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Advances in Brief

Therapeutic Implications of Enhanced G_0/G_1 Checkpoint Control Induced by Coculture of Prostate Cancer Cells with Osteoblasts[1]

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Abstract

Osteoblastic metastases are common in lethal prostate cancer. Effective therapy for bone metastases is lacking. Thus, developing an appropriate *in vitro* screening system is critical to prioritize which of the newly developed agents should undergo additional expensive and time-consuming *in vivo* evaluation in bone metastases animal models. In the past, such *in vitro* screening evaluated the response of prostate cancer cells to chemotherapeutic agents in monoculture without the presence of osteoblasts. In such monoculture, prostate cancer cells have a high (*i.e.*, >90%) proliferative growth fraction. In contrast, the growth fraction (*i.e.*, mean: 7.1 ± 0.8%; median: 3.1%) in 117 metastatic sites of prostate cancer obtained from 11 androgen ablation failing patients at "warm" autopsy was found to be >10-fold lower. To better mimic the lower growth fraction observed clinically, LNCaP human prostate cancer cells were cocultured with membrane-separated hFOB human osteoblasts. Such coculturing significantly lowered the growth fraction of the LNCaP cells (*i.e.*, from >90 to <30%) without enhancing their low rate (*i.e.*, <5%) of apoptosis. This lowering of the growth fraction was documented using flow cytometry, Ki-67 immunohistochemistry, and 5-bromo-2-deoxyuridine incorporation. Using RNase protection assays, it was documented that coculture with osteoblasts causes enhanced p53, p27, and p21 expression leading to a decrease in the number of LNCaP cells entering the cell cycle (*i.e.*, enhanced number of LNCaP cells in G_0-G_1 and a decrease in S and G_2-M and thus the growth fraction). This osteoblast-induced enhanced G_0-G_1 checkpoint control affected the chemosensitivity of LNCaP cells. This was documented by coculturing LNCaP cells with hFOB cells to condition the medium for 3 days to lower the growth fraction to <30% before exposing the LNCaP cells for 48 h to various concentrations of Taxol, doxorubicin, or thapsigargin (TG). In standard high (*i.e.*, >90%) growth fraction cultures (*i.e.*, cultures in the absence of osteoblast-conditioned medium), there was a dose-dependent and significant ($P < 0.05$) increase in apoptosis of LNCaP cells exposed to Taxol or doxorubicin. In contrast, even the highest dose of Taxol (1 μM) did not enhance apoptosis of lower growth fraction LNCaP cells cultured in osteoblast-conditioned medium. Similarly, only the highest concentration of doxorubicin (1 μM) enhanced apoptosis in lower growth fraction cells. In contrast, 100 nM TG induced high levels of apoptosis in both lower and high-growth fraction LNCaP cultures. These results demonstrate that the osteoblast/LNCaP coculture system is a better *in vitro* screen than monoculture to identify proliferation-independent agents for the treatment of prostate cancer bone metastases, and TG is such an agent.

Introduction

Prostate carcinoma is the most common male malignancy in the United States and the second leading cause of cancer-related death (1). A significant event contributing to morbidity and mortality associated with prostate cancer is the development of skeletal metastases. Medical or surgical castration can only provide remission, and most patients with advanced prostate cancer metastases to the bone will relapse (2).

Salvage cytotoxic therapy has not been effective in patients with hormone-refractory prostate cancer, in part because of the growth of chemotherapy-resistant tumors in osteoblastic lesions (3). An effective therapy requires a better understanding of the mechanisms involved in the development of chemotherapy resistance of prostate cancer bone metastasis.

The growth of prostate cancer cells in bone lesions can be influenced by growth factors derived from bone marrow, osteoblasts, or from bone matrix products released by osteoclastic resorption. These factors include morphogenetic proteins, heparin-binding fibroblast growth factor, platelet-derived growth factor, insulin-like growth factors I and II, nerve growth factor, TGF-β-1,[3] various cytokines, and other not yet identified paracrine factors (4).

