KOREA. J. ORTHOD. 1995. 25(6) : 715-722

EFFECTS OF NITRIC OXIDE SYNTHASE INHIBITORS ON OSTEOCLAST-LIKE CELL FORMATION

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Orthodontic tooth movement in response to orthodontic force results from actions of osteoclasts and osteeoblasts in the cell level. Convincing evidence has now been provided to support the view that osteoclasts are derived from mononuclear cells that originate in the bone marrow or other hematopoietic organs and they migrate to the bones via vascular routes. Nitric oxide(NO), which accounts for the biological properties of endothelium-derived relaxing factor(EDRF), is the endogenous stimulator of soluble guanylate cylase. The discovery of the formation of nitric oxide(NO) from L-arginine in mammalian tissues and its biologioal roles has, in the last 7 years, thrown new light onto many areas of research. Data from experiments in vitro showed that N-metyl-L-arginine(L-NMA) and L-nitro-L-arginine(L-NAME) are competitive inhibitors of nitric oxide synthase.

This study suggest that the multinucleated cells in our culture have characteristics of osteoclasts and that the potential bone cell activity of nitric oxide in vitro may be mediated in part by stimulation of marrow mononuclear cells to form osteoclast-like cells. Bone marrow cells were obtaineed from tibia of 19-days old chick embryo. After sacrifice, tibia was quickly dissected and the bone were then split to expose the medullary bone. The cells were attached for 4 hours and the nonadherent cells were collected. Marrow cells weere cultured in 96-well plate in medium 199. To examine the number of TRAP-positive multinucleated cells(MNCs), 10^{-8} M Vit-D_3 and various concentration of L-NMA and L-NAME weere added at the beginning of cultures and with each medium change. After 7 days of culture, tartrate-resistant acid phosphatase(TRAP) staining was performed for microscopic evaluation. Cells having more than three nuclei per cell were counted as MNCs.

The obsrved results were as follows ; 1. 1,25-dihydroxyvitamine D_3 stimulated the osteoclast-like multinucleated cells in cultures of chick embryo bone marrow. 2. Nitric oxide synthase inhibitors(NOSI ; N-NMA, N-NAME) stimulated the osteoclast-like cells in cultures of chick embry bone marrow. 3. 1,25-dihydroxyvitamine D_3 and nitric oxide synthase inhibitors did not appear to have additive effect on the generation of TRAP-positive MNCs.

These results guggest that nitric oxide synthase inhibitors may stimulate the osteoclast-like multinucleated cell formation and fusion in cultures of chick bone marrow.

Key Words : Nitric oxide synthase inhibitors, Osteoclast-like multinucleated cells

Nitric Oxide(NO), which accounts for the biological properties of endothelium-derived relaxing factor(EDRF), is an endogenous stimulator of soluble guanylate cyclase, as originally defined by Dale(1933)[9]. The discovery of the formation of nitric oxide(NO) from L-arginine in mammalian tissues and its biological role[30] has, in the last 7 years, thrown new light onto many areas of research. Subsequent researches have progressed on to two directions, one toward understanding how NO participates in vital physiology, the other toward elucidating the biochemistry of nitric oxide syntheses (NOS).

The demonstration of the synthesis of NO from a guanidino nitrogen atom of L-arginine by vascular endothelial cells and activated macrophages led to the identification of the L-arginine-NO pathway in many other cells and tissues[9]. The vascular endothelium [30,25]

[25], brain[18,5,38], plateletes[31] and adrenal gland[27] contain constitutive Ca^{2+} / calmodulin-dependent enzymes. There are at least two types of NOS. One type is constitutive, cystosolic, Ca^{2+}/ calmodulin response to receptor or physical stimulation. The other enzyme is induced after activation of macrophages, endothelial cells, and a number of other cells by cytokines and once expressed, synthesizes nitric oxide for a long period. Also, the NO acts as a transduction mechanism to regulate vascular tones, platelet activation, neurotransmission, and has many other functions.[30,20,18,5]

NOS are soluble and they require NADPH to form citrulline as a co-product N-substituted analogs of L-arginine have been extremely useful in demonstrating the participation of NOS in intact cells[16], organs[35], and organisms.

Data from experiments *in vitro* indicate that L-NMA and L-NAME are competitive inhibitors of NOS[22].

