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Paracrine Factors Produced by Bone Marrow Stromal Cells Induce Apoptosis and Neuroendocrine Differentiation in Prostate Cancer Cells

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Abstract

Background—Preferential bony metastasis of human prostate cancer (PCa) cells contributes to disease mortality and morbidity. Local factors in bone stromal extracellular matrix microenvironment affect tumor growth through paracrine interactions between tumor and stromal cells.

Methods—Using co-culture and medium transfer, we used several methods to assess interactions between PCa and bone stromal cells using three PCa cell lines: PC3, LNCaP, and the LNCaP derivative, C4-2B.

Results—Co-culture of LNCaP and C4-2B cells with bone marrow stromal cell lines, HS27a and HS5, decreased cell number, as did culture with conditioned medium (CM) harvested from these two cell lines suggesting a soluble paracrine factor was responsible. PC3 cell growth was unaffected. CM harvested from bone stromal cell lines triggered apoptosis in LNCaP and C4-2B cell lines, but not in PC3 cells. Surviving C4-2B cells grown in bone stromal cell CM over several days were growth arrested, suggesting presence of a growth inhibitor. Apoptosis induced by CM was dose-dependent. Flow cytometry demonstrated that over a five day culture period in stromal cell CM, LNCaP and C4-2B cell lines, but not PC3 cells, underwent greater apoptosis than parallel cultures in SF medium. The LNCaP and C4-2B cells showed morphology and biomarker expression consistent with transdifferentiation towards a neuroendocrine phenotype after exposure to stromal cell CM.

Conclusions—The reactive bone stromal microenvironment initially is hostile to PCa cells producing widespread apoptosis. Activation of transdifferentiation in a subset of apoptotic resistant cells may support phenotypic adaptation during disease progression in bone, eventually favoring lethal disease.

Keywords

prostate cancer; bone metastasis; bone stroma; apoptosis; neuroendocrine differentiation

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Introduction

Prostate cancer (PCa) is one of the leading cancers diagnosed in men and a major cause of male-related cancer death. Bone is nearly twice as likely to be involved in PCa metastasis as are other organs, a phenomenon clearly related to the tumor microenvironment (1). When PCa metastasizes to bone, the patient five year survival rate drops to near 30% from virtually 100% when disease remains confined to the prostate (2). Bone PCa cell lesions are characterized as osteosclerotic, with major phenotypic and genotypic changes occurring including development of androgen independence and transdifferentiation to other mesenchymal and neuroendocrine differentiated (NED) phenotypes (3–5). The rationale behind the preference of PCa cells for growth in bone marrow remains unclear. The “Seed and Soil” hypothesis explains the nonrandom trends of breast cancer metastasis and it also has been used to describe the behavior of metastatic PCa cells (6). This model suggests that there are homing factors in the bone microenvironment to support growth of PCa cells, but there is very little convincing evidence that PCa cells are specifically attracted to bone (7). Rather, mounting evidence suggests that bone enriched growth factors, cytokines, proteases and components of ECM in bone marrow provide a hospitable environment for docking circulating PCa cells (8–10). Recent data in our laboratory and others (11–12) showed that the initial interactions between bone stromal and PCa cells are not always positive ones. The complex bone microenvironment may, in fact, initially resist the newly resident PCa cells that can arrive there via either vascular, perineural or lymphatic routes, leading to latency which is typical of patients with delayed reoccurrence of PCa in bone. In such cases, it is most likely that the genetic signature of proximal PCa cells and bone stromal cells co-evolve to eventually support growth of bone metastases (13–14). Such adaptive changes could allow a subpopulation of PCa cells to survive in the bone and eventually support the re-emergence of lethal disease. It is noteworthy that the presence of cells with a NED phenotype in PCa tumors in bone is associated with resistance to apoptosis and poor patient prognosis (15).

In this work, we used two osteoblastic PCa cell lines: LNCaP and its subline C4-2B, to simulate the paracrine interactions occurring between PCa cells and bone stromal cells when they first encounter one another in the bone microenvironment. Other cancer cell lines, including the osteolytic PCa cell line, PC3, and mesenchymal cells served as specificity controls. The important findings from these studies indicate that soluble factors produced by bone marrow stromal cells initially mount a defense against PCa cells arriving in the bone environment, but that this resistance may be overcome by transdifferentiation of a subpopulation of the cancer cells, specifically toward an NED phenotype which ultimately favors the development of a lethal disease phenotype (16).

