EFFECT OF LIPOPOLYSACCHARIDE AND INTERFERON-γ ON THE FORMATION OF OSTEOCLAST-LIKE MULTINUCLEATED CELL FROM CHICKEN BONE MARROW CELLS IN VITRO

Hong-Kyun Oh, Jung-Keun Kim, Jae-Hyun Lee
Dept. of Periodontolog, School of Dentistry, Dankook University

I. Introduction

Osteoclasts are derived from mononuclear hematopoietic stem cell[1] and are the major cells responsible for regulation of serum calcium concentration by bone resorption[2]. The formation of osteoclast can be distinguished in three stage[3]. In early stage, the early osteoclast progenitors do not express tartrate-resistant acid phosphatase and are proliferated[4]. At the second stage, the early and late form of osteoclast precursors can be separated base upon their acquistion of tartrate-resistand acid phosphatase positivity[5,6]. In maturation stage, the mature multinucleated osteoclasts are formed by fusion of late precursor. Many researches for examining the formation of osteoclast-like multinucleated cells(MNCs) were developed[7,8,9].

Osteoclast has a morphological features which enable to resorb bone. It is a large, multinucleated, polarized cell and have rich mitochondria, rough endoplasmic reticulum, lysosomal enzyme, multiple Golgi complex, coated transport vesicle. It's surface is divided into three different functional area[2]. Basolateral membrane acts as active transport of the products of bone resorption to extracellular fluidm, and clear zone is a site to attach tightly to the bone surface that is to be resorbed. At last, ruffled border is sites that carries out the resorption process itself.

Lipopolysaccharide (LPS) is a component of gram negative bacterial cell wall. In human periodontal disease, LPS is related to pathway for bone resorption[10]. It was suggested that LPS enhanced osteoclast formation in vitro[11,12,13].

Interferon-γ is a glycoprotein secreted by activated lymphocyte, fibrolbast and monocytes[14,15,16]. The function of IFN-γ were known to have antiviral and immuno-defence effect[16,17]. It has been shown that interferon-γ inhibited collagen osteoclast and $1.25(OH)_2$ vitamin D_3-stimulated osteoclast production in osteoblast cultures, and increased alkaline phosphatease activity in human osteoblast[18,19,20]. Joshihara R. et al. suggested that interferon-γ decreases DNA synthesis in human osteoblasts[21].

Many studies suggest that immune cells in the bone marrow play a role in bone formation and resorption. Studies with monokines and lymphokine have been shown that interleukin-1[22,23,27], tumor necrosis factor α and β[24] stimulate osteoclastic bone rersorption in vitro. It has been shown that interferon-γ inhibited

bone resorption stimulated by cytokines, such as tumer necrosis factor α,β, interleukin-1[25,26,27]. It was also suggested that prostaglandin-mediated and parathyroid hormone-induced bone resorption was inhibited by interferon-γ in cultured neonatal mouse calvaria[28].

In this study, we studied the effect of interferon-γ(IFN-γ) and lipopolysaccharide(LPS) on the formation of osteoclast-like multinucleated cell from chicken bone marrow cell in culture.

II. Materials and methods

1. Isolation and purification of mononucleated cell from chick embryo

16~19 days old white leg horn chick embryo was used to collect the osteoclast precursors. Eggs were disinfected with 70% betadine solution and washed with 70% alcohol solution. After sacrifiece, both tibia were quickly seperated and placed into ice-cold 60mm culture dish with medium 199, containing 10% fetal bovine serum(FBS, Gibco). Longitudinal section of tibia was performed to exposure the medullary bone. Splitted tibia were removed by vibrating for 3 to 5 minutes. The resluting cell suspension was filtracted by 8 μm pore size filter to removed bone fragment and already differentiated osteoclast. The filtrated cell suspension was centrifused to collecte the bone marrow cell and then serum free media were sucked and media change with M-199 containg 10% FBS were followed. The cell suspension was incubated to attach differentiated osteoclast at 37℃. After 2hrs non-adherent cells were collected.

