. LNCaP cells [6], expressing the functional androgen receptor (AR), are the androgen-dependent (responsive) cancer type. The growth inhibition induced by BFA in these cells was shown to result primarily from the post-translational down-regulation of the AR [8]. On the other hand, owing to the absence of a functional AR in two androgen-independent prostatic cancer cell lines, PC-3 [7] and DU-145 cells [16], the mechanisms of their BFA-induced growth inhibition were independent of the AR and different from that in LNCaP cells. The retinoblastoma protein (pRB), a principal cell cycle regulator for the G1-S phase progression [15], was found to be the essential factor modulated in BFA-induced PC-3 growth inhibition [9]. In DU-145 cells, BFA was capable of inducing growth inhibition leading to apoptosis [2], presumably through the up-regulation of WAF1 (p21) [5], which was a cyclin-dependent kinase inhibitor and also known to play a key role in the apoptotic process.

These studies thus demonstrate that, despite a possible difference in the growth regulatory factors/pathways, BFA is capable of inducing the growth cessation in both androgen-dependent and -independent prostatic cancer cells, implying its diverse/global influence over cell proliferation.

We were also interested in searching for a more specific prognostic marker, besides PSA (prostate-specific antigen), for disease progression. Cathepsin D (Cat.D), a lysosomal protease found in a variety of mammalian cells [1], is known to exist in three different forms resulting from post-translational processing [4]. Procathepsin D, the inactive 52-kDa precursor form, is first converted into a 48-kDa intermediate form, which is

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then processed to the active 31-kDa mature form (Fig. 1). Expression of these different Cat.D forms have been shown to be biologically and clinically significant in neoplastic progression [1, 11, 12]. We then reported that such Cat.D expression varied among prostate tissues including normal (NML), benign prostatic hyperplasia (BPH) and CAP specimens [3]. Thus, the differential expression of Cat.D appears to be a useful indicator for assessing prostate cancer status.

Despite all these studies on the growth regulatory mechanisms of BFA and Cat.D expression in prostate specimens, we have not integrated the data to address a possible link between a BFA-induced growth inhibition and a role of Cat.D. In this study, we therefore investigated the effects of BFA on cell growth and Cat.D in vitro to find a BFA-Cat.D relationship regarding prostate cancer proliferation. Prostate tissue specimens were also analyzed to validate the in vitro data and to elicit a significance of Cat.D in clinical setting.

### Materials and methods

#### Cell culture

The two androgen-independent human prostatic cancer cell lines, PC-3 cells established from a bone metastatic site [7] and DU-145 cells from a brain metastasis [16], and the androgen-dependent (responsive) LNCaP cells, derived from a lymph node metastasis [6], were obtained from the American Type Culture Collection (Rockville, Md., USA). 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 T-25 or T-75 flasks at the initial cell number of 1 x 10^6 cells in triplicates and incubated with varying concentrations of BFA (Epicentre Technologies, Madison, Wis., USA). Cell number was then determined at specified times by the trypan blue exclusion method.

#### Prostate specimen collection

The detailed procedures were described previously [3]. Briefly, a section from each of the fresh prostate tissues obtained either at radical prostatectomy or at transurethral resection was sent for histological examination. The remainder of the specimen was washed with phosphate-buffered saline (PBS) and stored at −80 °C. Following the pathology report, 25 non-cancerous specimens including normal (n = 15) and BPH (n = 10) specimens and 30 CAP specimens were used in this study.

#### Preparation of cell extracts

The previously described procedures [3, 9] were adapted. In the preparation of cell extracts from prostatic cancer cells, cell pellets were resuspended in lysis buffer and subjected to three cycles of freeze-thaw in liquid nitrogen. Cell extracts (supernatant) were obtained by centrifugation and stored at −80 °C. For prostate specimens, a small portion (~50 mg) of prostate tissue was first homogenized in lysis buffer using a tissue grinder. The homogenate was then subjected to freeze-thaw in liquid nitrogen, and cell extracts were collected and stored, as described above. Protein concentrations of cell extracts were spectrophotometrically determined using the protein assay reagent (Pierce, Rockford, Ill., USA).

#### Western immunoblot analysis

The procedures essentially followed the method described previously [9]. Briefly, an equal amount of proteins (7 μg) from each cell extract was first subjected to 10% SDS-PAGE, followed by protein transfer to a nitrocellulose membrane. The blot was incubated with the primary antibody against Cat.D (anti-Cat.D; Calbiochem, La Jolla, Calif., USA), followed by incubation with the anti-rabbit secondary antibody conjugate. The specific immunoreactive proteins were then detected by chemiluminescence. The intensities (arbitrary scales) of both procathepsin D and mature Cat.D in each sample were further quantified by scan densitometry (Silk Scientific, Oregon, Utah, USA). The relative amount of individual Cat.D form was expressed by the percentage (%) of the total Cat.D values (procathepsin D + mature Cat.D) normalized to 100%.

#### Statistical analysis

Differences in cell numbers between control and BFA-treated cells were assessed using the unpaired Student t test. A value of P < 0.05 was considered significant.

### Results

#### Effects of BFA on cell growth and Cat.D in prostatic cancer cells

The growth inhibitory activity of BFA was first examined in PC-3, DU-145, and LNCaP cells. These cells were cultured with 10, 20, or 30 ng/ml of BFA and cell numbers were determined at 72 h. The growth of all three cell lines was profoundly inhibited by BFA, resulting in approximately 75, 85, and 90% reduction in cell numbers with 30 ng/ml BFA in PC-3, DU-145, and LNCaP cells, respectively (Fig. 2).

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**Fig. 1** Processing of cathespin D. Sequential processing of procathepsin D (52 kDa), the intermediate form (45–48 kDa), and the mature form (31 kDa) is illustrated.