Introduction

Bone is the predominant site of prostate cancer metastasis, and patients with advanced-stage prostate cancer commonly develop metastatic bone lesions (1). Unfortunately, the pathophysiology of skeletal metastasis is not yet completely understood (2). One major obstacle to better understanding skeletal metastasis is the unusual complexity of the tumor microenvironment in bone (3), due to multiple constituent cell types. Emerging evidence supports that cells in the bone marrow microenvironment are actively involved in prostate cancer metastasis (4).

Bone marrow–derived myeloid lineage cells are critical regulators of tumor progression and metastasis (5–10). Yang and colleagues showed that expansion of Gr-1+CD11b+ myeloid cells directly promotes tumor angiogenesis (6) via increased production of matrix metalloproteinase (MMP)-9 (7). Myeloid cells (expressing surface markers CD11b and/or Gr-1) are a major component of undifferentiated bone marrow cells, and ultimately differentiate into monocytes, macrophages, and granulocytes (10). Parallel to the tumorigenic roles of myeloid cells, monocyte macrophages also have been shown to participate in tumor metastasis (11–13). All of these data collectively support the critical roles of myeloid lineage cells in prostate cancer progression and bone metastasis. However, it is not clearly understood how the alterations in the bone marrow occur, which could provide clues for therapeutic approaches.

In clinical settings, chemotherapeutic drugs and/or irradiation perturb the bone marrow microenvironment, leading to alterations in marrow cellular composition. Although chemotherapy and irradiation are both bone marrow suppressive, the subsequent recovery process may lead to temporary spikes of certain cell types, including monocytes and neutrophils (14, 15). Therefore, net effects of bone marrow–suppressive agents could have pro- or antitumorigenic effects. Interestingly, priming the murine host with cyclophosphamide, a bone marrow–suppressive chemotherapeutic drug, promoted subcutaneous tumor growth and metastasis in several mouse models (16–19). Cyclophosphamide is a DNA-alkylating drug commonly included in chemotherapeutic regimens against breast and lung cancers and non-Hodgkin’s lymphoma. In addition, cyclophosphamide is used in the conditioning regimen for recipients of myeloablative bone marrow transplantation, to enhance engraftment and suppress the host immune reaction. Intriguing data showing opposite prometastatic effects of chemotherapeutic drugs remain poorly investigated.

Cyclophosphamide Creates a Receptive Microenvironment for Prostate Cancer Skeletal Metastasis

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To our best knowledge, the effects of cyclophosphamide on skeletal metastasis have never been reported. Given that prostate cancer has been shown to use similar strategies as hematopoietic stem/progenitor cell homing and that prostate cancer has long been known to home typically to bones enriched with red marrow (20), we hypothesized that alterations induced by cyclophosphamide in the bone marrow microenvironment would contribute to prostate cancer colonization in the bone and/or subsequent tumor growth. In the current study, we investigated prometastatic effects of bone marrow suppression in a prostate cancer skeletal metastasis model and explored the underlying mechanisms that could be used to design methods of therapeutic intervention.

Materials and Methods

Cells
Luciferase-labeled PC-3 cells (PC-3Luc) were established from the PC-3 cell line (American Type Culture Collection; ATCC), as previously described (20). PC-3Luc cells were regularly authenticated and matched short tandem repeat DNA profiles of the original PC-3 cell line (last tested on May 9, 2009).

Mouse models of prostate cancer
All experimental protocols were approved by the University of Michigan Institutional Animal Care and Use Committee. For a skeletal metastasis model, the procedure described by Park and colleagues was followed (21). Briefly, 2 × 10^6 PC-3Luc cells were injected into the left heart ventricle of male athymic mice (Harlan Laboratories). For an orthotopic bone tumor model, 1 × 10^5 PC-3Luc cells were injected in the proximal tibiae as described (21).

Ex vivo murine bone marrow microvascular angiography
Mice were anesthetized and perfused sequentially with heparin-supplemented Ringer’s lactate (9 minutes), formalin (9 minutes), and MICROFIL (Flow Tech, 7 minutes) via the intracardiac route. Following polymerization, femurs were dissected, decalcified, and scanned by microcomputed tomography (µCT).