2. Bone marrow cell culture and treatment.

The bone marrow cells were cultured in M-199 (Gibco) buffered with 25mM HEPES, containing 10% heat-inactivated fetal bone serum(FBS), penicillin (100 units/ml) and streptomycin (50μg/ml) (Gibco) at 8×10^5 cells /well in 24 well plates(Falcon). The cultures were fed by replacing 0.5ml old medium with fresh M-199 the next day. All cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37℃.

To examine the number of TRAP (+) multinucleated osteoclat-like cell, 50, 205, 500U/ml of interferon-γ were added at 7 days of culture. And then, maintained for 7 days.

3. Tartrate-resistant acid phosphatase (TRAP) staining and cell couning

After 7 days of treatment, the medium was removed. The cells were washed with PBS. and then fixed with citrate-acetone-formadlehyde fixative for 10 sec. The cells were rinsed 3 times in prewarmed DDW. and then, tartrate-resistant acid phosphatase (TRAP) staining was performed using an acid phosphatase kit (Sigma, st. Louis, MO). Fast Garnet GBC base solution(0.25ml) and sodium nitrite solution (0.25ml) were added into microtube. The solution was mixed by gentle inversion. Prewarmed D.D.W.(18ml), diazotized fast garnet GBC solution(0.4ml), naphthol AS-BI phosphate solution(0.2ml), acetate solution(0.8ml) and tartrate solution(0.4ml) were added into 50ml tube and mixed by gentle inversion. The mixing solution was added 0.8 ml/well to TRAP(+) stain in 24 well plates. The 24-well plates incubates for 1 hr. in 37℃ incubator protected from light with shaking. After 1 hr., the plates were rinsed thoroughly in D.D.W. and air dried.

After staining, TRAP(+) multinucleated cells were evaluated microscopically. The cells

having more than three nuclei per cell were counted as osteoclast-like multinucleated cells (MNCs).

4. Statistical method.

Sample mean and standard error were calculated for all results. The paired Student's t-test were used to compare the results.

III. Results

1. The effect of LPS on the formation of TRAP(+) MNCs in the chick bone marrow cell culture.

After 0, 0.1, 0.5, and 1 μg/ml of LPS were added in the M-199 and chick bone marrow cells were cultured for 7 days. 0.1 μg/ml of LPS showed increased tendency on the number of TRAP(+) MNCs. Otherwise, 0.5 and 1.0 μg/ml of LPS showed decreased number of TRAP(+) MNCs significantly ($P<0.05$, $P<0.01$). (Table 1 and Fig. 1)

Table 1. The effect of LPS on the formation of TRAP(+) MNCs

Treatment		No. of TRAP(+) MNCs/well
LPS (μg/ml)	0	14.00 ± 3.25
	0.1	21.00 ± 4.18
	0.5	4.50 ± 1.59*
	1.1	3.20 ± 1.59**

Value are Mean ± SE *$P<0.05$, P**<0.01
MNCs : mulitucleatd cekks

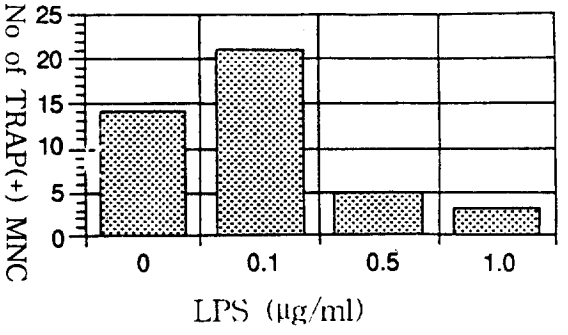

Fig. 1. The eff of LPS on the formation of TRAP(+) MNCs

2. The effect of IFN-γ on the formation of TRAP(+) MNCs in the chick bone marrow cell culture

After 0, 50, 250, and 500 U/ml of IFN-γ were added in the M-199 and chick bone marrow cells were cultured for 7 days. The number of TRAP(+) MNCs were decreased by the addition of 50 and 500 U/ml of IFN-γ (Table 2 and Fig. 2).

