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A proteome study of secreted prostatic factors affecting osteoblastic activity: identification and characterisation of cyclophilin A

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Abstract

Prostate cancer cells metastasise to bone causing a predominantly osteosclerotic response. It has previously been shown that PC3 cells secrete factors which stimulate the mitogenic activity of human bone marrow stromal (hBMS) cells. Some of these mitogens have been found to be proteins with a molecular weight between 20 and 30 kDa. Even though a number of investigations have been performed to identify the osteoblastic mitogenic factor or factors produced by prostate cancer cells, it is still unknown what causes the mitogenic activation of osteoblasts. Therefore, the aim of this study was to characterise the protein profile of conditioned medium (CM) from PC3 cells in the molecular weight range of 5–30 kDa using proteome analysis. A protein profile of the CM from PC3 cells was performed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Thirty protein spots with molecular weights ranging from 5 to 30 kDa were analysed by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS). One of these spots was identified as cyclophilin A. We examined whether cyclophilin A alone or in combination with insulin-like growth factor-I (IGF-I) had any effects on the proliferation or differentiation of hBMS cells. Cyclophilin A at 1, 10, and 100 nM and cyclophilin A at 10 nM combined with 10 ng/ml IGF decreased the proliferation of hBMS cells up to 49±30, 38±29, 50±8 and 60±16%, respectively [mean (treated/control)±standard error of the means (SEM)] of control. IGF-I did not significantly affect these decreases. Cyclophilin A alone or in combination with IGF-I did not have any effect on differentiation (assessed by measuring the activity of alkaline phosphatase (ALP)). In conclusion, these results suggest cyclophilin A is not involved in the osteosclerotic effects seen when prostate cancer metastasises to bone.

Keywords: Proteome analysis; Prostate cancer; Bone metastases; Osteosclerotic; Cyclophilin A

1. Introduction

Prostate cancer is a disease frequently found among men in the western world. In 70–80% of patients with metastatic prostate cancer, bone is the target for the metastases [1,2]. Such metastases are associated with an increased bone destruction [3,4]. However, the overriding characteristic is increased osteoblastic activity leading to accelerated formation of dense sclerotic bone, which is a common feature of prostate cancer [5]. Understanding the underlying molecular mechanism by which osteoblastic metastases are formed is of great interest. Investigations have focused on identification of growth factors, which are secreted by prostate cancer tissue and initiate osteoblastic stimulation. Histomorphometrical studies have suggested that osteoblastic metastases are caused by soluble factors secreted by the metastasising prostate cancer cells. These factors stimulate the process of bone formation on trabecular surfaces without the requirement for prior bone resorption [6]. Extracts of human prostate cancer, human benign prostatic hyperplasia (BPH), normal adult, and normal prepubertal prostate tissues were found to exhibit mitogenic activity in several osteoblast-like cell bioassay systems [7–10]. Furthermore, using reverse-phase high performance liquid chromatography, several peptides with molecular weights between 6 and 24 kDa were purified, all exerting mitogenic activities for osteoblast-like cells. Prostate cancer tissue contains an osteoblastic

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mitogen or mitogens, which act independently or synergistically with known growth factors [9]. The human prostate cancer cell line, PC3, has been extensively used in studies on prostate cancer in vitro. This androgen-unresponsive cell line was established from metastatic tumour tissue obtained from lumbar vertebra of a human prostate cancer patient in 1979 [11]. When inoculating these cells into nude mice, osteolytic lesions were much more pronounced than osteoblastic lesions. The reason why these cells did not produce osteoblastic lesions in vivo is not clear. An explanation could be that PC3 cells produce parathyroid hormone-related peptide (PTHrP) and transforming growth factor α (TGF-α), both stimulators of the osteolytic response [12,13]. However, it has been shown that PC3 cells secrete a factor or factors which stimulates the mitogenic activity of osteoblasts [14–16]. The PC3-derived mitogens were found to be proteins with molecular weights between 20 and 30 kDa [14,15]. When PC3 conditioned medium (CM) was tested in combination with a maximally effective concentration of either basic fibroblast growth factor (FGF), insulin-like growth factor-I (IGF-I), IGF-II, or transforming growth factor (TGF-(β)), it produced an additive effect suggesting that PC3 CM stimulates the proliferation of osteoblasts by a mechanism that is independent of these bone mitogens [14].