Materials and Methods

Cell lines

The LNCaP series of PCa cell lines serve as an excellent cell culture model of PCa progression from androgen sensitivity to insensitivity (17). The androgen independent C4-2B cell line was derived from androgen sensitive LNCaP tumors grown in castrated mice. Unlike their LNCaP parents, C4-2B cells can spontaneously metastasize to bone. PC3 cells, a lytic PCa cell line that more closely resembles breast cancer than PCa when grown in bone, also were used in these studies (16)). HS27a cells and HS5 cells, which represent the two major phenotypes of human bone marrow stromal cells (11,18–19) were purchased from ATCC (Manassas, VA). HS27a, HS5, and PC3 cells were maintained in DMEM (GIBCO/Invitrogen Cat. No.11965, Carlsbad, CA) with 10% (v/v) heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) as described (19). Stocks of LNCaP cells and C4-2B

(subline 4) cells were maintained in T-medium (Invitrogen Cat. No. 02-0056DJ, Grand Island, NY) containing 5% (v/v) heat inactivated FBS as described (10). DU145, T47D, PrEC and MG63 were maintained in DMEM with 10% FBS. All cells were transferred into DMEM at the start of the experiments.

Co-Culture

LNCaP cells, C4-2B cells and PC3 PCa cells were seeded at 1×10^5 in 6-well plates. HS27a cells and HS5 cells were mixed [1:1] and seeded in the culture inserts [0.4 μ m] (NUNC Cat. No. 140640, Rochester, NY) at 1×10^5 each for a total of 2×10^5 stromal cells. After the HS27a/HS5 stromal cells attached, medium was removed and the inserts were placed on top of the LNCaP cells, C4-2B cells or PC3 cells in the 6-well plates in the same serum free (SF) DMEM medium surrounding the PCa cells. Media were changed every other day. At the designated time points over a five day co-culture period, cells were detached using trypsin-EDTA (Invitrogen) and counted using a hemocytometer.

Conditioned medium (CM)

HS27a cells and HS5 cells were grown separately in DMEM with 10% (v/v) FBS (Invitrogen) as described above in T-75 flasks until confluent. Cells were rinsed once with 1 X PBS (Invitrogen) and then switched into SF DMEM. Conditioned media (CM) were harvested every two days for two weeks and then all media were pooled. Cells that detached during this culture period were removed along with the medium. The CM from the two lines were mixed together in equal proportions and then filtered through a 0.22 μ m pore size SteriCup (Millipore Billerica, MA) to remove cells and cell debris. Filtered CM were aliquotted and stored frozen at -80°C until use.

WST assay

All cells for WST assay were seeded into 96 well plates at 1×10^3 cells/well. WST assays were performed using Cell Proliferation Reagent WST-1 (Roche, Cat No. 11644807001, Indianapolis, IN). After 30 min, absorbance was measured at 450 nm using a Dynex MRX ELISA plate reader.

Apoptosis assay

All PCa cells for apoptosis assays were seeded into 24 well plates at 1×10^5 cells/well. Cells were harvested using trypsin-EDTA after treatments as indicated for individual experiments. Cell pellets were collected and apoptosis assays were performed using Cell Death Detection ELISA^{plus} (Roche, Cat. No. 11920685001, Indianapolis, IN), which monitors DNA fragmentation.

FACS assay

All PCa cells were grown in T-25 flasks in T-medium containing 5% (v/v) FBS until confluent, after which medium was replaced with stromal cell CM mixed with fresh DMEM [1:1]. Cells were harvested using trypsin-EDTA at individual treatment time points as indicated in the figures then cell pellets were collected. Using a test population of 1×10^6 cells, the percentage of cells undergoing apoptosis under these conditions was assessed using a FACSCalibur (Becton Dickinson, San Jose, CA) and a commercial kit [Vybrant FAM Caspase 3 and Caspase 7, Invitrogen, Cat. No. V35118] that measures the presence of active caspases 3 and 7. Labeled cell fractions were analyzed using fluorescence activated cell sorting (FACS) software CellQuest v3.3 (Becton Dickinson).