A remarkable fact about prostate cancer is that its lethality is not attributable to a high rate of malignant proliferation but enhanced resistance to apoptosis (5). Previous studies have documented that the proliferative growth fraction of human prostate cancers is usually <10% (5). This is consistent with the low response rates when prostate cancer patients are treated with the commonly used chemotherapeutic agents, the cytotoxicity of which is dependent on high rates of cell division (5, 6). Because of this limitation, novel agents need to be developed which can induce the apoptotic death of androgen-independent prostate cancer cells in a proliferation-independent manner. Historically, screening for agents against prostate cancer has involved the use of *in vitro* cell culture models. In these *in vitro* systems, the proliferative growth fraction of the cancer cells is usually >90%, limiting their usefulness in screening for cell proliferation-independent agents. Attempts have been made to lower the growth fraction of these *in vitro* cultures by means of serum or nutrient deprivation. Unfortunately, such manipulation itself induces the apoptotic death of these cancer cells (7, 8). Thus, an alternative screening system is needed, in which prostate cancer cells can be maintained in a lower proliferative state without undergoing programmed cell death.

We have developed such an *in vitro* lower proliferation/low apoptosis state using an osteoblast/prostate cancer coculture system. In this coculture system, the androgen-sensitive LNCaP human prostate cancer cell line and the conditionally immortalized hFOB human osteoblastic cell line were used. The human hFOB osteoblastic cell line has been conditionally immortalized by the expression of a temperature-sensitive SV-40-large T antigen (9). At the permissive temperature of 34°C, these cells express large T antigen inducing their continuous proliferation (9). In contrast, at 37°C, T antigen is de-

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[3] The abbreviations used are: TGF, transforming growth factor; BrdUrd, 5-bromo-2-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; TG, thapsigargin.

graded, and the cells stop proliferating without undergoing apoptosis (9). We have found that a significant decline in the growth fraction of LNCaP prostate cancer cells (*i.e.,* going from 90 to <30%) can be achieved without induction of apoptosis by culturing LNCaP cells in media preconditioned by coculture of LNCaP with hFOB cells at 37°C.

In this study, we documented that this growth fraction reduction involves specific molecular changes which enhance the G_0-G_1 checkpoint. In addition, we studied the responsiveness to both standard and novel chemotherapeutic agents of the androgen-sensitive LNCaP prostate cancer cells in either high or lower growth fraction cultures induced by exposure to osteoblast preconditioned media.

Materials and Methods

Determination of the Growth Fraction in Clinical Samples and Cultured Prostate Cancer Cells. Tissue was obtained from 117 separate metastatic sites of prostatic cancer from a total of 11 androgen ablation failing patients at "warm autopsy" (*i.e.,* within 4–6 h of death). This tissue was fixed in 10% buffered formalin and embedded in paraffin, and a 5-μm section was cut. As described previously (5), these sections were deparaffined, rehydrated, and processed for immunohistochemical staining using monoclonal antibody (Immunotech, Marseille, France), which recognizes the Ki-67 antigen expressed in cell nuclei when they are in the proliferation cycle (10). Using these Ki-67 stained slides, the growth fraction (*i.e.,* percentage of prostate cancer cells expressing Ki-67 in the nuclei) was determined, as described previously (5). For determination of the growth fraction for cultured LNCaP cells, these cells were harvested by trypsinization, fixed in 10% buffered formalin, and cytospun onto glass slides. These cytospun glass slides were processed for Ki-67 expression, and growth fraction was determined as described for tissue sections.

Determination of Percentage of Cells in S Phase. The percentage of both monocultured and cocultured LNCaP cells in S phase was determined by two methods. The first was by flow cytometry based on DNA content, as described previously (11). The second was by incorporation of BrdUrd into new synthesized DNA using cell proliferation kits obtained from Amersham (Buckinghamshire, United Kingdom), according to the manufacturer's instructions.