Neutralizing antibodies
Anti-mouse CCL2 antibody (C1142, Janssen) and anti-mouse interleukin (IL)-6 antibody (R&D Systems) were provided by Janssen, LLC. C1142 is a rat/mouse chimeric antibody specific for mouse C-C chemokine ligand (CCL)2/MCP-1 and does not cross-react with human CCL2 or mouse MCP-5 (23–25). Non-specific IgG from mouse serum (Sigma-Aldrich) was used as a control antibody.

Flow cytometry
Bone marrow cells were collected by flushing femurs and tibiae. Lungs, liver, and kidney were digested in complete Dulbecco’s Modified Eagle’s Medium supplemented with 0.5 mg/mL collagenase (Sigma-Aldrich). One million cells were used for flow cytometry (BD Bioscience).

Complete blood counting with white blood cell differentials
Blood cell counting was carried out in the University of Michigan Unit for Laboratory Animal Medicine, using a Forcyte automatic hematology analyzer (Oxford Science).

Quantitative PCR
The mRNA samples were prepared from the flushed bone marrow cells, followed by real time-polymerase chain reaction (RT-PCR) for CD31 and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Applied Biosystems).

Statistical analyses
Experimental skeletal metastasis experiments were analyzed using linear mixed models. The primary outcome was the natural log transformed bioluminescence measurement. Fixed covariates in the model included the groups in the experiment and time (weeks) and the interaction between group and time. The repeated measures aspect of the model, due to multiple measurements over time within each mouse, was adjusted for using a single order autoregressive correlation structure. Contrasts were used to test the pairwise comparisons of interest. Analyses were completed using SAS (SAS Institute) with a type I error of 5%.

All other statistical analyses, including Kaplan–Meier analyses of metastasis-free mice, Student t tests comparing 2 groups, and Mann–Whitney U tests of samples failing to distribute normally, were conducted with GraphPad Prism.

Results
Cyclophosphamide enhanced experimental prostate cancer skeletal metastasis in vivo
Cyclophosphamide has been shown to promote subcutaneous tumor growth and experimental metastasis in various animal models (16–19). Initially, the effects of cyclophosphamide on prostate cancer skeletal metastasis were investigated. The experimental design is schematically shown in Fig. 1A. The serum half-life of cyclophosphamide is less than 17 minutes (in mice) and 6.5 hours (in human), and mice were allowed 7 days of recovery to insure that the drug was completely cleared, to avoid any direct antitumor effects of cyclophosphamide (26, 27). Interestingly, mice primed with cyclophosphamide developed significantly larger tumors in the hind limb bones after 7 days (Fig. 1B). Cyclophosphamide-treated mice exhibited increased tumor bioluminescence in the mandible also, but the effects were variable and not statistically significant until day 35 (Fig. 1C). Because hind limb skeletal metastases are more clinically relevant, and also murine mandibles are significantly different from human (e.g., continuous eruption of incisors), the hind limb skeletal metastases were the focus of subsequent investigation. Cyclophosphamide-primed mice developed hind limb metastases at an earlier time point (i.e., increased incidence of metastases on day 7, 14, and 21; Fig. 1D), compared with the saline-treated group that developed detectable hind limb metastatic lesions only after 14 days. These data suggest that the larger tumor size on day 42 in the hind limbs of cyclophosphamide-treated mice (Fig. 1E) is
Figure 1. Priming mice with a single administration of cyclophosphamide (CY) enhanced experimental prostate cancer skeletal metastasis. A, schematic representation of the experimental design. Male athymic mice were divided into 2 groups and treated with saline or CY. Following 7 days of recovery, PC-3Luc cells were injected into the left heart ventricle (n = 18 for saline control and n = 13 for CY group). Metastatic tumor growth was monitored by weekly in vivo bioluminescence imaging for 6 weeks. B, hind limb metastatic tumor size was measured by weekly in vivo bioluminescence imaging. Data are medians with interquartile range. Asterisks represent statistical significance (linear contrasts P < 0.01). C, mandibular metastatic tumor size was measured. Data are median ± interquartile range. Asterisks represent statistical significance (linear contrasts P < 0.01). D, percentage of hind limb metastasis-free mice plotted in a Kaplan-Meier curve. Lesions emitting more than 1 × 10^5 photon/sec were considered as metastases, and statistical significance was determined by log-rank test (P < 0.01). E, representative histologic images of metastatic bone tumors. Tumor-bearing hind limb tibiae were dissected, followed by hematoxylin and eosin staining. The presence of metastatic tumor cells was confirmed microscopically. Tumor perimeter is indicated by dotted lines in lower magnification images (×4; top). Higher magnification images (×20; bottom) show tumor, bone, and bone marrow (denoted T, B, and BM, respectively).
attributable to the early events following tumor cell inoculation.