In this study, we used two proteome analysis to examine the proteins in the CM of PC3 cells. A protein profile was performed on the CM from PC3 cells by two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE). It was not possible to create a control 2D-PAGE since prostate cells secrete osteoblastic mitogens irrespective of whether they are normal, benign or cancer cells. Instead, we analysed the proteins in the CM from PC3 cells in the molecular weight range of 5–30 kDa by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS). The aim was to identify factors affecting proliferation and/or differentiation of human bone marrow stromal (hBMS) cells. Among the proteins and peptides examined, cyclophilin A was identified.

Cyclophilin A is an 18 kDa protein, which belongs to the family of peptidylproline *cis-trans*-isomerase (immunophilins) and binds selectively to the immunosuppressant cyclosporine A, whereby its activity is inhibited [17]. As an enzyme, cyclophilin A participates in the folding of peptide chains, specifically involving the amino acid proline [18]. Experiments have revealed that cyclophilin A is present in the cytoplasm of various eukaryotic cells (reviewed in Ref. [17]). In principal, cyclophilin A is an intracellular enzyme, but it has been reported that cyclophilin A can be secreted by vascular smooth muscle cells (VSMCs) in response to oxidative stress. Furthermore, it was demonstrated that cyclophilin A stimulates extracellular signal-regulated kinase (ERK1/2) activation, increases proliferation, and inhibits apoptosis in VSMCs [19]. These observations forced us to examine whether cyclophilin A modulates the proliferation and/or differentiation of hBMS cells.

2. Materials and methods

Recombinant human IGF-I was purchased from GroPep Research Products, Bie & Berntsen A/S (Aarhus, Denmark). Lymphoprep™ (density 1.007±0.001 g/ml, 20 °C) was purchased from In Vitrogen (Copenhagen, Denmark). Minimum Essential Medium (MEM) (Eagle), phosphate-buffered saline (PBS) with and without Ca^{2+} and Mg^{2+}, and fetal calf serum (FCS) were purchased from GIBCO/BRL (Copenhagen, Denmark). Bovine serum albumin (BSA), cyclophilin A, and alkaline phosphatase (ALP) kit were purchased from Sigma-Aldrich (Copenhagen, Denmark). Immobiline pH Gradient (IPG) buffer 3–10 was obtained from Plus One, Amersham Pharmacia Biotech (Copenhagen, Denmark). Linear dry IPG strips (pH 3–10), ExcelGel XL sodium dodecyl sulphate (SDS) 12–14% gradient gels, Excel SDS Buffer Strip, and [methyl-^{3}H]-thymidine (specific activity 37.0 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Copenhagen, Denmark).

2.1. Cell culture

PC3 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in MEM containing 10% FCS (v/v).

Cultures of hBMS cells were established as previously described in Ref. [20]. In short, bone marrow was aspirated from the posterior iliac spine of nine healthy volunteers (two females and seven males) aged 27–47 years (after informed consent and approval from the regional ethical committee). Low-density molecular cells were isolated by centrifugation in a Lymphoprep™ density gradient. The cells were plated (10^6 cells/ml) in MEM containing 10% FCS (v/v) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed regularly until the cells reached confluence. Cells were trypsinised and seeded in 96-well culture plates. The concentration of the plated cells depended on the assay type and is indicated in the relevant sections below.

2.2. Purification of proteins from PC3 CM

PC3 cells were grown to approximately 80% confluence, washed once with 0.1% BSA–MEM (w/v), and incubated for 48 h with 0.1% BSA–MEM (w/v). CM from three flasks (75 cm^2) was pooled and centrifuged to remove dead or released cells. Proteins in the CM were precipitated using 25% trichloroacetic acid (v/v) for 30 min on ice and pelleted by centrifugation. The

pellet was washed in ice-cold 96% ethanol and dissolved in 1 ml of a solution containing 9 M urea, 2% Triton X-100 (v/v), 0.13 M dithiothreitol (DTT), 2% IPG buffer 3–10 (v/v), 8 mM phenylmethansulphonyl fluoride (PMSF), and traces of bromophenol blue. The protein samples were stored at −80 °C until analysed by 2D-PAGE.