RNase Protection Assays. Cells were harvested by trypsinization and pelleted. Total RNA was extracted from the pelleted cells using a Qiagen RNeasy Kit (Valencia, CA), as per manufacturer's recommendations. RNA concentration of the samples was determined using a GeneQuant spectrophotometer (Pharmacia, Peapack, NJ). RiboQuant RPA template sets (BD PharMingen, Franklin Lakes, NJ) were labeled with $\alpha(^{32}P)$UTP for use as probe, as per manufacturer's recommendations. The labeled probe was centrifuged through a 20-kDa membrane cutoff microspin column (Roche Biochemicals, Indianapolis, IN) to remove excess free label, and the retained radioactivity was quantitated in a Beckman scintillation counter. The probe was diluted to a working concentration of 1×10^7 cpm/5-ml hybridization buffer. Five μg of total RNA sample was hybridized with 5×10^5 cpm of probe (250 μl volume) for 16 h at 72°C in a thermal cycler using reagents from an Ambion (Austin, TX) PRA II kit. After digesting unbound RNA and excess probe with a single-stranded RNase as directed, the resulting labeled double-stranded fragments were separated on a 5% acrylamide gel using an 8″ Bio-Rad vertical gel apparatus (Hercules, CA). The gels were dried for 1 h at 60°C using a Bio-Rad gel dryer and exposed to Kodak X-OMAT film for 4–24 h depending on template set and RNA preparations.

Cell Culture and Cell Viability Assay. The human prostate cancer cell line, LNCaP, and the human fetal osteoblastic cell line, hFOB, were obtained from the American Type Culture Collection. The LNCaP cell line was cultured in RPMI medium supplemented with 10% fetal bovine serum. The conditionally immortalized human fetal osteoblastic cell line, hFOB, was maintained in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium containing 10% fetal bovine serum supplemented with geneticin (300 μg/ml) at 34°C, the permissive temperature for the expression of the large T antigen. 10^5 LNCaP cells/well were seeded into Costar 6-multiwell plates. The following day, LNCaP cells were incubated for 3 days at 37.5°C in a 1:1 mixture of DMEM/Ham's F12 medium and RPMI media as monoculture or cocultured with hFOB osteoblasts. For the cocultures, hFOB osteoblasts were seeded into porous (1 μm) inserts (Falcon) at a density of 10^5 cells/insert, and the inserts were maintained at 39°C for 2 days to allow osteoblasts to reach confluence and growth arrest. After the 2-day period, the inserts containing a growth arrested, and the confluent layer of osteoblasts were then placed into separate LNCaP cells containing wells of Costar 6-multiwell plates for coculture experiments. After 3 days of coculture at 37.5°C, the osteoblast containing inserts were removed, and chemotherapeutic agents (*i.e.,* doxorubicin, Taxol, and TG) were added to the unchanged medium at concentrations of 10, 100, or 1000 nM. After 48 h of incubation with various compounds, cells were trypsinized, and 100-μl samples were diluted 1:1 with 0.2% trypan blue dye. A hemocytometer was used to count the total number of viable (*i.e.,* trypan blue excluding) cells from each well. TG was provided by LC Services Corp. (Woburn, MA). Taxol and doxorubicin were obtained from Sigma Chemical Co. (St. Louis, MO).

Apoptosis Assay. After the treatment of LNCaP cells for 48 h with various concentrations of different chemotherapeutic agents, cells were washed with sterile saline, fixed in methanol, and incubated with 1 μg/μl DAPI (Sigma Chemical Co.) in saline to stain nuclear DNA. The percentage of apoptotic nuclei was determined by evaluating nuclear morphology by epifluorescence microscopy.

Statistical Analysis. The data obtained were analyzed by one-way ANOVA. Values represent the mean ± SE. $P < 0.05$ was considered significant.