A single dose of cyclophosphamide significantly disrupted bone marrow vascular integrity

Cyclophosphamide has been found to cause damage to endothelial cells, potentially promoting tumor cell seeding in the metastatic target organs (28). These data are consistent with the observation in Fig. 1 showing outgrowth of metastatic tumors at earlier time points in the cyclophosphamide-treated hosts. Consequently, an experiment was designed to test whether a single dose of cyclophosphamide could perturb endothelial integrity in the bone marrow, which might in turn lead to increased extravasation of tumor cells immediately after inoculation. Because immunohistochemistry can only provide 2-dimensional images of selected cross-sections, a technique to reconstruct 3-dimensional vascular structures enclosed in calcified tissues was used (ref. 22; Fig. 2A). In Fig. 2B, this technique clearly showed 3-dimensional structures of microvessels in the epiphyses and the central sinusoidal vessels in the diaphyses of saline controls. In sharp contrast, a single dose of cyclophosphamide very obviously disrupted vascular integrity and continuity (Fig. 2C). Quantification of the images in Fig. 2B and C showed that bone marrow vascular volume was
significantly reduced by cyclophosphamide (Fig. 2D). CD31 (an endothelial-specific marker) gene expression in bone was significantly suppressed with cyclophosphamide administration (Fig. 2E), but not in lungs, liver, and kidney (Supplementary Fig. S1). In addition, bone marrow endothelial cells treated with 4-hydroperoxycyclophosphamide (4-HC, a metabolite of cyclophosphamide with in vitro biologic activity) had significantly increased apoptosis (Fig. 2F). Taken together, cyclophosphamide-induced vascular disruption led to altered endothelial cells in the bone marrow.

Cyclophosphamide treatment did not cause systemic inflammation

We next ruled out the possibility that cyclophosphamide promoted metastasis by systemic inflammation secondary to the bone marrow suppression. Cyclophosphamide-treated mice had significantly reduced body weight, compared with the saline control groups, and the effects lasted more than 2 weeks (Supplementary Fig. S2A). However, cyclophosphamide-treated mice regained body weight with a similar trend to the saline-treated controls. In addition, cyclophosphamide-treated mice did not show any significant lethargy or signs of systemic inflammation, the latter often signaled by increased circulating levels of C-reactive protein (Supplementary Fig. S2B).

Cyclophosphamide pretreatment promoted orthotopic prostate tumor growth in bone

The potential role of disrupted bone marrow vascular integrity secondary to cyclophosphamide treatment in the increased metastatic tumor growth in the bone was further tested using an orthotopic approach (Fig. 3A). This approach was designed to circumvent the effects of vascular disruption that could contribute to initial tumor cell seeding. PC-3 tumors grew larger after 6 weeks in the cyclophosphamide-treated bone marrow, than in control (Fig. 3B and C), suggesting that alterations in the cyclophosphamide-treated murine bone marrow, not a specific compromise of vascular integrity, were responsible for promoting tumor growth and/or metastasis.