Western blotting

Lysates from confluent cultures of PCa cells treated with and without CM were obtained using RIPA buffer (1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15M NaCl, 0.01M Na₃PO₄ (pH7.2), 2mM EDTA, 50mM NaF). LDS sample buffer and reducing agent (NuPAGE) were added to 30 µg of total cellular protein before boiling for 10 min at 95°C, and subjected to SDS-PAGE. Protein was transferred to a PVDF membrane using NuPAGE Transfer Buffer (NP0002), and blocked for 3 hrs in PBS containing 0.1% Tween 20 (v/v), and 4% (w/v) BSA. Anti-NSE (Dako Cytomation, 0873, clone B13S/NCVIH14, Denmark) was applied at a 1:1000 dilution overnight at 4°C. Anti-actin (Sigma Aldrich, A2066) was applied at a 1:5000 dilution overnight at 4°C. A horseradish peroxidase-linked anti-mouse antibody (GE Healthcare Life Sciences, NA931V) was used for detection of anti-NSE, and a horseradish peroxidase-linked anti-rabbit antibody (GE Healthcare Life Sciences, NA9340V, GE Healthcare Life Sciences) was used for detection of anti-actin. Secondary antibodies were applied at a 1:5000 dilution for 30 min at room temperature. After washing with PBS-T, protein was detected using enhanced chemiluminescent detection reagent (Millipore, Immobilon Western Chemiluminescent HRP Substrate, WBKLS0500), followed by exposure to X-ray film (Denville Scientific, Hyblot CL, E3018). The membrane was stripped between incubation with primary antibodies for 15 min at room temperature (Thermo Scientific, Restore PLUS Western Blot Stripping Buffer).

Immunofluorescence assays for NED

PCa cells (1×10^4) were grown in 8-well chambers (Lab-Tek Cat. No. 155411 Rochester, NY) and treated with CM prepared as described above. After five days, cells were washed three times to remove unattached cells, and the cells remaining on the plate were incubated with primary antibody to NSE (Dako, Cat. No. 0873, 1:1000) at 37°C for one hour. After this time, the secondary antibody was added for 30 min at room temperature in PBS. Draq 5 (Biostatus Limited) 1:1000 dilution then was added for five min as a nuclear counterstain. Cells were washed again three times in PBS and photographed using a confocal microscope (Zeiss LSM 510 VIS attached to an Axiovert 100M).

Quantification of cellular processes

LNCAp and C4-2B cells were plated the day before beginning of experiments at 1×10^4 cells per cm² in 60 mm plates. Cells were treated with DMEM/5% (v/v) FBS or HS-5 CM/5% (v/v) FBS for 72 hrs. Photomicrographs were taken from five random regions of each plate at 100X magnification using a Nikon eclipse inverted microscope (TE2000-U, Nikon, Tokyo, Japan). Length of processes was measured semi-manually in Adobe Photoshop by measuring the length of the straight line connecting the end of the longest process to the cell body. Number of processes and branch points were counted manually. Statistical analyses were performed using student t-test. All experiments were repeated a minimum of three times.

Results

LNCAp and C4-2B cells, but not PC3 cells, exhibited decreased cell proliferation when co-cultured with bone stromal cell lines

To study how the bone microenvironment affects PCa cell growth, we used a Transwell system to co-culture two mixed bone stromal cell lines, HS27a and HS5, with one of the PCa cell lines, LNCAp, C4-2B, or PC3. As shown in Figure 1, all three PCa cell lines either maintained or increased cell number over the first three days in SF medium. Between days 3 and 5, the growth of both LNCAp and C4-2B cells declined in the absence of serum (Figure

1A,B). In contrast, the growth of the PC3 cells continued over the five day period (Figure 1C). Surprisingly, when co-cultured with HS27a/HS5 stromal cells in SF medium, the numbers of the LNCaP and C4-2B cells declined significantly by days 3 and (Figure 1A,B; $p < 0.01$). No such decline was evident in the PC3 cells co-cultured with the bone stromal cells (Figure 1C). This suggested that the bone stromal cells secreted factor(s) into their environment that limited the growth of the two osteoblastic PCa cell lines, LNCaP and C4-2B, but not that of the osteolytic PC3 cells.