2.3. 2D-PAGE

First-dimensional isoelectric focusing (IEF) was performed using linear pH 3–10 IPG strips. The IPG strips were rehydrated for 15–20 h at room temperature (RT) in 400 μl of protein sample. IEF was carried out sequentially at 500 V for 0.01 h, 500 V for 3 h, 3500 V for 5 h, and 3500 V for 20 h in a gradient mode at 22 °C. Prior to protein separation in SDS-PAGE, the IPG strips were equilibrated twice for 15 min in 10 ml of equilibration solution [0.1% Tris–HCl (w/v), pH 6.8, 5.5 M urea, 0.3% glycerol (v/v), 35 mM SDS, and 65 mM DTT]. DTT was replaced by iodoacetamide (14 mM) and traces of bromophenol blue in the second equilibration solution. The equilibrated IPG strips were transferred to ExcelGel XL SDS 12–14% gradient gels. Electrophoresis was performed at 15 °C using Excel SDS Buffer Strips for horizontal SDS electrophoresis at a maximum voltage of 1000 V, 20 mA for 45 min, and continued in the same conditions for approximately 2 h with the IPG strips removed. The proteins were visualised by silver staining as previously described in Ref. [21]. Briefly, the gels were fixed in 45% methanol with 5% acetic acid for 30 min and placed in double-distilled water overnight. The gels were sensitised with 0.02% sodium thiosulphate (w/v) for 2 min, incubated in 0.1% silver solution (w/v) for 30 min, and developed with 0.04% formalin (v/v) and 2% sodium carbonate (w/v) within 10 min. The development was stopped by the addition of 1% acetic acid.

2.4. In-gel tryptic digestion of proteins within an acrylamide gel matrix

Thirty silver-stained protein spots with molecular weights ranging from 5 to 30 kDa were analysed. The spots were excised from the stained gel and 100% acetonitrile was added. When the gel pieces had shrunk, the acetonitrile was removed and 0.1 M ammonium bicarbonate was added. After 10–15 min of rehydration, this solution was removed and the gel pieces were dried under vacuum. The gel pieces were reswelled in 30 μl of 10 mM DTT in 0.1 M ammonium bicarbonate for 45 min at 56 °C to reduce the proteins. Excess liquid was removed and replaced by the same volume of freshly prepared 55 mM iodoacetamide in 0.1 M ammonium bicarbonate. After 30 min of incubation in the dark, the iodoacetamide solution was removed and the gel pieces were washed with 0.1 M ammonium bicarbonate/100% acetonitrile (1:1 v/v). The washing solution was discarded and 80 μl of 100% acetonitrile was added to shrink the gel pieces. The acetonitrile was removed and the gel pieces were completely dried under vacuum. The dried gel pieces were rehydrated in 15 μl of freshly prepared digestion buffer (50 mM ammonium bicarbonate and 15.5 ng/μl trypsin) on ice for 45 min. The remaining supernatant was removed and replaced by 15 μl of 50 mM ammonium bicarbonate to keep the gel pieces wet during enzymatic cleavage at 37 °C, overnight. The supernatant was removed and the peptides were recovered from the gel by addition of 25 μl of 5% formic acid (v/v) and incubated for 10 min. 25 μl of 100% acetonitrile was added and after 10 min of incubation the supernatant was removed. The extraction procedure was repeated twice and the supernatants were pooled. The samples were dried under vacuum and the lyophilised samples were dissolved in 20 μl of 5% formic acid (v/v).

