Homeostasis as Regulated by Activated Macrophage. VIII. LPSw (a Lipopolysaccharide from Wheat Flour) Can Regulate Bone Resorption of Chick Embryo

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The effect of LPSw (a lipopolysaccharide from wheat flour) on the bone resorption of 18-d chick embryonic calvaria was examined in an organ culture following the method of Raisz. Bone was prelabeled in culture medium containing ^{45}Ca and chased in a cold medium. On addition of test samples, labeled calcium was released indicating the grade of bone resorption. LPSw (10—100 ng/ml) stimulated bone resorption, showing an effect comparable to parathyroid hormone (PTH) (1 U/ml). PTH at 1 U/ml decreased the total amount of calcium and phosphorus, while LPSw did not. LPSw is thus assumed to stimulate bone resorption more actively than PTH.

Keywords homeostasis; macrophage; bone resorption; osteoclast; osteoblast; parathyroid hormone; lipopolysaccharide (LPS); LPSw

Introduction

As reported in previous papers of this series,[1] LPSw (a lipopolysaccharide (LPS) from wheat flour) has shown a therapeutic effect on various intractable diseases in experimental animals and sometimes in human patients. We have assumed that the effect was ascribable to activation of macrophages to the primed stage ready for endogenous production of tumor necrosis factor (TNF).[2] The present report describes the effect of partially purified LPSw on bone resorption.

In bone resorption osteoclast, known as a macrophage-like cell, degrades the bone matrix and releases calcium, in synchrony with the activation of osteoblast which supplies components of bone resorption. Therefore, the fate of prelabeled ^{45}Ca in bone matrix is a good indicator of bone resorption.

In this experiment, 18-d chick embryonic calvaria were prelabeled with ^{45}Ca and chased in a cold medium following the method of Raisz.[3] Release of ^{45}Ca indicates the grade of bone resorption depending on samples placed in the culture medium. LPSw has shown a more remarkable effect on bone resorption than that of parathyroid hormone (PTH).

Materials and Methods

Estimation of Bone Resorption as Observed by ^{45}Ca-Release A slightly modified method of Raisz[3] was employed using calvaria of 18-d chick embryo. Calvaria was prelabeled during incubation for 2 h in a culture medium (BGJb-HW2) containing ^{45}Ca (0.5 μCi/ml). After washing with cold phosphate buffered saline (PBS), the calvaria was transferred to a chase medium and was incubated for 24 h to remove the physicochemical contaminant ^{45}Ca. A test sample was then added to the chase medium, incubated for 24 h, and the radioactivity of ^{45}Ca released into the medium was measured. After the incubation, remaining ^{45}Ca in calvaria was released by 1 N HCl overnight and the radioactivity of ^{45}Ca was measured in a liquid scintillation counter. Bone resorption is expressed as percentage of released ^{45}Ca:

$$\text{release \% of } ^{45}Ca = \frac{[^{45}Ca \text{ released into medium}] \times 100}{[^{45}Ca \text{ released}] + [^{45}Ca \text{ remaining in calvaria}]}$$

Effect of samples is expressed as follows:

$$\text{T/C ratio} = \frac{[\text{release \% of } ^{45}Ca \text{ in treated groups}]}{[\text{release \% of } ^{45}Ca \text{ in the control group}]}$$

Bone Formation Femur of 9-d chick embryo or calvaria of 18-d chick embryo was incubated in BGJb-HW2 for 4 d. Bone formation was examined by estimating the content of Ca and P accumulated in bone. The medium was changed every other day.

To control for individual deviations, pair mate cultures were carried out, taking one bone for control and the contralateral bone from the same embryo for the test sample.

Quantitation of Ca and P The quantity of calcium in bone was estimated colorimetrically by the *o*-cresolphthalein complexone method.[4] Phosphorus content in bone was estimated colorimetrically using the molybdate blue method.[5]

LPS The procedure for LPS preparation from wheat flour (LPSw) was described previously.[1a] In this report, partially purified sample was used. Content of LPSw in this crude sample as expressed by its specific activity on the basis of Limulus reaction was 0.1%. The molecular size of LPSw on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was less than 5 kilodalton (kDa).[1a] LPS from *Escherichia coli* (*E. coli*) 0127:B8 was purchased from Difco Lab. (Detroit, U.S.A.). LPS from *Bordetella pertussis* (*B. pert.*) was purified by the conventional method of Westphal *et al.*[6]

Chemical Reagents Synthetic human (1-34) PTH was purchased from Peptide Institute Inc. (Osaka, Japan). Muramyl dipeptide (MDP) was a gift, and lipid A (LA-15-pp (506)) was purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). BGJb-HW2, slightly modified BGJb,[7] was made by ourselves. ^{45}Ca as $CaCl_2$ (4—50 Ci/g calcium) was purchased from New England Nuclear (Boston, U.S.A.). Reagents to measure Ca content were purchased from Iatron Lab. Co. (Tokyo, Japan). Phospho-B-test Wako to measure inorganic phosphorus was purchased from Wako Pure Chemicals Co. (Osaka, Japan).