Table 2. The effect of IFN-γ on the formation of TRAP(+) MNCs

Treatment		No. of TRAP(+) MNCs/well
LPS (μg/ml)	0	14.17 ± 3.16
	50	9.67 ± 3.53
	250	13.50 ± 5.01
	500	8.00 ± 2.43

Value are Mean ± SE MNCs : multinucleated cells

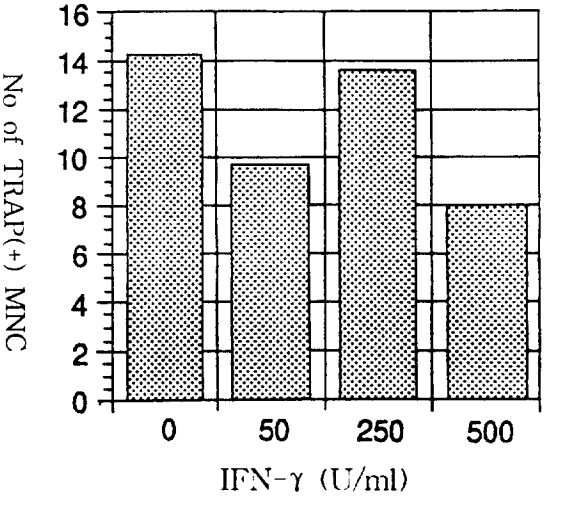

Fig. 2. The effect of IFN-γ on the formantion of TRAP(+) MNCs in the chick bone marrow cell cluture.

3. The effect of LPS and IFN-γ on the formation of TRAP(+) MNCs in the chick bone marrow cell culture

After 0.1 μg/ml of LPS and various concentration of IFN-γ were added in the M-199, the chick bone marrow cells were cultured for 7 days. Te number of TRAP(+) MNCs were decreased significantly by 250 and 500 U/ml of IFN-γ ($P\vee0.05$) (Table 3 & Fig. 3).

Table 3. Effect of LPS and IFN-γ on the formation of TRAP(+) MNCs

Treatment		No. of TRAP(+) MNCs/well
LPS (μg/ml)	0	25.83 ± 4.91
	50	22.83 ± 5.77
	250	12.00 ± 2.79
	500	13.00 ± 3.14