2.5. Peptide mapping by MALDI-TOF MS

The peptides were purified in microcolumns, which were prepared for each sample individually as previously described in Ref. [22]. A slurry of 100–200 μl of chromatographic resin Poros 50R2 was prepared in 5% formic acid/100% acetonitrile (1:1 v/v) and placed into a GelLoader tip (Eppendorf). The packed column was washed using 15 μl of 100% acetonitrile and equilibrated using 15 μl of 5% formic acid (v/v). The peptide sample (3 μl) was loaded together with 15 μl of 5% formic acid (v/v). The column was washed using 15 μl of 5% formic acid (v/v). The sample was eluted directly on the MALDI target by 1 μl of matrix solution, which consisted of α-cyano-4-hydroxycinnamic acid (HCCA) suspended in 100% acetonitrile. Mass measurements performed in the MALDI-TOF instrument (Bruker Reflex) were obtained from spectra resulting from 200 laser shots after internal calibration using the monoisotopic mass of two autolysis tryptic fragments at mass-to-charge ratio (m/z) 842.51 and 2211.10. The proteins were identified by a database search using "MASCOT" and "ProFound" software (www.matrixscience.com and www.proteomics.com).

2.6. Proliferation assay

The mitogenic effects of PC3 CM and cyclophilin A on hBMS cells were examined by [^{3}H]-thymidine incorporation. To obtain CM, approximately 80% confluent PC3 cells were incubated with 0.1% BSA-MEM for 48 h. CM was collected and centrifuged to remove dead and released cells, and used immediately. The hBMS cells were plated into 96-wells at 10^4 cells/well. After 24 h, the hBMS cells were serum-deprived by replacing the medium with 0.1% BSA–MEM (w/v) for 24 h before

treating them with PC3 CM or 0.1% BSA–MEM (w/v) in the presence of cyclophilin A alone (1, 10, and 100 nM) or in combination with 10 ng/ml IGF-I. After 24 h, 2.5 μCi [^{3}H]-thymidine was added to each well. The hBMS cells were labelled for 24 h, washed three times with PBS and dissolved in OptiPhase "HiSafeII" scintillation fluid (100 μl/well). The incorporation of [^{3}H]-thymidine was measured in a TRILUX scintillation counter from Applied Biosystems (Copenhagen, Denmark).

2.7. Differentiation assay—ALP activity

The hBMS cells were plated into 96-wells at 2×10^4 cells/well. After 24 h, the medium was changed and the cells were allowed to grow for 48 h in the presence of 10% FCS (v/v), washed once with 0.1% BSA–MEM (w/v), and further incubated for 96 h with PC3 CM or 0.1% BSA–MEM (w/v) in the presence of cyclophilin A alone (1, 10, and 100 nM) or in combination with 10 ng/ml IGF-1. CM from PC3 cells was prepared as described earlier. The ALP activity was measured using an ALP activity kit according to the manufacturer's recommendations. The measurements of ALP activity were corrected for cell number variation as estimated by the methylene blue assay [23].

3. Results

3.1. 2D-PAGE of secreted proteins from PC3 cells and identification of cyclophilin A by MALDI-TOF MS

We examined the proteins in the CM from PC3 cells with molecular weights ranging from 5 to 30 kDa by proteome analysis using 2D-PAGE and mass spectrometry. A silver-stained protein profile of the CM from PC3 cells was obtained by 2D-PAGE (Fig. 1a). Three gels were performed independently to ensure that the obtained protein profiles were reproducible. Thirty protein spots in the pI range 3–10 and with molecular weights ranging from 5 to 30 kDa (Fig. 1b) were excised and analysed by MALDI-TOF MS after in-gel tryptic digestion. The MALDI-TOF mass spectrum obtained for spot number 26, a peptide mass fingerprint, is shown in Fig. 2. The database search result for this peptide mass fingerprint is summarised in Table 1 and allowed the identification of cyclophilin A. In a similar way, galectin-1 was identified in spot number three. This latter protein will not be discussed any further in this paper. Protein spots number 1, 2, 4, 9, 13, 19, 20, 21 and 22 were identified as fragments of BSA derived due to the proteolytic degradation of BSA in the culture medium. The remaining protein spots were not readily identified by MALDI-TOF MS mostly due to background noise from the BSA and because the amounts of proteins in the individual spots were too small to allow further analysis.