Convincing evidence has now been provided to support the view that osteoclasts are derived from mononuclear cells that originate in the bone marrow or other hematopoietic organs and they migrate to the bones via vascular routes. Burger et al.(1982)[6] reported that HNC to possess typical properties of true osteoclasts, for example, expression of tratate-resistant acid phosphatase response to osteotropic hormones such as calcitonine(CT), 1,25-dihydroxy-vitamin D_3 [1,25$(OH)_2D_3$], parathyroid hormones (PTH), or Prostaglandin E_2(PGE_2), and the capacity to resorb devitalized bone. Vit D_3, which is known to increase the number of nuclei per osteoclast in cultured fetal rat bones, stimulates the rate of fusion of the preosteoclasts into osteoclasts as well as TRAP activity of the preosteoclasts in long-term cultures of bone marrow cells[1,3,17,36,19].

This study suggest that the potential bone cell activity of NO *in vitro* may be mediated in part by inhibition of marrow mononuclear cells to form osteoclasts-like multinucleated cells.

MATERIALS AND METHODS

1. Isolation and purification of mononuclear cells

Bone marrow cells were obtained from tibia of 18 to 19 days old chick embryo. After sacrifice, tibia was quickly dissected and placed into ice-cold minimum essential medium(MEM, Gibco), pH 7.4, containing 10% fetal bovine serum(FBS, Gibco).

The bones were then split to expose the medullary bone. The bone marrow cells were removed by shaking in the serum free MEM. The resulting cell suspension was centrifuged at 800xg at 10° C for 6 minutes and resuspended in the medium for culture. The cells were then attached for 4 hours and the nonadherent cells were collected by washing gently in the fresh medium. The resulting suspension was filtered through 50μm nylon mesh to seperate the cells from the fragments of bone and other tissue debris generated during the preparation[10] This crude fraction, highly enriched in large cells, was then filtered through a 8μm nylon mesh to remove the macrophage retained on the filter and recover the mononuclear cells in the filtrate.

2 Bone marrow cultures

Marrow cells were cultured in medium 199(Gibco) buffered with 25mM HEPES containing 10% heat-inactivated fetal bovine serum(FBS), penicillin (100 units/ml), streptomycin(50μg/ml)(Gibco) with 3.3 X 10^6cells/ml in 96-well plates(Falcon). Cultures were fed every 3 days by replacing 50μl of old medium with fresh medium. All cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37° C. To examine the number of TRAP-positive MNCs, in selected experiments, various concentrations of L-NMA, L-NAME, and 10^{-8}M 1.25$(OH)_2D_3$ were added at the beginning of cultures and each time medium was changed, and they were maintained for 7 days.

3. Tartrate-resistant acid phosphatase(TRAP) staining and counting procedures.

After 7 days of culture, cells were fixed with citrate-acetone-formaldehyde fixture for 30 seconds, and cytochemical staining for tartrate-resistant acid phosphatase(TRAP) was performed[11] using an acid phosphatase kit(Sigma, st. Louis, Mo). After staining, a detailed light microscopic analysis was performed. Cells having more than three nuclei per cell were counted as osteoclast-like multinucleated cells

Table 1. Effect of 1.25[OH]$_2$D$_3$ on the formation of TRAP(+)MNCs in the chick bone marrow cell culture

Treatment	No. of TRAP(+) MNCs/well
CONTROL	73.67 ± 5.49
1.25[OH]$_2$D$_3$(10^{-8})	113 ± 5.51**

Values are Mean ± SE(n=3)
**P<0.01, compared to control
MNCs : multinucleated cells

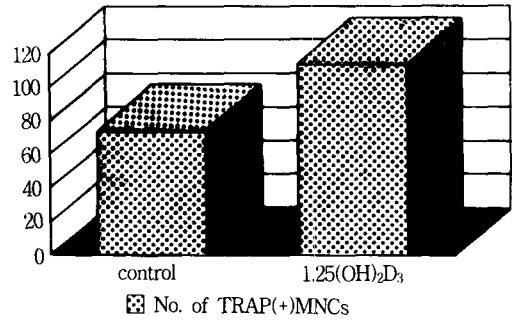

Fig 1. Effect of 1.25[OH]$_2$D$_3$ on the formation of TRAP(+)MNCs in the chick bone marrow cell culture

(MNCs), and the results were expressed as mean ± SE of quadruplicate cultures.

4. Statistical Methods

The mean ± SE was calculated for all results. Results were compared by using a paired Student's *t* test. Level of statistical significance was established at P<0.05 and P<0.01.