Conditioned medium harvested from bone stromal cell lines decreased proliferation of LNCaP and C4-2B cells, but not PC3 cells

To study further how the bone stromal cells affect PCa cell growth, we harvested bone stromal cell conditioned medium (CM) and used these in all our later studies. The PCa cell lines were treated with CM or SF medium over a five day time culture period. The WST assay was used as an index of cell proliferation and data were collected every other day. As shown in Figure 2, all cell lines grew in the same manner in SF medium as they did in complete serum-containing medium over the first three days. The WST activity of the LNCaP cells and C4-2B cells (Figure 2A, B) then dropped between day 3 and day 5 time points when cells were grown in SF medium, but continued to grow in the presence of FBS. PC3 cells grew fastest over the first three days, and reached a growth plateau when grown in FBS containing medium before the day 5 time point. When grown in CM, the LNCaP and C4-2B cells displayed noticeably less proliferation at the day 3 time point ($p < 0.01$) compared to cells grown in SF medium. This downward trend continued through day 5. The PC3 cells grew at the same rate in CM as they did in SF medium. This indicated to us that an unknown soluble factor(s) present in the CM harvested from bone stromal cells inhibited growth of PCa cell lines LNCaP and C4-2B, but did not alter the growth of PC3 cells. It also indicated that co-culture was not needed to see the negative effect of the bone marrow stromal cells. Therefore, further work was conducted with CM.

CM harvested from bone stromal cell lines induced apoptosis of LNCaP and C4-2B cells

To study why the cell numbers of LNCaP and C4-2B cells were lower when co-cultured with bone stromal cell lines or when cultured with CM harvested from bone stromal cells, we tested how these treatments affected apoptosis in LNCaP and C4-2B cells. All cells were grown in CM for 2 days after which DNA fragmentation was assessed by ELISA assay. As shown in Figure 3A, both LNCaP cells and C4-2B cells demonstrated considerable apoptosis when grown in CM compared to SF medium. In contrast, PC3 cells grown in CM showed no more apoptosis than seen when they are grown in SF medium. The lack of apoptosis in SF alone showed that apoptosis in LNCaP and C4-2B cells could not be attributed solely to nutrition depletion (Figure 1A). To study the specificity of the apoptosis effect, we also tested various other cell lines (Figure 3B). DU145 is another osteolytic PCa cell line that was isolated from a brain metastases. T47D is a breast cancer cell line. MG63 is an osteoblastic cell line derived from an osteosarcoma and PrEC represents a normal prostate epithelial cell line. None of these cells showed significant apoptosis when grown in bone marrow stromal cell CM than they did in SF medium.

To determine if the factor in CM that negatively affected growth of PCa cells was common to all stroma, we next tested if CM harvested from other stromal cells would cause apoptosis in the three PCa cells. Neither the CM harvested from foreskin fibroblasts (Figure 4A) nor prostate stromal cells (Figure 4B) induced apoptosis in LNCaP cells or C4-2B cells. This suggested that the CM harvested from bone stromal cell lines specifically induced apoptosis in LNCaP and C4-2B cell lines, but not other cell lines.

The apoptosis induced by CM is dose dependent

In all the CM experiments described above, we mixed CM half and half (v/v) with new SF medium and treated cells with the mixture. This was done to ensure the continuous presence of nutrients present in cell culture medium. To study if the negative effect of CM was dose dependent, we performed a dilution experiment. We mixed different ratios of CM with new SF medium and supplied these mixtures to C4-2B cells as growth medium. As shown in Figure 5, the more CM that was present in the mixture, the greater the amount of apoptosis that was seen. This indicates that the factor(s) in CM that negatively impacts growth of C4-2B cells functions in a concentration dependent manner.