Results

Differences between the Growth Fraction in Androgen-independent Prostate Cancer Metastases from Patients *Versus* LNCaP Cells *in Vitro*. Previously, we reported that the mean proliferative growth fraction in androgen-independent prostate cancer bone metastases was 4.8 ± 0.8% (range 0.2–9.2%; Ref. 5). These previous data were based on 10 specimens from the Johns Hopkins Department of Pathology Archival Repository. In the present studies, 117 additional metastatic sites were harvested at "warm" autopsies from 11 androgen ablation failing prostate cancer patients. The distribution of growth fraction in the multiple metastatic sites from each patient is presented in Fig. 1*A*. Fig. 1*B* presents the composite frequency distribution for the growth fraction for the entire 117 samples. These analyses demonstrated that the median growth fraction was 3.1%, and the mean was 7.1 ± 0.8%. There were only 2 samples (*i.e.,* 1.7%) with a proliferative growth fraction >30% (*i.e.,* 30.4 and 32.9%). Thus, >98% of these lethal prostate cancer metastases had a growth fraction <30%, with the majority being <5% (Fig. 1, *A* and *B*). In contrast, the growth fraction for LNCaP human prostate cancer cells growing *in vitro* under standard culture conditions was 90.5 ± 5.0% based on Ki-67 expression. This high-growth fraction was confirmed using both flow cytometry and BrdUrd incorporation to identify the percentage of LNCaP cells in the S phase of the cell cycle. These results demonstrate that by flow cytometry, 21.7 ± 0.2%, and by BrdUrd incorporation, 36.2 ± 3.7%, of LNCaP cells in standard monoculture are in S phase. These S-phase data are consistent with a continuously cycling cell population with a 90% growth fraction. These results demonstrate that under standard conditions, LNCaP high-growth fraction cultures are not representative of the lower growth fractions characteristic of clinical (*i.e.,* lethal) prostate cancers from patients.

Effect of Osteoblast Conditioning on Cell Cycle Distribution and Cell Cycle/Apoptotic Modulators in LNCaP Cells. To lower the growth fraction, LNCaP cells were grown in the presence of media conditioned by coculture with osteoblasts. The high (*i.e.,* >90%) growth fraction of LNCaP cells (*i.e.,* percentage of cells expressing Ki-67) was profoundly decreased when these cells were exposed to media conditioned by membrane-separated coculture with osteoblasts (Fig. 2). This lowering of the growth fraction of LNCaP cells induced by osteoblasts conditioning was not attributable to enhancement of apoptosis. This was documented using DAPI nuclear staining to detect LNCaP cells undergoing apoptosis in both osteoblast-conditioned

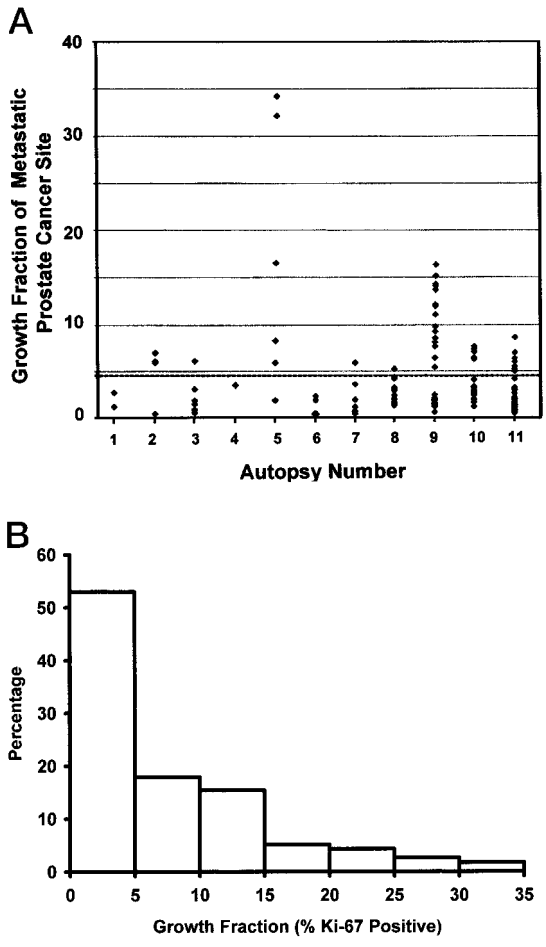

Fig. 1. Distribution of proliferative growth fraction in various metastatic sites obtained from 11 androgen ablation failing prostate cancer patients at warm autopsy. ····, the mean growth fraction (*i.e.*, 4.7%) from the previous study (5); *B*, combined growth fraction distribution for all 117 metastatic sites.