RESULT

In control cultures without treatment, the number of TRAP-positive MNCs was approximately 74 cells/well. Treatment with 1,25(OH)$_2$D$_3$ resulted in increased MNCs formation compared to the control group (Table 1). Our experimental cultures with inhibitors (L-NMA, L- NAME) alone had increased the number of TRAP- positive MNCs compared to the control group(Table 2. 3).

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Table 2. Effect of NOSI(NMA) on the formation of TRAP(+) MNCs in chick bone marrow cell culture.

Treament	No. of TRAP(+) MNCs/well	
	(-) 1.25[OH]$_2$D$_3$	(+) 1.25[OH]$_2$D$_3$
CONTROL	73.67± 5.67	113 ± 5.51
NMA 5㎛	187.33±25.71**	221.33±38.08*
NMA 50㎛	208 ± 9.17**	2051 ± 7.16**
NMA 500㎛	209 ±16.64	273 ±42*

Values are Mean ± SE(n=3)
**P<0.01, compared to control
* P<0.05, compared to control
MNCs : multinucleated cells

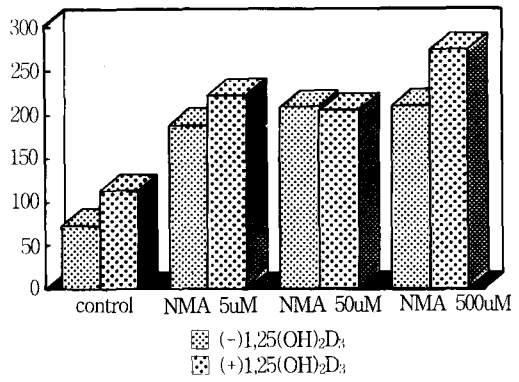

Fig 2. Effect of NOSI(NMA) on the formation of TRAP(+) MNCs in chick bone marrow cell culture.

was increased from 0.1μM to 1μM, TRAP-positive MNCs increased in three-fold. However, in the presence of 10mM NAME and 10^{-8}M 1.25(OH)$_2$D$_3$ cytotoxic effect was apparent(Table 3). Also, the L-NMA concentration in the medium increased TRAP-positive MNCs compared to the control group(Table 2).

In the presence of 0.1mM NAME without 1.25 (OH)$_2$D$_3$, no stimultion of differentiation and fusion of osteclast-like precusor was seen.

Two distinct morphological types of MNC were formed in the chick bone marrow culture system(Fig. 11) One with a smooth cellular margin, which we termed smooth-margined MNC, and the other type displaying irregular spike-like cells were observed in these cultures(Fig. 4.5.10.11). Stellate MNCs demonstrated more intense TRAP staining than the smooth-margined MNCs.

Table 3. Effect of NOSI(NAME) on the formation of TRAP(+) MNCs in chick bone marrow cell culture.

Treament	No. of TRAP(+) MNCs/well	
	(-) $1.25[OH]_2D_3$	(+) $1.25[OH]_2D_3$
CONTROL	73.67± 5.49	113 ± 5.51
NAME 0.1㎛	88.33± 5.04	275.33±13.98*
NAME 1㎛	199.67±19.1**	275.33±15.19**
NAME 10㎛	92 ±14.22	75 ±14.53**

Values are Mean ± SE(n=3)
**P<0.01, compared to control
* P<0.05, compared to control
MNCs : multinucleated cells

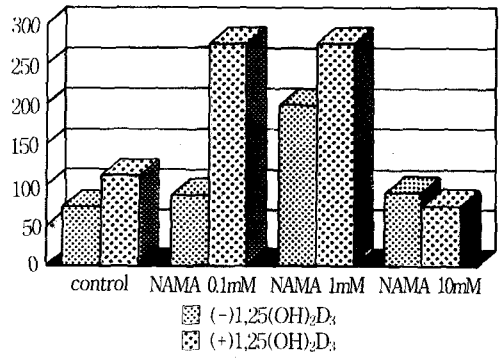

Fig 3. Effect of NOSI(NAME) on the formation of TRAP(+) MNCs in chick bone marrow cell culture.