Value are Mean ± SE * $P<0.05$ MNCs : multinucleated cells

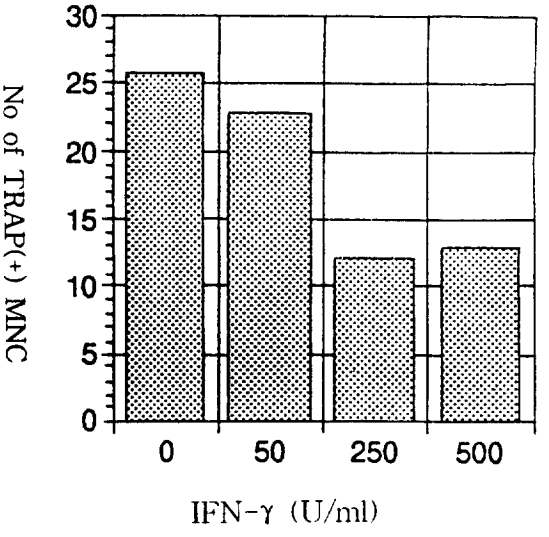

Fig. 3. Effect of LPS and IFN-γ on the formation of TRAP(+) MNCs

Ⅳ. Discussion

The present study demonstrates that TRAP (+) MNCs formation were affected by LPS and IFN-γ. We cultured undifferentiated bone marrow cells as osteoclast precursors from white leg horn chick embryo. After 0, 0.1, 0.5, and 1 μg/ml of LPS were added in the M-199, the chick bone marrow cells were cultured for 7 days. 0.1 μg/ml of LPS showed a increased tendency of the number of TRAP(+) MNCs. This result was similar to study of Nishihara et al. and Shuto et al.[11,12]. The effect of paraformaldehyde-fixed murine macrophage P388D1 cells on osteoclast-like cell formation was investigated by Nishihara et al[11]. When mouse marrow cells were cocultured with paraformaldehyde-fixed P388D1 cells stimulated with LPS, many TRAP(+) cell formation was achieved. Also, Shuto et al[12]. suggested that LPS enhanced osteoclast formation in mouse bone marrow culture. But, Orcel et al[3]. suggested that LPS caused a tripled increase in the osteoclast surface, a 4.5 times increase in the number of osteoclast, but no change in the number of TRAP(+) MNCs. Otherwise, in this study 0.5 and 1.0 μg/ml of LPS showed decreased formation of TRAP (+) MNCs significantly ($P<0.05$, $P<0.01$), it might the result of cell toxicity due to high dosage of LPS. The TRAP(+) multinucleated cells do not have similarity in contrast to control group in terms of cell shape and integrity.

After 0, 50, 250, and 500 U/ml of IFN-γ were added in the M-199 and the chick bone marrow cells were cultured for 7 days. the number of TRAP(+) MNCs was decreased by 50 and 500 U/ml of IFN-γ. When the 0.1 μg/ml of LPS and various concentration of IFN-γ were added in the M-199, and the chick bone marrow cells were cultured for 7 days, the INF-γ showed a decreased tendency on

LPS-induced TRAP(+) multinucleated cell formation. Especially, when treated with 250 and 500 U/ml of IFN-γ, the number of TRAP (+) MNCs were decreased significantly ($P<0.05$). Naoyuki et al[29]. suggested that IFN-γ inhibited TRAP(+) formation and significantly inhibited 1.25 D_3-stimulated MNCs formation. The 1.25-dihydroxy vitamine D_3 significantly increased both MNCs formation and the number of nuclei per MNCs at 10^{-8}M concentration. Also, 100 U/ml of IFN-γ actively inhibited these effect of $1.25-D_3$ and inhibited the growth granulocyte-macrophagy colony forming cells. Additionally it inhibited MNC formation by parathyrpoid hormone or Interleukin-1. The study suggested that IFN-γ inhibits bone resorption in part by inhibiting osteoclast formation[29]. Yuko et al[30]. suggested that murine IF-γ usually did not affect basal[45]. Ca release and amost completely inhibited bone resorption induced by PTH, PTH-rp and interleukin-1. In periodontal disease that feature chronic inflammation increased bone resorption and bone loss occur. Horten et al[31]. substantiated the possibility that immune cells release product to stimulate bone resorption. It was suggested that interleukin-1[22,23,27], tumor necrosis factor α and β[24] stimulate osteoclastic bone resorption in vitro. Gowen et al[25,26]. suggested that interferon-γ inhibited bone resorption stimulated by cytokines, such as tumor necrosis factor α β, and interleukin.

IFN-ρ inhibits osteoclastic bone resoption, but the action made responsible for this effect is controvesial. 1) Interferon-γ suppress precursors of multinucleated cell[32]. Broxameyer et al[33]. suggested that interferon-γ suppress the growth of myeloid progenitor cells, the probable precusors of multinucleated cell. Therefore, interferon-g decreased the number of multinucleated cell. 2) Interferon-γ inhibits fusion of precursors to form multinucleated cell[25,26,34]. Takahashi et al[29]. has shown that interferon-γ decreases the number of multinucleated cell due to inhibit fusion of precursors to form multinucleated cell. Also, it decreased the number of nuclei per multinucleated cell. But, this report can not evaluate whethe inhibit fusion of precursors or suppress precursors of multinucleated cell.

