Abstract

Imaging analysis of bone destruction and bone invasion of squamous cell carcinoma using tissue clearing techniques.

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[Introduction]

More than 90% of oral malignancies are oral squamous cell carcinomas arising from the epithelium of the oral mucosa, which are known to invade the jawbone in many cases. It has been reported that osteoclasts induction by cancer cells is closely related to bone metastasis and bone destruction observed in many cancers, including oral squamous cell carcinoma. The induction of osteoclasts is thought to be mediated by local factors released from cancer cells and osteoblasts. On the other hand, the possibility of direct bone destruction by cancer cells has also been reported. However, the relationship between cancer cells and host cells in the microenvironment causing destruction of the bone in oral squamous cell carcinoma is still not well understood. Therefore, elucidation of the mechanisms of bone destruction in squamous cell carcinoma cells is expected to be an important for considering surgical treatment.

[Methods]

In this study, calvaria were stained by the intraperitoneal administration of calcein (CL), alizarin red (AR) and tetracycline (TC), fluorescent dyes that bind to Ca2+ in bone formation. In these experiments, the mouse model for cancer bone invasion was generated by the administration of CL and AR to 4-week-old male C3H mice, and subsequent transplantation of mouse squamous cell carcinoma cells (SCC7, $1x10^6$ cells) just above the calvaria, and then TC was administered 2 to 14 days later. The calvaria removed from these mice were fixed in formalin and cleared with SCALEVIEW-S4, after which the fluorescence of the calvaria was analyzed three-dimensionally using confocal laser microscopy. In this experiment, the loss of the Calcein and Alizarin Red layers administered before SCC7 implantation was considered for bone resorption, while the loss of the Tetracycline layer administered after SCC7 implantation was considered for the suppression of bone formation. Autofluorescence was also used to visualize cells including SCC7.

[Results]

Observation of the appearance showed a swelling at the transplant site at day 7 after transplantation of the cancer cells. Tumor tissue could also be seen in the removed calvaria from day 7, and mobility of the tumor tissue was observed at day 10, but from day 14 the tumor tissue adhered to the calvaria in all mice. Confocal laser microscopy of the calvaria on day 7 showed a loss of the Alizarin Red layer, but no loss of the deeper Calcein layer, suggesting that bone resorption occurred from the bone surface towards the depth in this area. In addition, a large loss of the tetracycline layer suggests a

widespread suppression of bone formation as early as day 7 after SCC7 transplantation. In the calvaria at day 10 after SCC7, when bone destruction was more advanced, autofluorescence showed that cancer cells had penetrated through the Calcein layer and invaded deeply. In the large bone defects in the calvaria at day 14, where bone destruction had progressed further, loss of Alizarin Red layer with remaining Calcein were observed in addition to areas of the loss of Calcein layer with remaining Alizarin Red layer. It was considered that bone disruption of a horizontal direction was the cause of this mixing in these large bone disruption. TRAP staining for examining the distribution of osteoclasts in the calvaria revealed that the staining was observed in the sutures and bone marrow, while the staining was absent in the plate-like areas separated from the sutures in the calvaria of normal mice. In contrast, marked TRAP-stained areas were observed on the plate-like areas near the parietal tuberosity as well as at the sutures in SCC7-transplanted mice. Interestingly, TRAP-positive areas directly under the cancer formation were observed only at the margins of the bone defect, whereas prominent TRAP-positive areas were found on the opposite side of the cancer formation. Observation of Calcein fluorescence in TRAP-stained calvaria showed that TRAP-positive cells and the loss of fluorescence in small perforated bone resorption sites located away from the tumor tissue. On the other hand, no clear TRAP-positive cells were observed in the bone resorption area immediately below the tumor tissue, and weak TRAP staining was observed at the bone resorption margins. Confocal laser microscopy showed a loss of the Calcein layer in a wider area around the bone resorption area.

Analysis using GFP mice showed that the cluster of tumor cell was surrounded by angiogenesis derived from the host with green fluorescence. Protruding osteocyte-like cells and cancer cells were also observed in bone resorption areas where the Alizarin Red layer was lost. These findings suggest that the dissolution of calcium (demineralization) may have occurred in areas where bone matrix remained. Second harmonic generation (SHG) of two-photon laser microscopy was therefore used to visualize the distribution of collagen in the region of Alizarin Red layer was lost in SCC7-implanted GFP mice. The results showed that large defects in the Alizarin Red layer were observed around areas of bone resorption where host-derived cells, which appeared to be osteoclasts, had invaded. Collagen fibers and protruding osteocyte-like cells remained in the area. This indicates that demineralization may occur prior to collagen degradation in cancer-induced bone destruction. This is different from common bone resorption by the resorption fossa of osteoclasts, suggesting that cells other than osteoclasts may be involved.

To elucidate the molecular mechanisms of bone destruction and bone invasion caused

by cancer, we performed a comprehensive gene expression analysis by NGS using samples of human-derived tongue squamous cell carcinoma cells (SAS) transplanted into nude mice. Analysis of tumor tissues revealed increased gene expression of myosin, MMP, calpain, and cathepsin in bone-infiltrated cancer cells. In addition, gene expression of keratin, extracellular matrix, IL20, Notch signaling, and TNFα was increased in SAS-infiltrated bone.

[Conclusions]
Three-dimensional structural analysis of bone destruction using tissue clearing technology provides evidence that demineralization (Ca2+ resorption) precedes the loss of bone matrix (such as collagen) in the bone destruction by cancer. In addition, the results of NGS suggested the involvement of local factors including cathepsin, myosin, TNFα, IL20 and Notch in the bone invasion of cancers. Furthermore, the increased expression of proteolytic enzymes, such as MMPs, in cancer cells raised the possibility of direct bone destruction by cancer cells.