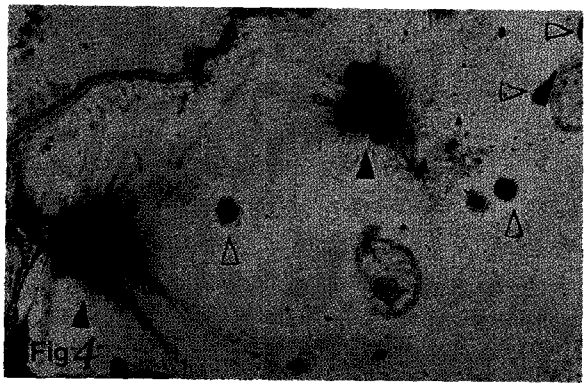

Fig. 4. TRAP-positive mono- and multi-nucleated cells in chick bone marrow cell culture(x40)

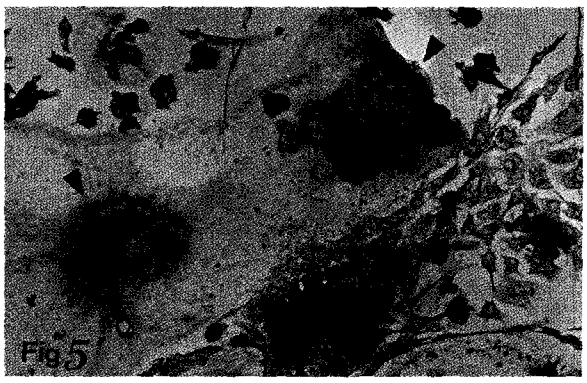

Fig. 5. Fusion of TRAP-positive multinucleated cell in chick bone marrow cell culture(x40)

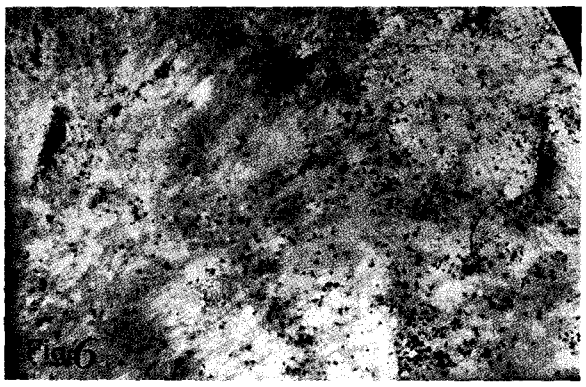

Fig. 6. TRAP stain in control group. TRAP-positive mononucle- ated cells in chick bone marrow cell culture(x10)

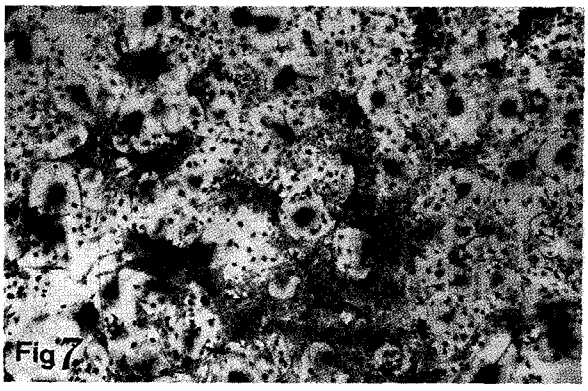

Fig. 7. TRAP stain of chick bone marrow cells cultured with $1.25[OH]_2D_3$. TRAP-positive multinucleated cells were shown(x10)

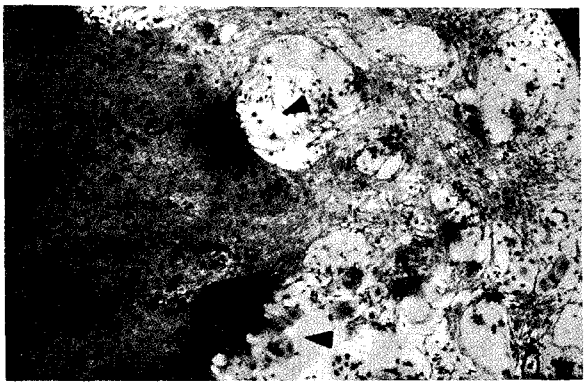

Fig. 8. Fuion of TRAP-positive multinucleated cells in chick bone marrow cell culture.(x10)

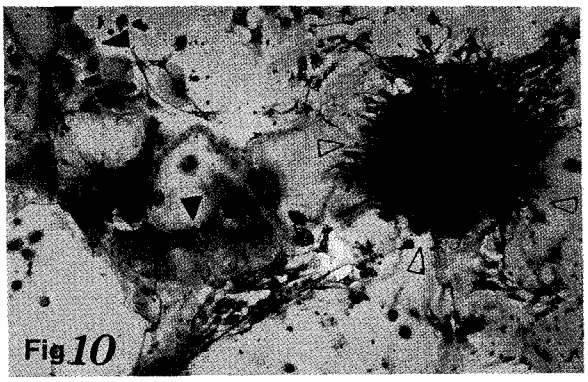

Fig. 10. Variable fusion of TRAP-positive multinucleated cells in chick bone marrow cell culture.(x40)