FACS analysis showed increased apoptosis of LNCaP and C4-2B cells, but not of PC3 cells

Next, we performed a FACS analysis to determine the percentage of cells undergoing apoptosis in the presence of CM. We cultured the various PCa cell lines either with CM or SF medium. At various time points, the cells were harvested and labeled with fluorescent caspase 3 and 7 antibodies that only recognize the active forms of caspases 3 and 7. As shown in Figure 6A, the peaks represent the fluorescent cell population. The greater the shift of the peak to the right, the more fluorescent apoptotic cells in the entire population. Both LNCaP and C4-2B cells showed a fluorescent shift to the right when treated with CM (shown as arrow) compared to SF medium. The percentage of fluorescent cells in M1 region when treated with SF medium were normalized to 1% and then the same M1 region was used for the corresponding CM treated cells. As shown in Figure 6B, both LNCaP and C4-2B cells demonstrated a 6 to 8 percentage point increase of cell death at the day 3 time point and up to 10% increased cell death at the day 5 time points. In contrast, the PC3 cells had no fluorescence shift over the five day time points, indicating that they were not undergoing measurable apoptosis.

LNCaP and C4-2B cells underwent NED differentiation when grown in CM

When LNCaP and C4-2B cells were grown in CM, their cell morphology changed obviously. As shown in Figure 7, after treatment with SF medium for 5 days, all the PCa cells maintained an epithelial cell cobblestone morphology. After treatment with CM for 5 days, many of both the LNCaP and C4-2B cells developed long and narrow processes. When stained with the NED marker NSE, the processes reacted strongly, positive (arrows). Western blot data (Figure 8A) showed that both LNCaP and C4-2B cells had more NSE expression when cultured with CM from HS5/HS27a cells than those with serum-free medium alone. This was not seen with PC3 cells that had higher basal levels of NSE than did either of the other two cell lines. C4-2B cells also increased NSE in response to CM from HS5 cells alone (not shown). In some cases, LNCaP cells cultured with HS5 CM did not show a similar increase in NSE, suggesting that progression may favor the ability of the LNCaP series to undergo NED in response to bone stromal factors. In the PC3 cells treated with CM, neither the morphology nor the NSE levels changed. As shown in Figure 8B, the process length of both LNCaP and C4-2B cells were increased when grown in CM. The change was more significant for the C4-2B cells than for the LNCaP cells.

Discussion

It long has been recognized that PCa preferentially metastasizes to bone. Previous theory described this process as a “Seed and Soil” effect similar to that described by Paget (20–21). The bone microenvironment is very rich in growth and nutritive factors, and during late stage disease bone marrow provides a supporting growth condition for the lethal metastatic PCa cells that arrive there (22). Studies have shown that factors in human bone marrow can stimulate growth of the PCa cell lines PC3 and DU145, both of which cause lytic bone

degradation (23). Unlike the tumors associated with these cell lines, prostatic tumors typically stimulate osteoblastic activity and produce lesions described as osteosclerotic or osteoblastic (24–26). To test if factors produced by the bone microenvironment favor osteoblastic PCa cell growth in co-culture, we used LNCaP and its sublines, which share similarities with human PCa progression including loss of androgen sensitivity and progressive invasiveness (17).

We first co-cultured LNCaP and C4-2B cells with the mixed bone stromal cell lines HS27a and HS5, which represent the two major types of bone marrow stromal cells in the cancer niche. As initially described, HS-27a supports cobblestone area formation by early hematopoietic progenitors, whereas HS-5 secretes multiple cytokines that support the proliferation of committed progenitors (27). The culture insert method we employed allows cells in two different compartments to exchange soluble factors without forming cell-cell contacts. In contrast to what we expected, LNCaP and C4-2B cells both failed to show increased cell growth when co-cultured with bone marrow stromal cells. On the contrary, the number of PCa cells dropped after 3 days. A similar effect was seen when these cells were treated with CM harvested from HS27a and HS5 cells where the cell number dropped considerably after 3 days, continuing through 5 days. These results suggested to us that the bone stromal cell conditioned environment has an inhibitory affect on PCa cell growth, at least in the initial period when the epithelial-derived PCa and mesenchymal stromal cells are introduced to one another. This result is not unlike the reported findings of Lang *et al.* (28) who reported that CM from fresh human red bone marrow did not affect growth of PC3 cells.