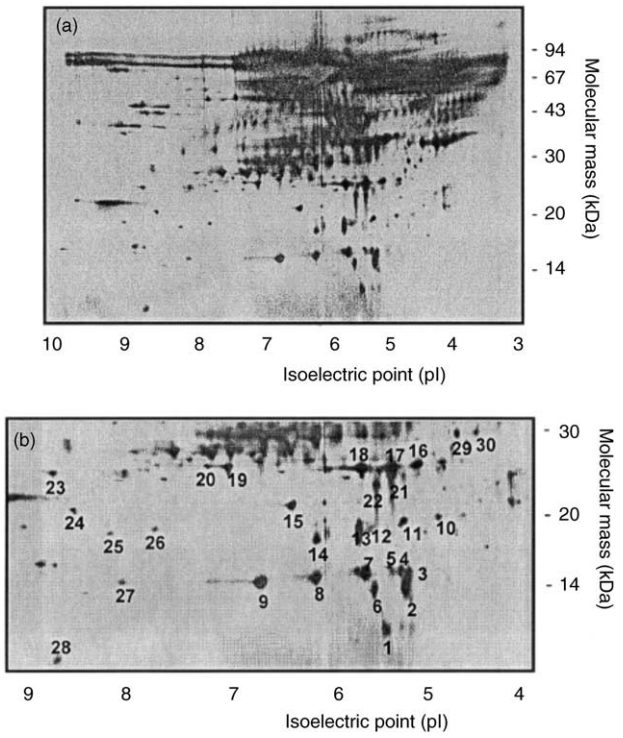

Fig. 1. (a) 2D-PAGE protein profile of CM from PC3 cells. Proteins in the CM were resolved by 2D-PAGE and silver stained. (b) A limited area, presented on the panel A, was analysed by MALDI-TOF MS.

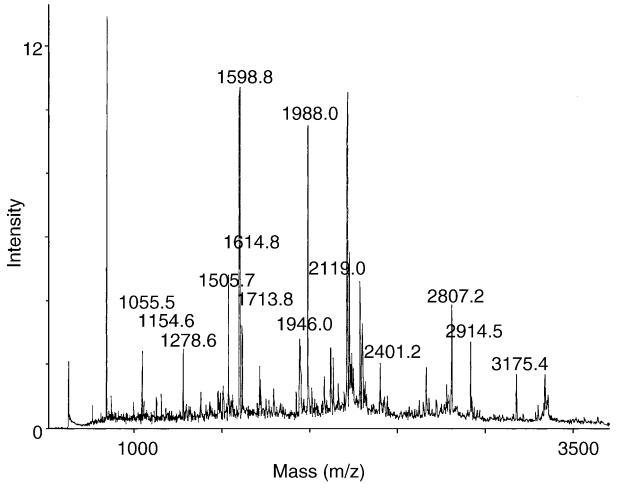

Fig. 2. Peptide mass fingerprint obtained for spot number 26. Proteins in the CM from PC3 cells were separated by 2D-PAGE and visualised by silver staining. Spot number 26 was excised from the gel and subjected to in-gel tryptic digestion. Peptide fragments were analysed by MALDI-TOF MS.

3.2. The effect of cyclophilin A on proliferation of hBMS cells

Since it has been proposed that the mitogen derived from PC3 CM stimulates the proliferation of osteoblasts by a mechanism independent of different bone mitogens [14] and that we identified cyclophilin A in the CM from

Table 1
Identification of a 18 kDa protein by MALDI-TOF MS

Fractions	Mass submitted	Mass matched	Peptide sequence
1	1054.530	1054.533	[20]VSFELFADK[28]
2	1153.555	1153.563	[83]FEDENFILK[91]
3	1277.568	1277.574	[134]EGMNIVEAMER[144]
4	1504.720	1504.737	[132]VKEGMNIVEAMER[144]
5	1597.763	1597.737	[56]IIPGFMCQGGDFTR[69]
6	1613.784	1613.732	[56]IIPGFMCQGGDFTR[69]
7	1945.005	1944.994	[1]XVNPTVFFDIAVDGEPLGR[19]
8	2806.219	2806.315	[92]HTGPGILSMANAGPNTNGSQFFICTAK[118]

Protein sequence of cyclophilin A as retrieved from ProFound software search result (www.proteomics.com) (accession no. 3659980, NCBI Entrez). Matched peptides are underlined: [1]XVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHRIIPGF, [61]MCQGGDFTRHNGTGGKSIYGEKFEDENFILKHTGPGILSMANAGPNTNGSQFFICTAKTE, [121]WLDGKHVVFGKVKEGMNIVEAMERFGSRNGKTSKKITIADCGQLE.