Our results suggest that the number of TRAP(+) multinucleated cells was significantly increased by the addition of 0.1 μg/ml of LPS. The IFN-γ showed inhibitory effect on LPS-induced TRAP(+) MNCs number in chick bone marrow culture, especially in 250 and 500 U/ml of INF-γ. Further investigation must be performed to elucidate the action of LPS and IFN-γ.

V. Conclusion

This study was performed to evaluate the effect of lipopolysaccharide and interferon-γ on the osteoclast-like multinucleated cell formation from chicken bone marrow cells in culture. The results were as follows.

1. Treatment of 0.1 μg/ml LPS showed increased tendency on the number of TRAP (+) MNCs. otherwise 0.5 and 1.0 μg/ml of LPS showed decreased significantly on the number of TRAP)+) MNCs. ($P<0.05$, $P<0.01$)
2. 50 and 500 U/ml of IFN-γ showed tendency of decreasing the TRAP(+) MNCs formation
3. By the treatment of 0.1 μg/ml LPS and various concentration of IFN-γ, TRAP(+) MNCs number were decreased significantly by 250 and 500 U/ml of IFN-γ. ($P<0.05$)

On the basis of above results, it is concluded tha LPS increased TRAP(+) MNCs number

and IFN-γ decreased LPS-induced TRAP(+) MNCs number in chicken bone marrow cell.

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-국문초록-

세포 배양시 닭 골수세포로부터 파골세포양 세포형성에 지질다당류와 인터페론 감마가 미치는 영향

오홍균 · 김정근 · 이재현

단국대학교 치과대학 치주과학교실

파골세포는 조혈기관 단핵의 세포로부터 생성되어 골 흡수에 중요한 역할을 담당하며, 지질다당류는 그람음성균의 세포벽을 이루는 성분으로서 치주질환시 치조골 흡수에 관여한다고 알려져 왔다. 활성화된 림프구, 대식세포와 단핵세포로부터 생성되는 당단백질인 인터페론 감마는 파골세포에 의한 골흡수를 억제한다고 밝혀졌다.

이 연구 논문의 목적은 지질다당류와 인터페론 감마가 닭 골수의 미분화세포가 파골양세포로 전환되는데 어떠한 영향을 주는지를 알아보기 위함이다.

16~18일째의 닭의 배(chick embryo)에서 경골을 분리하고 횡절개하여 혈청없는 M-199 배양액에 보관했다. 이것을 9μm filter로 여과시켜서 이미 분화된 파골세포와 기타 다른 분화세포를 분리했다. 여기에서 파골세포의 전구세포를 얻어 LPS와 IFN-γ를 단독 또는 복합처리하고나서 4일 후에 tartrate resistant acid phosphatase (TRAP) Stain을 시행하고 TRAP 양성이며 핵이 세개 이상인 다핵의 세포형성을 관찰하여 세포를 계수하여 다음과 같은 결과를 얻었다.

1. 닭에서 분리해낸 미분화세포에 0.1, 0.5, 1.0 μg/ml의 LPS 농도를 처리하고 1주일간 배양한 결과, 0.1 μg/ml의 농도에서는 대조군에 비해 TRAP양성인 파골양세포가 증가하는 경향을 보였으며, 반면에 LPS는 0.5와 1.0 μg/ml의 농도에서 세포독성을 보였다. ($P<0.05$)
2. IFN-γ는 50, 500U/ml의 농도에서 대조군에 비해 TRAP양성인 파골양세포의 수가 감소하는 경향을 보였다.
3. INF-γ는 LPS에의해 유도된 TRAP 양성인 파골양세포의 형성을 감소시켰고 특히, 250, 500U/ml의 농도에서 유의성있는 감소를 보였다.

위의 결과로부터 LPS는 닭의 골수세포로부터 파골양세포의 형성을 증가시키며 IFN-γ는 LPS에의해 유도된 파골양세포수를 감소시킨다는 결론을 얻었다.

Key Word : Lipopolysaccharide, IFN-γ, Trap(+), Chicken Bone Marrow