Interestingly, the CM harvested from bone stromal cells not only inhibited PCa cell growth, but also induced their apoptosis. In the presence of CM, the LNCaP cells and C4-2B cells showed considerably more cell death than did cells grown in SF medium, and this effect was concentration dependent. The specificity of this effect was evident when we tested several other PCa cell lines. Neither PC3 nor DU145 cells underwent apoptosis in the presence of stromal cell CM. This difference suggests that LNCaP and its subline C4-2B are susceptible to factor(s) produced by bone marrow stromal cells that impact their survival and apoptotic pathways. The breast cancer cell line T47D did not demonstrate appreciable apoptosis after treatment with stromal cell CM. Like PC3 and DU145 cells, breast cancer cells typically form osteolytic lesions in bone (29). Additionally, neither an osteoblastic cell line, MG63, nor a “normal” prostate cell line, PrEC, underwent apoptosis in response to CM. This indicates that the active factor(s) in bone marrow stromal CM does not target local osteoblasts, nor is the factor detrimental to normal non-metastatic prostate cells, hence might be an effective tumor-specific targeting agent(s).

To determine if the factor(s) in bone marrow stroma that negatively impact growth of LNCaP and C4-2B cells are produced by mesenchymal cells in the prostate or by normal human fibroblasts, CM harvested from foreskin fibroblasts or prostate stromal cells was substituted for bone stromal CM. Both of these failed to induce apoptosis in the LNCaP and C4-2B cells, thus the inhibition of cell growth and apoptotic effect of bone stromal cell CM appears to be specific for osteoblastic PCa cells. The most likely explanation for all of these observations is that the receptors for the bone stromal factor(s) are acquired by metastatic PCa cells that can form osteoblastic lesions sometime during disease progression.

Interestingly, when grown in CM harvested from bone stromal cells, the surviving LNCaP and C4-2B cells demonstrated a striking change in cell morphology. The cells lost the typical epithelial cell cobblestone phenotype and cell-cell contact was greatly reduced. Cells became elongated to a spindle shape during which many formed long processes. This phenotypic change indicates that these cells undergo a transdifferentiation in the presence of

factor(s) produced uniquely by bone marrow stromal cells. Both LNCaP and C4-2B cells grown in CM stained positive for the NED marker NSE, indicating that the morphology change was accompanied by changes in gene expression that resembled the neuroendocrine cell phenotype. Western blots also showed an increased level of NSE in LNCaP and C4-2B cells after treatment with CM. It is well known that an increase in cells showing signs of NED in biopsy specimens is a negative prognostic indicator for PCa patients (30–31). The findings reported here in the co-culture models make it intriguing to speculate that the development of osteoblastic lesions in late stage PCa may involve both the well reported phenomenon of osteomimicry (3), and also an increase in the likelihood that PCa cells themselves will undergo neuroendocrine differentiation.

Conclusion

In figure 9, we propose a model of paracrine interaction between PCa cells and bone stromal cells that favors transdifferentiation of PCa cells under the influence of factor(s) produced by bone stromal cells. In our model, we propose that cross talk of bone stromal cells and PCa cells involving secreted factor(s) made by bone stromal cells induces apoptosis of a significant number of PCa cells. Surviving PCa cells undergo a series of gene expression and morphological changes that include NED, further adapting them to growth in the bone microenvironment leading to disease progression and acquisition of a lethal tumor burden. In this model, supported by our data and the earlier data (28), bone marrow is at best a growth static environment. Acquisition of a cell population through NED that allows cells to survive may be owed to their ability to mimic long lived neural cells (32).

If one accepts this model, it follows that the bone microenvironment initially is not a hospitable environment for PCa cells that have not been adapted previously to growth in bone. This is seemingly counterintuitive because advanced PCa is known to be a bone homing disease. Additionally, the findings are surprising because the C4-2B cell line was isolated from LNCaP cells first grown in castrate mouse model to select for cells capable of growing without androgen, then further selected for cells that can grow in bone (17). The clear susceptibility of C4-2B cells to factors produced by bone marrow cells may indicate that they lose, at least to some extent, their resistance to bone marrow stromal factors when maintained as cell culture stocks in standard growth medium. In our future work, we plan to study further the phenotypic and gene expression changes of the surviving PCa cells in the presence of bone marrow stroma. We also plan to identify the soluble factor(s) in the CM that induce the PCa cell apoptosis. Such information can have long term benefits that include the potential to develop new therapeutic targets that reduce the ability of osteoblastic PCa cells to grow in bone, which unlike osteolytic cancer cells may not be inhibited for the long term by current therapies that target bone resorption such as the bisphosphonates (33–34).