PC3 cells, we examined whether cyclophilin A (1, 10, and 100 nM) alone or in combination with IGF-I (10 ng/ml) could increase the proliferation of hBMS cells in the culture to the same degree as PC3 CM. IGF-I was used for co-stimulation, since high levels of plasma IGF-I were associated with an increased risk of developing malignant prostate cancer [24]. The proliferation was assayed by [^{3}H]-thymidine incorporation. As shown in Fig. 3, PC3 CM increased the proliferation of hBMS cells three-fold (302±22%, $P<0.05$). Cyclophilin A at 1, 10, and 100 nM and cyclophilin A at 10 nM combined with 10 ng/ml IGF decreased the proliferation of hBMS cells up to 49±30, 38±29, 50±8, and 60±16% ($P<0.05$), respectively.

3.3. The effect of cyclophilin A on the differentiation of hBMS cells

We next investigated whether treatments of hBMS cells by cyclophilin A would affect their differentiation. The activity of ALP of hBMS cells stimulated by cyclophilin A alone or in combination with IGF-I was measured. As shown in Fig. 4, CM from PC3 cells increased the ALP activity of hBMS cells up to 175±27% of the controls ($P<0.05$). Cyclophilin A alone had no effects on the activity of ALP. IGF-I alone increased the activity of ALP up to 128±11% of the controls ($P<0.05$).

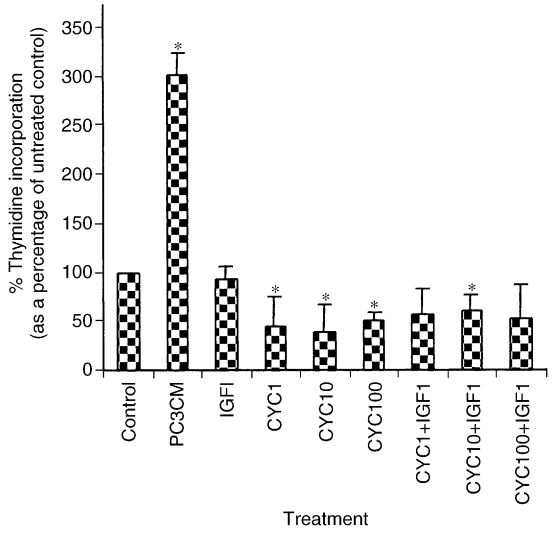

Fig. 3. Effect of CM and cyclophilin A on [^{3}H]-thymidine incorporation of hBMS cells. The hBMS cells were treated with 0.1% BSA–MEM alone (control), PC3 CM, IGF-I (10 ng/ml), cyclophilin A (CYC) alone (1, 10, and 100 nM) or in combination with IGF-I (10 ng/ml). After 24 h, 2.5 μCi/well [^{3}H]-thymidine was added. The hBMS cells were labelled for 24 h. Bars represent the means of nine experiments ($n=9$) + SEM. Significance was tested using the paired two-tailed t-test (*$P<0.05$).

