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Localization of osteopontin in resorption lacunae formed by osteoclast-like cells: a study by a novel monoclonal antibody which recognizes rat osteopontin

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Abstract The characteristics of a monoclonal antibody produced against osteoclast-like multinucleated cells (MNCs) formed in rat bone marrow cultures were examined immunohistochemically and biochemically. The in vitro immunization was performed using as immunogen the MNCs from rat bone marrow cell culture, which revealed many characteristics of osteoclasts. After screening and cloning of hybridomas, the monoclonal antibody HOK 1 was obtained. This antibody reacted weakly with stromal cells and intensely with both MNCs and their putative migratory traces on culture dishes. Immunofluorescent examination of paraffin sections revealed intense reactivity on the epithelium of the choroid plexus, the ileum and the proximal-convoluted tubules of the kidney, and also on bone cells such as osteocytes, osteoblasts, and osteoclasts. Western blotting using purified rat osteopontin verified that the antigen recognized by HOK 1 was osteopontin. Positive HOK 1 immunoreactivity was further observed in the resorption lacunae formed by a culture of MNCs on human tooth slices and on the surface of osteoclasts. The present data suggested that osteopontin is preferentially present on the resorption lacunae in resorbing calcified matrices and that osteoclasts under a specific state might trap this protein on their cell surface.

Introduction
Osteoclasts are cells of hematopoietic origin and are primary cells which resorb bone. These cells appear to have important roles in bone remodeling; however, the details of the regulation of bone remodeling remain ambiguous. It has been postulated that the proteins secreted by osteoclasts play important roles in bone remodeling. Nevertheless, only limited numbers of proteins have been identified as being secreted by osteoclasts. For example, it has been reported that tartrate-resistant acid phosphatase (TRAP; Ek-Rylander et al. 1991), cathepsin D (Goto et al. 1992), and cathepsin L (Rifkin et al. 1991) are secreted by osteoclasts.

Osteopontin, a phosphorylated protein, is a noncollagenous protein involved in bone (Prince et al. 1987). The protein, of which the main source in bone is thought to be osteoblastic cells, may perform some functions in calcium binding and nucleation of calcium hydroxyapatite in crystal formation with other proteins (Gorski 1992; Oldberg et al. 1986). Osteopontin may also provide a scaffold for osteoblasts or osteoblastic cells to attach and spread (Mark et al. 1988a; Somerman et al. 1989; Mckee et al. 1992, 1993). As osteopontin is an adhesive bone matrix protein containing a functional Arg-Gly-Asp-Ser cell-binding sequence (Oldberg et al. 1986), it is suggested that osteoclasts adhere to bone via a vitronectin receptor (Davies et al. 1989; Reinhold et al. 1990; Flores et al. 1992; Helfrich et al. 1992). Mark et al. (1987) previously reported that occasional immunoreactivity to osteopontin was detected in osteoclasts. Recently, Tezuka et al. (1992), Ikeda et al. (1992) and Arai et al. (1993) have demonstrated the expression of osteopontin mRNA in osteoclasts.

We are currently trying to obtain a panel of monoclonal antibodies that recognize osteoclasts. The use of rat bone marrow culture enabled us routinely to prepare multinucleated cells (MNCs) having numerous characteristics of osteoclasts. We have utilized these MNCs not only as the immunogen but also as the cells for antibody screening. We have obtained a novel monoclonal anti-
body, which recognized rat osteopontin but did not recognize it when this protein was involved in bone matrix. In this study, we found that osteopontin is preferentially present on resorption lacunae formed by osteoclasts and that osteoclasts might trap this protein on their surface.

**Materials and methods**

**Experimental animals**

For the formation of MNCs, male Sprague-Dawley rats (SD; Seiwa Experimental Animal Co., Fukuoka, Japan) of 4-8 weeks in age were used. One- to nine-day-old SD rats were used in the immunohistochemical study.

**Preparation of monoclonal antibody**

Bone marrow cultures were established according to the methods of Kukita et al. (1993). Briefly, SD rats were killed by ether anesthesia, and bone marrow cells were flushed from the femur and tibia after cutting both ends. Cells were seeded into 10-cm dishes (Becton Dickinson Labware, Lincoln Park, N.J., USA; 3×10^6 cells/dish) and cultured for up to 4 days in Alpha Minimum Essential Medium (aMEM; Gibco, Grand Island, N.Y., USA) containing 15% fetal bovine serum (FBS; Gibco) and counterstained with hematoxylin.

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The long bones were removed from 0- to 5-day-old SD rats, dissected free of adherent soft tissues and curetted with a scalpel blade into small amounts of aMEM containing 15% FBS. After 30 min, nonadherent cells were washed and cultured for more than 1 h on Lab Tek chambers (Nunc, Naperville, Ill., USA). Isolated osteoclasts were chilled on ice, washed with aMEM, and incubated with anti-osteopontin monoclonal antibody, HOK 1, on ice for 1 h. Subsequently the cells were fixed with PBS containing 2% paraformaldehyde for 20 min at room temperature. After rinsing, osteoclasts were stained using the Vectastain ABC-AP kit.

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