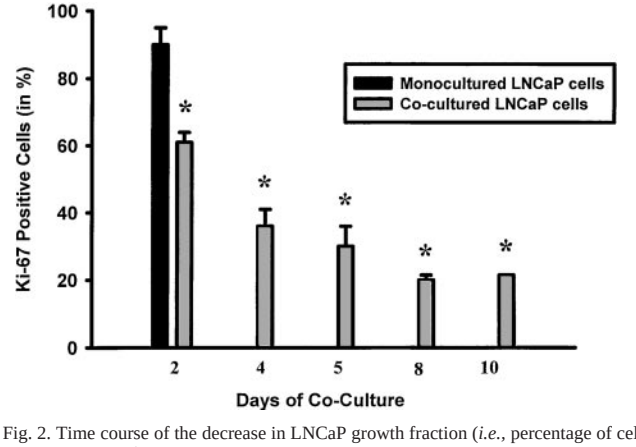

Fig. 2. Time course of the decrease in LNCaP growth fraction (*i.e.*, percentage of cells expressing Ki-67) after exposure to media conditioned by coculture with hFOB osteoblasts. *, $P < 0.05$ *versus* monoculture; values are mean ± SE.

versus -nonconditioned media (*i.e.*, under both conditions, <5% of LNCaP cells had apoptotic nuclear morphology). On the basis of the percentage of cells incorporating BrdUrd into the DNA, the percentage of LNCaP cells in S phase diminished by >50% ($P < 0.05$) from a value of 36.2 ± 3.7% to 17.2 ± 2.8% within 2 days of exposure to media conditioned by osteoblast coculture. During the next several days, the decrease in S phase continued, *e.g.*, by 4 days of exposure to media conditioned by osteoblast coculture, the percentage of LNCaP cells in S phase decreased to 6.2 ± 0.3%, based on flow cytometry analysis.

RNase protection assays documented that exposure of LNCaP cells to osteoblast-conditioned media for 4 days resulted in an increased expression of p53, p27, and p21, as compared with monocultured LNCaP cells (Fig. 3*A*). In contrast, there was no difference in the expression of bcl-2 family genes, such as *bclw*, *bcl-x*, *bid*, *bak*, *bax*, or *mcl1*, between LNCaP cells exposed to media conditioned by osteoblasts *versus* unconditioned media (Fig. 3*B*).

Effect of Decreasing Growth Fraction on Chemosensitivity of LNCaP Cells. Cell viability of high-growth fraction LNCaP cells (*i.e.*, grown in the absence of osteoblast-conditioned medium) exposed for 48 h to various concentrations of Taxol, doxorubicin, or TG was measured by trypan blue exclusion assay. These results demonstrate a dose-dependent decrease in cell viability for each of the test agents (Fig. 4*A*). Total number of viable cells in high-growth fraction LNCaP cultures exposed for 48 h to 100 nM Taxol, doxorubicin, or TG was significantly ($P < 0.05$) decreased by 88, 58, and 84%, respectively, as compared with controls (Fig. 4*A*). In contrast, dose responses to doxorubicin and Taxol were very different using osteoblast-conditioned media to lower the growth fraction during drug treatment. Even the highest dose of Taxol (1 μM) did not affect cell viability of these lower growth fraction LNCaP induced by exposure to osteoblast-conditioned medium (Fig. 4*B*). Similarly, only the highest concentration of doxorubicin (1 μM) significantly decreased the total number of viable prostate cancer cells exposed to osteoblast-conditioned medium, and this was only by 35%, as compared with controls (Fig. 4*B*). In contrast, TG decreased cell viability of both lower and high-growth fraction LNCaP culture significantly and to a similar extent (Fig. 4, *A* and *B*). DAPI nuclear staining revealed that only TG induced apoptosis of both LNCaP cells in osteoblast-conditioned *versus* -nonconditioned media (*i.e.*, 49.5 ± 6% *versus* 51.1 ± 5% of LNCaP cells had apoptotic nuclei by 48 h exposed to 100 nM TG under respective conditions). In contrast, the percentage of apoptotic nuclei after 48-h exposure to either 100 nM Taxol or 100 nM doxorubicin decreased from 48.2 ± 4% and 39.2 ± 5%, respectively, in high-growth fraction cultures to values of 5.2 ± 3% and 4.8 ± 3%, respectively, in lower growth fraction cultures induced by exposure to osteoblast-conditioned media.