Cyclophosphamide transiently expanded myeloid lineage cells

On the basis of the observation in Figs. 2 and 3, alterations induced by cyclophosphamide potentially contributing to tumor growth and/or metastasis were investigated. The changes of white blood cell (WBC) differential counts were further investigated serially after cyclophosphamide administration. Total WBC counts were significantly reduced 3 to 15 days after cyclophosphamide, indicating that cyclophosphamide suppressed bone marrow, and that the effects lasted more than 2 weeks (Fig. 4A). However, the WBC count was increased on day 7 compared with the day 3 cyclophosphamide group (Fig. 4A). Furthermore, neutrophil number was below detection on day 3 but significantly spiked on day 7, immediately followed by suppression (Fig. 4B). In addition, monocyte counts showed a similar pattern to neutrophils (Fig. 4C). Collectively, these data revealed that differentiated myeloid cells in the peripheral blood (i.e., monocytes and neutrophils) transiently increased during recovery from cyclophosphamide. Because both monocytes and neutrophils are differentiated from myeloid lineage cells in the bone marrow, the nature of the changes of myeloid lineage cells in the bone marrow was determined. Flow cytometric analyses of bone marrow cells from mice treated with cyclophosphamide after 3, 7, 10, and 15 days revealed that myeloid lineage cells (expressing CD11b) were significantly expanded 7 and 10 days after...
cyclophosphamide administration with suppression on days 3 and 15 (Fig. 4D). In contrast, there was no change in the numbers of CD11b+ cells in other organs such as kidney, lung, and liver (Fig. 4E–G). We next determined the serum protein levels of VEGF-A, IL-6, and CCL2. All 3 molecules have angiogenic properties and also promote myeloid cell proliferation and differentiation (29–31). All 3 serum cytokines were significantly increased by cyclophosphamide treatment (Fig. 4H–J).

Cyclophosphamide-induced skeletal metastases overlap temporally with bone marrow myeloid cell expansion

To assess the temporal impact of cyclophosphamide on myeloid cell populations, the effects of tumor inoculation at various times after cyclophosphamide treatment were evaluated. PC-3Luc tumor cells were inoculated into the systemic circulation 3, 7, and 15 days after cyclophosphamide treatment (Fig. 5A). The 7-day group had significantly more metastases,
then than the saline-treated control group, as observed previously. When tumor cells were injected at a later time point (i.e., 15 days after cyclophosphamide treatment), significantly fewer mice developed hind limb metastatic lesions, suggesting that levels of bone marrow myeloid cell populations correlate with hind limb metastases (Fig. 5B and C). The 3-day group had a similar metastatic pattern as the 7-day group (Fig. 5B) and increased tumor size compared with the 7-day group (Fig. 5C), potentially because of prolonged survival of tumor cells in the systemic circulation overriding the expansion of bone marrow myeloid cells.

Neutralizing host-derived murine CCL2, but not IL-6, inhibited cyclophosphamide-induced prostate cancer bone metastasis

These data described earlier collectively showed that cyclophosphamide provided an environment conducive to experimental prostate cancer skeletal metastasis, potentially mediated by increase of serum cytokines and/or expansion of myeloid cells. The causal relationship of alterations induced by cyclophosphamide and tumor metastasis was determined using the intracardiac metastasis model in combination with neutralizing antibodies. Mice were treated with neutralizing antibodies targeting mouse IL-6 or mouse CCL2 during the 7 day recovery phase after cyclophosphamide treatment (Fig. 6A). Consistent with the observation in Fig. 1B, cyclophosphamide treatment significantly enhanced the development and subsequent growth of experimental bone metastasis (Fig. 6B). Neutralizing IL-6 did not prevent development of metastases in cyclophosphamide-treated mice. However, neutralizing CCL2 significantly inhibited cyclophosphamide-induced prostate cancer metastasis in vivo (statistical comparison shown in Fig. 6C and D), indicating that the upregulation of CCL2 in response to cyclophosphamide contributed to the development and progression of metastasis. Moreover, administration of both antibodies against IL-6 and CCL2 had similar effects to the anti-CCL2 antibody alone group (Fig. 6B–D). Importantly, neutralizing antibodies were administered before the tumor cell inoculation, to exclude the possibility of direct effects of the drug on the tumor cells. Therefore, the effects of neutralizing antibody were mainly due to the changes exerted on the host microenvironment. However, preclinical pharmacokinetic studies showed that anti-CCL2 antibody can remain detectable in serum up to 10 days after administration, thus the possibility...
Figure 6. Neutralizing CCL2, but not IL-6, reverted cyclophosphamide (CY)-induced prostate cancer bone metastasis. A, schematic representation of the experiment. Male athymic mice were treated with saline (n = 10) or CY in combination with control IgG (n = 14; 10 mg/kg, i.p.), anti-mouse IL-6 (n = 11; 20 mg/kg, i.p.), anti-mouse CCL2 (n = 12; 10 mg/kg, i.p.), or a combination of anti-IL-6 and CCL2 antibodies (n = 12). Three doses were given 1 day before CY treatment and 3 and 6 days after CY treatment. On day 7 post-CY injection, PC-3Luc cells were injected into the left heart ventricle. Hind limb metastatic tumors were monitored by weekly in vivo bioluminescence imaging for 6 weeks. B, serial images from 5 representative mice from each group are shown. Week 4 (C) and week 6 (D) bioluminescence data were quantified and plotted. Tumor size was measured by photon/s from the hind limb lesions in each group. Data are median ± interquartile range, and statistical significance was determined by Mann–Whitney U test.
of direct effects may not be completely excluded (personal communication).