Results

Stimulative Effect of LPSw on Bone Resorption of 18-d Chick Embryonic Calvaria Bone resorption by LPSw as observed by release of ^{45}Ca was compared with that by PTH (Fig. 1). PTH at 1 U/ml stimulated the resorption of 18-d chick embryonic calvaria at a rate 1.5 times the control. LPSw stimulated the resorption similarly in a dose dependent manner, especially at concentration greater than 10 ng/ml.

Effect of LPSw on the Total Amount of Calcium and Phosphorus of 18-d Chick Embryonic Calvaria After 4 d of incubation, the total amount of calcium and phosphorus was decreased when PTH was added to the incubation medium (Fig. 2), while with the addition of LPSw, no decrease was observed. This suggests that PTH at this concentration induces a simple release of ^{45}Ca without maintaining the dynamic equilibrium of bone formation. LPSw at concentrations used did not exhibit a toxicity.

Effect of LPSw on the Total Amount of Calcium and

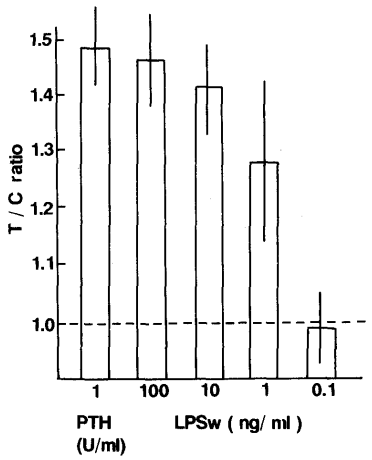

Fig. 1. Effect of LPSw on the Bone Resorption of 18-d Chick Embryonic Calvaria

Bone resorbing activity of 18-d chick embryonic calvaria treated with PTH and LPSw were determined. The results were expressed as T/C ratio as described in Materials and Methods.

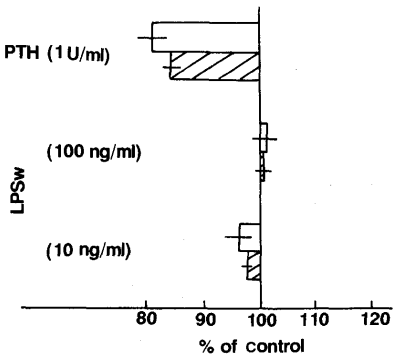

Fig. 2. Effect of LPSw on the Ca and P Content of 18-d Chick Embryonic Calvaria Cultivated *in Vitro*

Ca (□) and P (▨) content of 18-d chick embryonic calvaria were determined after 4 d of incubation. The results were expressed as % of the control.

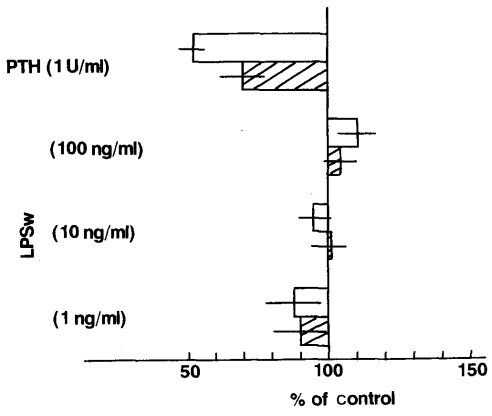

Fig. 3. Effect of LPSw on the Ca and P Content of 9-d Chick Embryonic Femur Cultivated *in Vitro*

Ca (□) and P (▨) content of 9-d chick embryonic femur were determined after 4 d of incubation. The results were expressed as % of the control.

Phosphorus of 9-d Chick Embryonic Femur Cultivated *in Vitro* Calvaria belongs to a bone showing only membranous ossification. Femur, which is composed of cartilage and bone and shows endochondral ossification, was also examined. Femur of 9-d chick embryo was incubated in the same way as above and the total amount of calcium and phosphorus was examined (Fig. 3). PTH caused a marked decrease, whereas LPSw did not.

