Tumor Necrosis Factor-α Produced by Osteoclasts Might Induce Intractable Pain in a Rat Spinal Metastasis Model of Breast Cancer

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Abstract:

Introduction: Causes of pain due to spinal metastases have been insufficiently investigated. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were the focus of this study. Both are known as proinflammatory cytokines associated with the pathophysiology of pain syndromes. It is well known that cancer cells produce these cytokines, but whether osteoclasts produce them as well remains unclear. We hypothesize that osteoclasts produce these cytokines; in other words, pain from spinal metastasis is stronger than pain from the primary tumor.

Methods: We made a rat spinal metastasis model of breast cancer (metastasis group) and models with a hole in the vertebral column (puncture group) and resected the vertebrae. Tartrate-resistant acid phosphatase (TRAP) staining was performed to confirm that osteoclasts increase in vertebrae with spinal metastasis. We then evaluated TNF-α and IL-6 expression using immunohistochemistry and real-time polymerase chain reaction (PCR).

Results: The results of TRAP staining showed that osteoclasts increase in metastatic vertebrae. The osteoclasts in the puncture models were TNF-α negative but were TNF-α positive in the metastasis model. The osteoclasts in the puncture models and metastasis model were both IL-6 positive. According to the real-time PCR results, TNF-α in vertebrae increased in the metastasis model, but IL-6 did not increase in the metastasis model compared with in the puncture model.

Conclusions: The number of osteoclasts is higher in the metastasis model. While TNF in the osteoclasts increased in the spinal metastasis model, IL-6 did not. This probably means that breast cancer affects TNF production in osteoclasts. This increase of TNF-α may lead to pain from spinal metastasis.

Keywords: osteoclast, TNF-α, IL-6, proinflammatory cytokines, spinal metastasis, breast cancer, pain

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Introduction

Medical advances have improved the life expectancy of cancer patients, with an increase in metastatic patients. A bone is one of the common sites of metastasis, causing refractory bone pain. In fact, 83% of patients, not all patients, who have bone metastasis experience pain. In particular, breast cancer is responsible for bone metastasis and 65%-75% patients who have advanced breast cancer suffer from bone metastasis. The spine is the most common site of metastases in breast cancer, and it can bring severe pain which impairs patients’ quality of life and is difficult to cure. However, pain from spinal metastasis also occurs when there are neither fractures nor nerve compression. It has recently been reported that osteoclasts cause pain through proton secretion. Tumor necrosis factor-α (TNF-α) is a known activator of osteoclasts and a mediator of neuropathic pain. In this study, we focused on TNF-α and interleukin-6 (IL-6), which are known as proinflammatory cytokines related to pain. We hypothesized that osteoclasts produce these cytokines so that pain from spinal metastasis is stronger than pain from the primary tumor.

Materials and Methods

Rat model with spinal metastasis of breast cancer

All animal procedures and protocols were approved by
the Ethics Committee of our University and were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (1996 revision).

We selected a rat model of spinal metastasis\(^{10}\), which was reported in the study by Mantha. We used 42 eight-week-old female Sprague-Dawley rats weighing 200-250 g. First, we cultured rat breast adenocarcinoma cell line CRL-1666 in 10 cm dishes. We then injected the CRL-1666 subcutaneously to make subcutaneous tumors in six donor rats. Secondly, the remaining 36 rats were anesthetized with a mixture of 0.15 mg/kg body weight (b.w.) medetomidine, 2.0 mg/kg b. w. midazolam, and 2.5 mg/kg b.w. butorphanol and were treated aseptically throughout the experiments. A midline dorsal longitudinal incision was made over the lumbar spine. When the needle point reached a depth of 1.5 mm, the needle 1 mm left of the midline in this L6 vertebra. We put the vertebrae into liquid nitrogen to make frozen specimens. The frozen specimens were crushed with Cell Destroyer\(^{5}\) (Biomedical Science, Tokyo, Japan). Then the RNA of the crushed specimens was extracted using Isogen\(^{5}\) (Nippon Gene, Tokyo, Japan). We performed real-time polymerase chain reaction (PCR) for TNF-\(\alpha\) and IL-6.