An alternative chemotherapeutic drug, docetaxel, did not promote skeletal metastases

To further determine the causal role of cyclophosphamide-induced myeloid cell expansion to the development of skeletal metastasis, the effects of docetaxel, a chemotherapeutic agent commonly included in prostate cancer treatment regimens, were tested. In contrast to cyclophosphamide-mediated pro-metastatic effects, pretreatment of mice with docetaxel decreased hind limb skeletal metastasis (Fig. 7B). In addition, CD11b+ cell enumeration in the docetaxel-treated bone marrow revealed similar but significantly blunted alterations in CD11b+ cells in comparison with the effects of cyclophosphamide (Fig. 7C). Docetaxel-induced myeloid cell expansion (59.1% ± 12.1%) at day 7 was not sufficient enough to increase myeloid cells (neutrophils and monocytes) in the peripheral blood (Fig. 7D–F).

Discussion

Multiple mechanisms have been proposed to explain why bone provides a congenial metastatic microenvironment. For example, bone is enriched with cytokines and growth factors that promote tumor cell proliferation, migration, and survival (32). In addition, bone houses the primary hematopoietic organ (i.e., bone marrow), containing multiple types of progenitor cells and hematopoietic cells of various tumorigenic potential. Previously, Schneider and colleagues showed that expansion of bone marrow cellularity before inoculation of prostate tumor cells significantly promoted skeletal metastasis (20), suggesting bones with increased cellularity constitute a more congenial microenvironment for metastasis. In this context, it is reasonable to expect that cytotoxic chemotherapy and/or irradiation may impact skeletal metastasis.

This study showed for the first time that alterations induced by cyclophosphamide, a common chemotherapeutic drug, enhanced prostate cancer skeletal metastasis. Furthermore, we showed that the prometastatic effects of cyclophosphamide

Figure 7. Docetaxel pretreatment did not promote the development of hind limb skeletal metastasis. A, schematic representation of the experiment. Male athymic mice were treated with saline or docetaxel. Following 7 days of recovery, PC-3Luc cells were injected into the left heart ventricle (n = 10 for saline control and n = 12 for docetaxel group). Hind limb metastatic tumors were monitored by weekly in vivo bioluminescence imaging for 6 weeks. B, hind limb metastatic tumor size was measured by weekly in vivo bioluminescence imaging. Data are medians with interquartile range. Asterisks represent statistical significance (linear contrasts P < 0.01). C, docetaxel induced myeloid cell expansion similarly but to a lesser extent than cyclophosphamide (CY). Data are mean ± SD, and asterisks represent statistical significance (*, P < 0.01 and **, P < 0.05 by the Student t test). D–F, male C57BL6/J mice (n = 10/group) were treated with saline or docetaxel (40 mg/kg, i.p.) followed by complete blood counting (CBC) with WBC differential counting 7 days after treatment. The numbers of WBC (D), neutrophils (E), and monocytes (F) are plotted. Data are mean ± SD. N.S., not significant (P > 0.05 by the Student t test).
were significantly reversed by suppression of CCL2, which suggests the causal role of bone marrow myeloid lineage cell expansion. We showed that a single dose of cyclophosphamide administration increased myelogenic cytokines, and correspondingly expanded the myeloid cell population in the bone marrow, as well as the numbers of monocytes and neutrophils transiently in the peripheral blood.