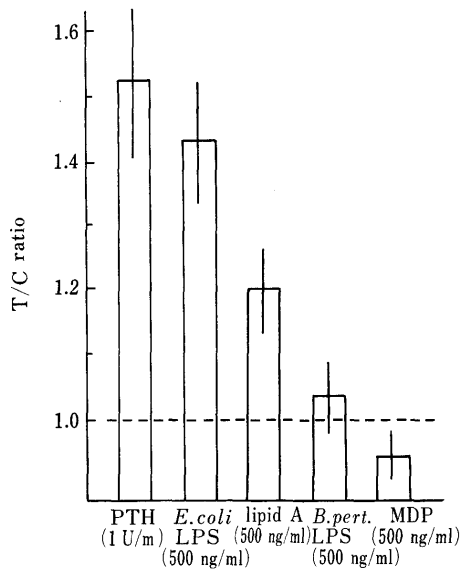

Fig. 4. Effect of PTH, *E. coli* LPS, Lipid A, *B. pertussis* LPS and MDP on Bone Resorption of 18-d Chick Embryonic Calvaria

Bone resorbing activity of 18-d chick embryonic calvaria treated with various LPSs were determined. The results were expressed as T/C ratio as described in Materials and Methods.

Effect of Other LPSs Derived from Bacteria on Bone Resorption of 18-d Chick Embryonic Calvaria Effect of other LPSs derived from bacteria including lipid A and MDP on bone resorption of 18-d chick embryonic calvaria was examined as compared with that of PTH. Each sample was tested at doses of 50 to 500 ng. The effect at 500 ng/ml is shown in Fig. 4, since results were similar at other doses. LPS of *E. coli* had a marked effect comparable to that of PTH (1 U/ml) whereas lipid A at this concentration produced a smaller but significant effect. However, LPS of *B. pertussis* and MDP displayed no effect.

Discussion

Cells participating in the metabolism of bone tissue are the so-called bone cells involving osteoblasts, osteoclasts, osteocytes and precursors of all these cells. These cells work together by communicating to maintain the dynamic equilibrium between bone formation and resorption. Events occurring in this network of bone cells are very similar to those in inflammation or immune cascade. The formation or activation of osteoclast ascribable to macrophages can be stimulated by cytokines excreted from osteoblast.[8]

Bone formation which follows bone resorption induced by osteoclast takes place after breakdown of the bone matrix followed by release of BMP (bone morphogenic protein), TGF-β (tansforming growth factor β) or IGF (insulin-like growth factor) from bone matrix.[9] The cytokines then activate osteoblast to form bone. Activation of osteoclast may also cause excretion of a cytokine which activates osteoblast. A good start in obtaining a homeostatic equilibrium in bone metabolism may thus be the appropriate activation of osteoclast.

In our previous reports,[1] we showed that LPSw has marked therapeutic effects on various intractable diseases

by its activation of macrophages to the primed stage ready for endogeneous production of TNF. In addition, LPSw can be applied orally or percutaneously. Thus percutaneous or oral administration of LPSw is the best way yet known to activate macrophages to aid in the cure of various diseases.

In this experiment, we have shown the stimulative effect of LPSw on bone resorption of 18-d chick embryonic calvaria as compared with that of PTH in an organ culture. While PTH showed an inhibitory effect on bone formation as estimated by calcium and phosphorus accumulation of 18-d chick embryonic calvaria and 9-d chick embryonic femur, LPSw did not. Although the experiment was in organ culture and the mechanism of action of PTH is different, LPSw may be useful for treatment of bone diseases such as osteoporosis. Perhaps most important is the fact that LPSw can be given either orally or percutaneously without harm.

The mechanism of action of LPSw remains to be ascertained. However, our present work demonstrated that activation of osteoclast is a potential site of action. As long as LPSw can activate macrophages and thereby appropriately maintain homeostasis, the activation of osteoclast will ignite the whole cytokine network of bone cells described above. The reaction may be mild, since effective doses cover a wide range (1 to 100 ng/ml), though it is observed in *in vitro* organ culture.

Other LPSs derived from bacteria are not as suitable for further therapeutical use. LPS of *E. coli* cannot be used orally or percutaneously.[1b)]

Thus, LPSw may be effective for therapeutic use in various bone diseases, since it can maintain the homeostasis of the network of bone cells which stimulated molecular turnover of bone matrix.

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