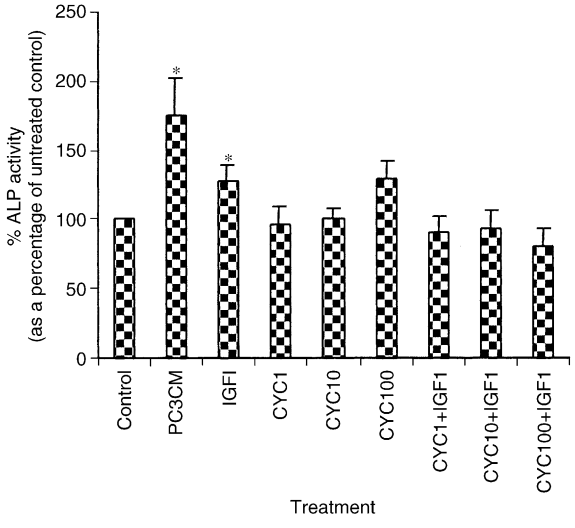

Fig. 4. Effect of CM and cyclophilin A on ALP activity of hBMS cells. The hBMS cells were treated with 0.1% BSA–MEM alone (control), PC3 CM, IGF-I (10 ng/ml), cyclophilin A (1, 10, and 100 nM) or in combination with IGF-I (10 ng/ml). After 96 h, the activity of ALP was measured. Bars represent the means of eight experiments ($n=8$) + standard errors of the means (SEM). Significance was tested using the paired two-tailed t-test (*$P<0.05$).

4. Discussion

A number of studies have shown that prostate cancer tissue contains a factor or factors that exerts mitogenic effects on osteoblast-like cells [7–9,25]. In addition, CM from the human prostatic cancer cell line, PC3, exerts mitogenic effects on human osteoblast-like cells [14]. A number of factors have been proposed as candidates for such an effect. These are: endothelin-l [26,27], high molecular weight urokinase type plasminogen activator (HMW-uPA) [28], and prostate-specific antigen (PSA) [29]. However, it still remains unclear what causes the mitogenic activation of osteoblasts. Therefore, we sought to examine peptides or proteins with molecular weights ranging from 5 to 30 kDa in CM from PC3 cells by proteome analysis using 2D-PAGE and MALDI-TOF MS.

In this study, we demonstrated that PC3 cells secrete cyclophilin A. Cyclophilin A is an immunophilin exerting peptidylproline *cis-trans*-isomerase activity, which is inhibited by cyclosporine, an immunosuppressant. It has been reported that secreted cyclophilin A is present in biological fluids such as synovial fluids [30] and culture medium supernatants from lipopolysaccharide-stimulated RAW 264.7 murine macrophages [31] and from FGF-stimulated mouse embryo 3T3 fibroblasts [32]. It was demonstrated that cyclophilin A possesses functions usually ascribed to cytokines and growth factors [31,33–35]. Cyclophilin A is a secreted growth factor in response to oxidative stress in VSMCs and it increases the proliferation of VSMCs [19]. In another study, it was shown that the proliferation of human embryonic brain cells was increased in response to cyclophilin A [36]. Based on these investigations, we hypothesised that cyclophilin A could be involved in the increased response of proliferation and differentiation in hBMS cells by PC3 CM. However, in this study we demonstrated that cyclophilin A decreases the proliferation of hBMS cells. This decrease was not significantly affected by IGF-I. In addition, cyclophilin A alone or in combination with IGF-I did not have any effect on the differentiation of hBMS cells.

The cytosolic concentrations of cyclophilin A in normal cells has been compared with the concentrations in tumour cells. The concentration of cyclophilin A was greater than normal in T-cell acute lymphocytic leukaemias and in mucosal cells from colonic tumours [37,38]. To our knowledge, cyclophilin A has not previously been correlated to prostate cancer. However, it has been shown that another immunophilin, FKBP51, is upregulated by androgens in the androgen-dependent human prostate cancer cell line, LNCaP [39]. These observations suggest that cyclophilin A could play a role in the progression of prostate cancer. Prostate cancer cells may secrete cyclophilin A, which participates in autocrine and paracrine stimulation of growth, either via increasing the proliferation of prostate cancer cells and/or inhibiting the proliferation of cells in their surroundings such as osteoblast-like cells.

In conclusion, 2D-PAGE-based proteome analysis of CM from the human prostate cancer cell line, PC3, demonstrated significant amounts of cyclophilin A. However, cyclophilin A was not involved in the proliferation or the differentiation of hBMS cells and, therefore, may not be involved in the osteosclerotic response of prostate cancer metastases.

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