The unexpected ‘opposite’ protumorigenic effect of such a chemotherapeutic drug is not a novel observation in other nonskeletal sites. There have been several reports of chemotherapy-induced metastasis and/or tumor growth (18, 19, 33, 34). Most notably, Carmel and Brown showed that pretreatment of the host with cyclophosphamide, among many other chemotherapeutic drugs including actinomycin D, vinblastine, bleomycin, methotrexate, and 5-fluorouracil, resulted in the most prominent metastatic effects in a syngeneic sarcoma lung metastasis model (17). While most of the previous studies focused on an experimental pulmonary metastasis model, our data expanded the earlier observations by showing the prometastatic effects of chemotherapy in a skeletal metastasis model (Fig. 1 and Supplementary Fig. S3). Data in the present study suggest that chemotherapeutic drugs with strong bone marrow suppression may have the adverse effect of promoting bone metastasis, a finding that has not been extensively investigated. Cyclophosphamide is not a standard chemotherapeutic drug for patients with prostate cancer, but recently low-dose metronomic administration of cyclophosphamide is in clinical trials as an antiangiogenic therapy in prostate cancer (35, 36). In addition, cyclophosphamide is widely used for treatment of breast cancer, which also has a strong propensity for skeletal metastasis. Consequently, the effects of varying dosages and administration scheduling of cyclophosphamide on bone metastasis warrant extensive further studies.

The findings concerning the mechanisms involved in chemotherapy-enhanced metastasis have clinically therapeutic implications. We showed that the numbers of bone marrow myeloid cells and myelomonocytic cells in the peripheral blood are significantly increased after cyclophosphamide administration, but not after docetaxel administration, potentially mediated by the increase of myelogenic cytokines. During the recovery phase after bone marrow suppression, spikes of monocytes and neutrophils are frequently observed in patients, and clinically considered a favorable prognostic sign (37). Data in the present study confirmed an abrupt increase of neutrophils and monocytes shortly after cyclophosphamide administration. Moreover, significant increases in CCL2, IL-6, and VEGF-A, all of which are potent myelogenic factors, were observed simultaneously or before the expansion of myelomonocytic cells, supporting the roles of these factors in the expansion of CD11b+ myeloid cells in the bone marrow. Results of this work confirmed that neutralizing CCL2, but not IL-6, significantly inhibited the prometastatic effects of cyclophosphamide. It should be noted that anti-CCL2 antibody is specific to the murine host–derived CCL2, and does not cross-react with prostate cancer-derived human CCL2, and that the neutralizing antibody was administered in only 3 dosages before tumor cell inoculation. Collectively, these data suggest that neutralizing CCL2 reconditions the premetastatic host microenvironment induced by chemotherapy.

Although the present data show the efficacy of anti-CCL2 antibody in the cyclophosphamide-induced prostate cancer bone metastasis model, increased expression of CCL2 (and subsequent expansion of myeloid cells) may not be the only mechanism of promoting metastasis after cyclophosphamide treatment. The first alternative explanation for the prometastatic effects of cyclophosphamide is that it could be mediated by the effects on bone cells. Given that inhibition of osteoclasts reversed the effects of granulocyte macrophage colony—stimulating factor (GM-CSF) on metastasis in a mouse model (38), it is possible that the effects of CCL2 neutralizing antibody in these results were, in part, mediated by inhibition of osteoclastogenesis. Second, while our results failed to confirm the causal role of cyclophosphamide-induced endothelial damage in metastasis, the possibility still remains for further investigation. Cyclophosphamide is currently being tested for efficacy as antiangiogenic therapy, and disruption of endothelial barrier function can promote extravasation of tumor cells in the metastatic microenvironment. Previously, Shirota and Tavassoli showed that cyclophosphamide induces endothelial damage detectable by electron microscopy, and destroys the integrity of bone marrow sinus endothelium (indicated by red blood cells in the extravascular space), leading to enhanced engraftment of bone marrow transplantation (28). Therefore, cyclophosphamide effects on metastasis may be varied in different doses and schedules (i.e., metronomic low dose) or different tumor models.

In conclusion, this study showed that priming the murine host with cyclophosphamide altered the bone microenvironment, leading to promotion of prostate cancer bone metastasis. In addition, suppression of host CCL2 by antibody treatment significantly reduced the adverse effects of cyclophosphamide.

Disclosure of Potential Conflicts of Interest
L.A. Snyder and J.A. Nemeth are employed by Janssen, LLC. K.J. Pienta is the consultant/advisory board member for Curis. L.K. McCauley had a commercial research grant from Centocor. No potential conflicts of interest were disclosed by the other authors.

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