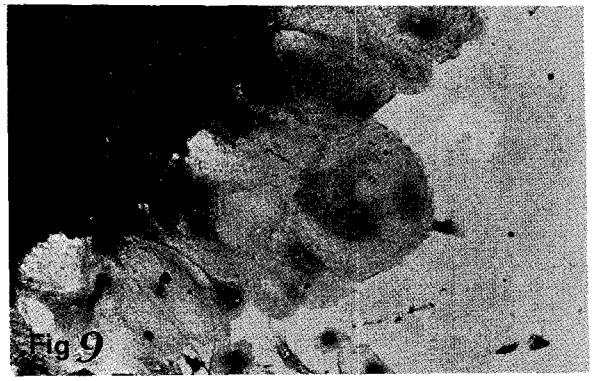

Fig. 9. Fuion of TRAP-positive multinucleated cells in chick bone marrow cell culture.(x40)

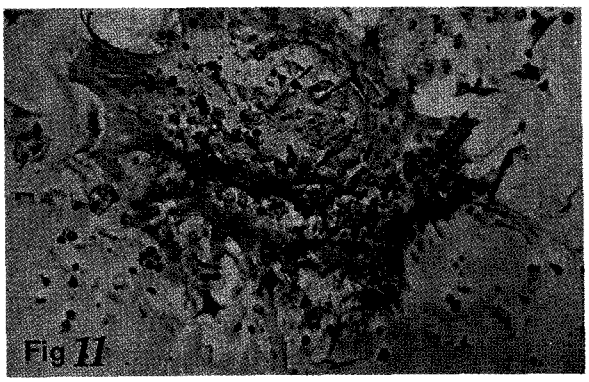

Fig. 11. TRAP-positive multinucleated cells in chick bone marrow cell culture(scanning electron-microscopic view)(x200)

DISCUSSION

It is well known that osteoclasts, the principal cells responsible for bone resorption, are multinucleated giant cells formed by the fusion of mononuclear precursors derived from hematopoietic progenitor cells.[11,7,26]

An assay of the differentiation of osteoclastic lineage cells are as follows[40] : 1)The multinucleated cells formed exhibited tartrate- resistant acid phosphatase(TRAP) acitivty ; TRAP is known as a marker enzyme of osteoclasts 2)The formation of TRAP-positive multinucleated cells is markedly stimulated by osteotropic hormones such as $1,25(OH)_2D_3$ and PTH hormone. Calcitonin strikingly inhibits the formation of TRAP- positive multinucleated cells induced by both osteotropic hormones. 3)As in the case of authentic osteoclasts, TRAP-positive multinucleated cells are formed by fusion of mononuclear precursors. 4)Perhaps most important, when marrow mononuclear cells were cultured on calcified dentin slices in the presence of $1,25(OH)_2D_3$, the TRAP-positive multinucleated cells resorbed dentin by creating resorption lacunae.

Formation of the multinucleated cells in the cultures is appropriately modulated by the osteotropic hormones such as PTH, 1,25 Vit-D_3 and calcitonin. Number of multinucleated cells is increased in the presence of PTH and $1,25(OH)_2D_3$, and decreased or inhibited by calcitonin. Similar modulation of osteoclast numbers is reported after the administration of these hormones *in vivo* or in organ culture.[41,12] The

bone resorption-stimulating hormone, $1,25(OH)_2D_3$, is directly or indirectly involved in the differentiation and fusion of mononuclear precursors to form multinucleated cells.[36.26.40.41.12] Also, $1,25(OH)_2D_3$ is a powerful stimulator of bone resorption [24.25] and increases the number and activity of osteoclasts both *in vitro* and *in vivo*.[15.34]

The demonstration in 1987 of the formation of NO by an enzyme in vascular endothelial cells[28] opened up what can now be considered a new area of biological research. NO, which accounts for the biological properties of EDRF, is an endogenous stimulator of the soluble guanylate cyclase.[9] Nitric oxide(NO) is a short-lived, gaseous radical that is the smallest biosynthetically derived secretory product of mammalian cells.