**Statistical analysis**

A statistical analysis of the number of TRAP-positive cells was performed using the Student \(t\)-test, and the statistical analysis of the relative expression of TNF-\(\alpha\) and IL-6 was performed using the Mann-Whitney \(U\)-test. \(P\)-values less than 0.05 were considered statistically significant. All of the results were expressed as the mean \(\pm\) standard error unless otherwise indicated.

**Results**

**TRAP staining and immunohistochemistry**

There were more osteoclasts in the L6 vertebrae of the metastasis model as compared to the puncture model \((P < 0.05)\) (Fig. 1, 2). This result indicates that CRL-1666 may...
F i g u r e 1. Tartrate-resistant acid phosphatase (TRAP)-staining of the L6 vertebrae. The arrowheads show TRAP-positive multinucleated cells. TRAP-positive multinucleated cells existing close to the trabecular bone in a L6 vertebra were regarded as osteoclasts. The scale bars are 100μm.

Figure 2. The number of TRAP-positive multinucleated cells in 1mm2. There were more osteoclasts in metastasis model compared with puncture model. (p<0.05)

induce osteoclast precursors to osteoclasts.

The osteoclasts in the puncture models were TNF-α negative, but TNF-α positive in the metastasis model (Fig. 3). The osteoclasts in the puncture models and metastasis model were both IL-6 positive (Fig. 4). This result implies that CRL-1666 increases TNF-α in the osteoclasts, but does not increase IL-6.

Real-time PCR

According to the results of real-time PCR, TNF-α in vertebrae increased in the metastasis model, but IL-6 did not as compared to TNF-α and IL-6 in the puncture model (P < 0.05) (Fig. 5). The results from immunohistochemistry and real-time PCR are consistent.

Discussion

Pain due to spinal metastases occurs even when there are not vertebral fractures, and several mechanisms have been reported. It is well known that bone resorption causes pain and that TNF-α induces osteoclast differentiation by stimulating osteoclast progenitors. Osteoclasts create an acidic microenvironment and cause bone pain via transient receptor potential vanilloid type 1 (TRPV1), an acid-sensing nociceptor.

The proinflammatory cytokines not only induce the differentiation of osteoclasts but also cause pain by themselves. Xu et al. in their study reported that the upregulation of TNF-α and tumor necrosis factor receptor (TNFR) 1 is essential for the initiation of neuropathic pain. Several studies provide that the epineural or intrathecal application of TNF-α causes a hyperalgesia, allodynia, hyperalgesia and changes in spinal cord neuronal responses to nociceptive stimuli and enhanced dorsal horn neuronal responses, including the acute responses to C-fiber stimulation, wind-up, and post-discharge in rats. It has also been documented that TTX-resistant sodium currents in acute TNF-α-mediated increase in nociceptors’ excitability depending on p38 MAPK. TNF-α also causes hyperalgesia through the sensitization of TRPV1 channels, related to acidity. As can be seen from these studies, TNF-α itself causes pain including bone cancer pain.

Previous studies have reported that the vertebral body is innervated by pain-related intravertebral sensory nerve fibers, and mechanical, thermal, and chemical stimuli are transmitted to the nerves. Assuming the microenvironment of spinal metastases, TNF-α, which increased in the vertebrae stimulates the nerves, and they may activate nociceptors and lower the threshold.
Moreover, the study by Geis reported that systemic antagonism of TNF significantly alleviated tactile hypersensitivity and spontaneous bone cancer-related pain behavior in the mouse model. This supports our theory that the production of TNF may lead to bone cancer pain.

**Limitations**

Several studies show that von Frey test, Gait analysis (Catwalk XT, Noldus) test, and other pain behavior tests are useful for evaluation of pain due to spinal metastasis\(^{22,26}\). We tried to evaluate pain by means of pain behavior with CAT WALK. However, the evaluation could not be completed because the rats of the metastasis model showed a significant decrease in activity due to possible pain and/or motor dysfunction. Considering that the primary purpose of the current study was to investigate the pain mechanism of spinal metastasis, our discussion was limited to the possible relationship between TNF-\(\alpha\) and pain.

To conclude, the increase of TNF-\(\alpha\) observed in our study may be one of the mechanisms of pain in the rat spinal metastasis model.

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