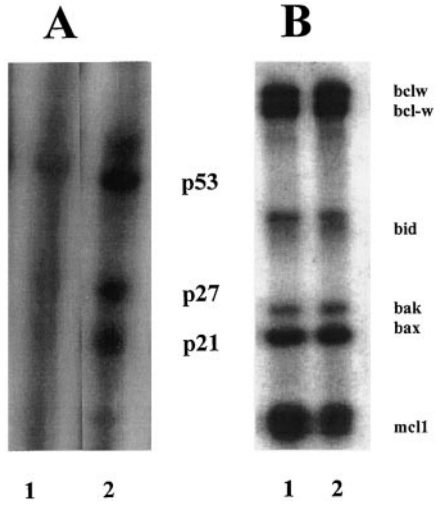

Fig. 3. RNase protection assay of LNCaP cells cultured alone (*column 1*) or exposed for 4 days to media cocultured by osteoblasts (*column 2*) for the expression of the following genes: *A*, *p53*, *p27*, and *p21*; *B*, *bclw*, *bcl-x*, *bid*, *bak*, *bax*, or *mcl1*.

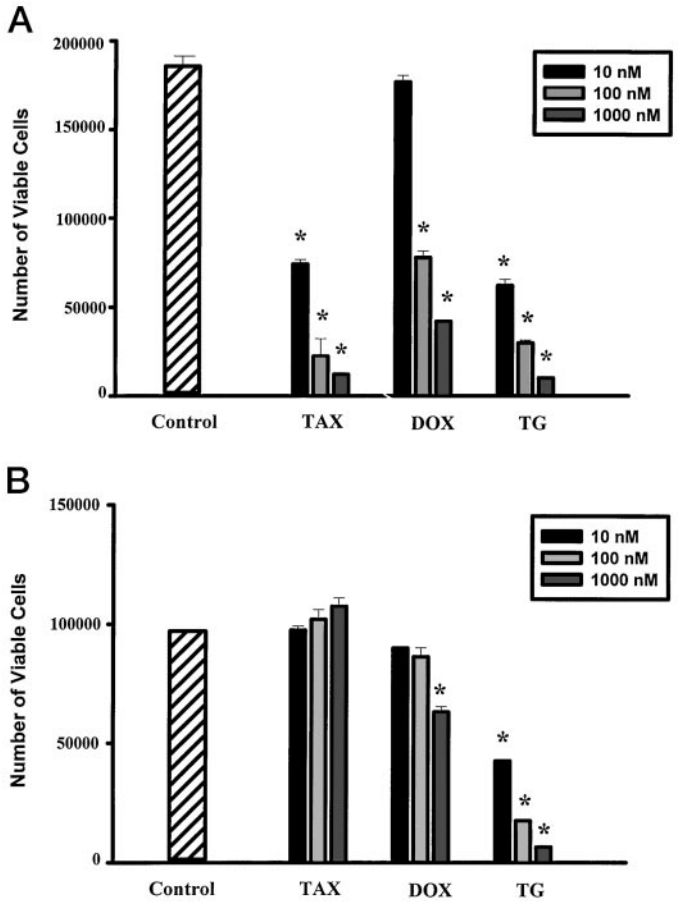

Fig. 4. Total number of viable LNCaP cells, as measured by trypan *blue* exclusion assay, cultured in the absence (*A*) or in the presence of osteoblast-conditioned medium (*B*) and exposed for 48 h to various concentrations of Taxol, doxorubicin, or TG. $P < 0.05$ *versus* control; values are mean ± SE. *TAX*, Taxol; *DOX*, doxorubicin.

Discussion

Prostate cancer frequently metastases to the skeleton producing painful osteoblastic lesions, which are associated with significant morbidity and mortality. This bone tropism involves the bi-directional paracrine interactions between prostate cancer cells and osteoblasts. These interactions enhance prostate cancer cell survival and proliferation of osteoblasts. Prostate cancer cells have been shown to produce peptides such as TGF-β, bone morphogenetic proteins, endothelin-1, and others with selective mitogenic activity for osteoblasts (12). On the other hand, osteoblastic cells release paracrine factors, such as neurotrophins, TGF-β, insulin-like growth factors, platelet-derived growth factors, various cyclins, and others, which cannot only enhance prostate cancer cell survival but also affect the sensitivity of prostate cancer cells to chemotherapeutic agents (4).