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Figure 1. Prostate cancer cell lines respond differently to co-culture with bone marrow stromal cells

Cells were counted after co-culture with a mixture of bone stromal cell lines HS27a and HS5 for 1, 3 or 5 days as described in the text. LNCaP (A) and C4-2B (B) demonstrated reduced growth when co-cultured with bone stromal cell lines compared to SF medium. PC3 (C) had the same growth pattern regardless of condition; ($p < 0.01$).

Figure 2. Bone stromal cell conditioned medium (CM) reduced LNCaP (A) and C4-2B (B) cell proliferation but not PC3 (C) cell proliferation

Cells were seeded into 96 well plates and treated with complete medium (10% (v/v) FBS), SF medium or bone stromal cell conditioned medium (CM). Media were replaced every other day. Cell growth was estimated by WST activity (see Methods and Materials) on days 1, 3 and 5; ($p < 0.01$).

Figure 3. Conditioned medium harvested from bone stromal cell lines triggered apoptosis in LNCaP cells and C4-2B cells, but not PC3 cells (A) or other tested cell lines (B) Cells were seeded into 24 well plates and then treated with SF medium or CM for 2 days. Apoptosis was measured by DNA fragmentation ELISA (see Methods and Materials). For individual experiments, the basal level of absorbance at 405 nm varied, so all CM treated cell cultures were compared to corresponding SF treated cell cultures; (* $p < 0.05$, ** $p < 0.005$).

Figure 4. Conditioned medium harvested from other stromal cell lines failed to induce apoptosis in LNCaP and C4-2B cell lines

(A.) Cells were treated with CM collected from cultured foreskin fibroblasts. (B.) Cells were treated with CM from cultured prostate stromal cells. Foreskin fibroblasts and prostate stromal cells were seeded into T-75 flasks until confluent, after which CM were harvested and filtered through 0.2nm filters. Prostate cancer cell lines were treated with CM for two days.

Figure 5. Apoptosis induced by conditioned medium (CM) from bone stromal cells is dose dependent

C4-2B cells are shown in the figure. Cells were treated with different ratios of SF:CM medium. A DNA fragmentation ELISA assay (see Materials and Methods) was used to measure the extent of apoptosis.

Figure 6. FACS assay showed increased apoptosis of LNCaP and C4-2B cells, but not of PC3 cells

(A.) FACS scattergrams showed increases of FAM-caspase 3&7 positive cells as “right shifts” (arrows). Cells were grown in T-25 flasks until confluent and then treated with CM or SF medium. Day 5 time points are shown in the figure. The number of cells that shifted into the M1 region was counted and plotted as shown in panel B. (B.) LNCaP and C4-2B cells showed increased cell death over 5 days in CM. The percent of SF induced cell death was standardized to 1.0 and relative CM increases in cell death were plotted.

Figure 7. Confocal microscopic images showed that surviving LNCaP and C4-2B cells undergo significant neuroendocrine differentiation (NED)

C4-2B cells, and to some extent LNCaP, but not PC3, underwent NED when grown in CM harvested from bone stromal cell lines. Cells were seeded into eight well microscopic growth chambers and then treated with CM or SF medium for 5 days. Cells were stained with NSE antibody for NSE (see text) and with Draq5 for nuclei. Magnification 40X.

A.

B.

Figure 8. Both LNCaP and C4-2B cells cultured with CM develop longer processes and increase expression of NSE

Cells were treated with HS5/HS27a CM for 72 hours (see Materials and Methods). Panel A: Western blot analysis showed increased expression of NSE in LNCaP and C4-2B cells, but not PC3 cells. See text for details. Panel B: Morphological quantification of cells showed a dramatic increase in process length; (* $p < 0.05$, ** $p < 0.005$).

Figure 9. Proposed model for paracrine stimulated transdifferentiation between bone stromal cells and PCa cells in the bone microenvironment

PCa cells in the bone microenvironment engage in paracrine cross talk with resident bone stromal cells. As a consequence of the cross talk, some PCa cells undergo apoptosis. The survivors undergo a series of adaptive changes to facilitate growth in the presence of stromal cells, including NED. This model suggests cell death and phenotypic change is induced by adaptive pressure from stromal cells during PCa cell metastasis to bone.