Production of nitric oxide(NO) from L- arginine has been demonstrated in the vascular endothelium and in other tissues and cell lines including EMT6 murine mammary adenocarcinoma cells.[24.2] There are two types of NOS, one is constituitive, calcium / calmodulin-dependent and the other one is inducible, calcium-independent which requires tetrahydrobiopterin. The constitutive enzyme, which produces nitric oxide for a short period in response to receptor or physical stimulation, is present in endothelial cells, platelets, adrenal gland and brain.[24] The biological actions of NO are vasodilation and inhibition of the soluble guanylate cyclase.[22.33] The inducible enzyme present in macrophages, vascular endothelium, vascular smooth muscle, Kupffer cells, hepatocytes and EMT6 adenocarcinoma cells produces NO for a long period following induction by an immunological stimulus.[35.2.39.8.32.42] These findings brought out three known families of inhibitors of NOS. The inhibitors first described, N^w-substituted analogs of L-arginine [16], have been extremely useful in demonstrating the participation of nitric oxide synthesis in intact cells, organs, and organisms. The most widely used analogs of this class are N^w-methyl- and N^w-nitro-L-arginine (NAME) in low micromolar range and they function as competitive, reversible inhibitors.

Since early 1980s several studies on osteoclast -like cell formation in long-term bone marrow cultures were reported.[41.42.37.13] However, the osteoclasts are relatively inaccessible and impossible to obtain in large numbers. Also, they are difficult to maintain in long-term cultures and these studies were performed in non-human species.

In many studies, mouse bone marrow cultures have been used to examine the effect of hormones and growth factors on the formation of osteoclasts from hemopoietic progenitor cells. The numbers of osteoclasts and osteoclast precursors formed *in vitro* were determined by counting cells that were positive for the enzyme tartrate-resistant acid phosphatase (TRAP) ; this enzyme is generally considered as a reliable marker.[14.21.43]

This study was performed in chick bone marrow cells. Three steps can be distinguished in the formation of osteoclasts : 1)early osteoclast progenitors do not express the tartrate-resistant form of acid phosphatase(TRAP) and are proliferative ; 2)osteoclast precursors, which are postmitotic cells, can be separated into an early and a late form based upon their acquisition of tartrate-resistant acid phosphatase positivity ; and 3)mature multinucleated osteoclasts, which are formed by the fusion of these late precursors.

Accurate counting, however, was not easy in cultures. We have applied our techniques to examine the effects of nitric oxide. We found in the various cell cultures multinuclear, TRAP-positive cells. TRAP-positive cells were often found in clusters in which the individual cells were difficult to count. With the TRAP -staining, the number of cells were stained (dark) red.

In our study multinucleated cells with several characteristics of osteoclasts were formed in cultures of chick bone marrow. Support for the osteoclast-like nature of these cells include the following ; 1)The multinucleated cells contained tartrate-resistant acid phosphatase, enzymes that are present in osteoclasts. 2)The multinucleated cells formed in these cultures were morphologically similar to osteoclasts under light microscopy, with centrally placed nuclei with prominent nucleoli and a cytoplasm that was generally granular but with a clear periphery(clear zone). 3)In the experimental cultures with $1,25(OH)_2D_3$, NOSI had increased the fusion of TRAP-positive MNCs.

Further studies are needed to see the effects of nitric oxide on the osteoclast-like multinucleated cell formation, attachment, morphology, and activities. They are feasible and they deserve intensive study.

CONCLUSION

Although it is now well established that osteoclasts are derived from mononuclear hematopoietic stem cells, there is a need to investigate the roles of nitric oxide and vit-D_3 in the generation of osteoclast-like MNCs from their precursor cells. Chick bone marrow cells were obtained from tibia of 19-days old chick embryo. Marrow cells were cultured in medium 199 containing 10% fetal bovine serum at 3.3 X 10^6 cells/ml in 96-well plates(100μl/well). To examine the number of TRAP-positive MNCs, various concentrations of L-NMA, L-NAME and 10^{-8}M Vit-D_3 were added at the beginning of cultures and with each medium change. After 7 days of culture, TRAP staining was performed for microscopic evaluation. Cells having more than three nuclei per cell were counted as MNCs. The observed results were as follows ;

1. 1,25-dihydroxyvitamine D_3 stimulate the osteoclast -like multinucleated cells in cultures of chick embryo bone marrow.
2. Nitric oxide synthase inhibitors(NOSI ; N-NMA, N- NAME) stimulate the osteoclast-like cells in cultures chick embryo bone marrow.
3. 1,25-dihydroxyvitamine D_3 and nitric oxide synthase inhibitors did not appear to have additive effect on the generation of TRAP-positive MNCs.

These results suggest that nitric oxide synthase inhibitors may stimulate the osteoclast-like multinucleated cell formation and fusion in cultures of chick bone marrow.

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