To study this latter process of osteoblast/prostate cancer interactions, the LNCaP human prostate cancer cell line was chosen as a model because it expresses androgen receptor, secretes prostate-specific antigen, and while being responsive to androgen stimulation, is androgen independent in its growth requirements (13, 14). Thus, LNCaP cells are representative of the type of androgen-independent prostate cancers which are commonly lethal to patients. The hFOB human osteoblastic cell line was selected as the other partner for these studies because it was conditionally immortalized with large T antigen, making its use in coculture experiments very convenient. In addition, when cultured at the restrictive temperature of 37°C or under confluent conditions, this cell line stops proliferating and instead expresses typical markers for differentiated osteoblasts, such as type I collagen and alkaline phosphatase, and produces extracellular matrix (9).

This study documents that exposure of LNCaP prostate cancer cells to media conditioned by coculture with hFOB osteoblasts increased the expression of p53, p27, and p21, leading to a decrease in the number of prostate cancer cells entering the cell cycle (*i.e.,* lowering the proliferative growth fraction). This lowering of the growth fraction from >90 to <30% is achieved without LNCaP cells undergoing enhanced apoptosis. This allows the routine screening of the sensitivity to chemotherapeutic agents of such lowered growth fraction LNCaP cells. This is critically important because analysis of 117 metastatic sites of prostate cancer obtained from 11 androgen ablation failing patients at autopsy demonstrated that <2% of these lesions had growth fraction >30% (Fig. 1). In such screening studies, the chemotherapeutic response was compared between LNCaP cells in high-growth fraction (*i.e.,* >90%) cultures maintained in standard media *versus* lower growth fraction (*i.e.,* <30%) cultures maintained in media preconditioned by coculture with osteoblasts. In these lower growth fraction cultures, inserts containing the osteoblasts were removed after 3 days of coculture, and the prostate cancer cells were then exposed for 48 h to osteoblast-conditioned medium in the presence or absence of chemotherapeutic agents. During the whole experimental period of time, prostate cancer cells were never in direct contact with osteoblasts, ensuring only paracrine interactions between these two cell lines. The osteoblast containing inserts were removed before initiation of drug treatments to avoid any possibility of complications of drug metabolism by the osteoblasts. These studies demonstrated that agents like Taxol and doxorubicin were highly effective in the high-growth fraction cultures but were much less effective in the lower growth fraction cultures. Thus, for these two agents, the osteoblast-induced enhanced G_0-G_1 checkpoint control decreased the chemosensitivity of LNCaP cells. In contrast, TG was equally effective as an inducer of LNCaP apoptosis in both high- and lower growth fraction cultures. We are presently studying the question, "How will factors secreted by osteoblasts affect the chemosensitivity of prostate cancer cell lines that are derived from bone metastasis and are androgen insensitive?" We are also in the process of identifying some of the osteoblast-derived paracrine factors, which might be involved in the development of chemotherapy resistance of prostate cancer cells.

Previously, we have documented that TG induces programmed cell death of androgen-dependent and -independent rodent and human prostate cancer cells through selective inhibition of the sarcoplasmatic reticulum/endoplasmatic reticulum Ca^{2+}-dependent ATPase pumps (15–20). Inhibition of sarcoplasmatic reticulum/endoplasmatic reticulum Ca^{2+}-dependent ATPase pumps by TG results in a three to four rise in intracellular-free calcium, within 20 min of exposure, attributable to depletion of endoplasmatic reticulum Ca^{2+}-pool, and a sustained elevation of Ca, because of the subsequent capacitive influx of extracellular calcium (15–20). Unlike the other drugs tested in this study, TG's induction of apoptosis is proliferation-independent and occurs subsequent to growth arrest (15–20).

In conclusion, our data demonstrate that exposure of LNCaP prostate cancer cells to media conditioned by coculture with hFOB osteoblasts results in a significant enhancement of the G_0-G_1 checkpoint control in LNCaP cells. This enhanced cell cycle control results in a lowering of the growth fraction, thus decreasing the chemosensitivity of LNCaP prostate cancer cells to Taxol and doxorubicin. The osteoblast/prostate cancer model described in this study is a more relevant *in vitro* screening system than monoculture in identifying novel cell proliferation-independent agents, such as TG, for the treatment of patients with prostate cancer metastasis.

Acknowledgments

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