대한침구학회지 제19권 제5호(2002년 10월)
The Journal of Korean Acupuncture & Moxibustion Society

원 저

Increased effects of Bee Venom on aromatase expression and activity in the human osteoblastic cells

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국문초록

인간 골아세포에서 aromatase 효소의 발현과 활성에 대한 봉독의 증강효과

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본 연구는 osteoblastic cells에서 estogen의 생합성을 유도하는 aromatase의 activity에 대한 蜂毒의 작용을 측정하여, 봉독치료시 Arthritis의 진행 억제 및 estogen에 의한 bone formation의 효과여부를 검증하기 위해 실행하였다.

사용된 세포주로는 Osteoblastic phenotype으로 분화가 유도되는 Human leukaemic cell line FLG 29.1 및 the primary first-passage osteoblastic cells (hOB cells)이며, 이들을 각각 배양하고 각각의 RNA를 isolation한 뒤 PCR 증폭을 하였다.

Aromatase에 대한 활성인자인 TPA와 TGF-β1 및 봉독을 이용하여 aromatase의 expression 및 activity에 대해 미치는 영향을 측정한 바, aromatase expression은 FLG 29.1 cell와 hoB cells에서, 50nM TPA 24시간 처리, 봉독 2 ~ 4시간 처리와 TGF-β1 3시간 처리로 유도한 결과 TPA와 TGF-β1의 경우는 서로 유사하였고, 봉독에서 상대적으로 높게 나타났다.

* 본 연구는 동국대학교 논문게재연구비 지원으로 이루어졌음.
· 접수 : 2002년 8월 24일 · 수정 : 2002년 9월 4일 · 채택 : 2002년 9월 14일
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Aromatase activity는 FLG 29.1 cell, hoB cells에서 24시간 incubation한 결과, 모든 실험에서 일정하게 선상증가를 보였다. 5㎕/㎖ BV에서 TPA와 TGF-β1보다 뚜렷하게 증가하였으며, o.5mM Bt2-cAMP, 50nM dexametasone처리에서는 유의성이 없었다.

Estrogen 생합성을 촉매하는 aromatase activity가 BV 처리에서 현저하게 증가하였기에, Rheumatis arthritis의 bone destruction에 대해 BV가 효과적인 역할을 할 것으로 보여진다.

Key words **: Bee venom(BV), Rheumatis arthritis(RA), bone distruction, aromatase activity, aromatase expression, estrogen**

I. Introduction

Rheumatoid arthritis(RA) is characterized by localized degeneration of articular cartilage mainly in weight-bearing joints, it is a systetemic inflammatory disorder characterized by inflammatory cell infiltration of proliferated synovial linings, and subsequent tissue erosion.

BV(Bee venom) contains at least 40 active substances such as peptide, enzymes, physiologically active amines, carbohydrates, lipids, amino acids, widely used in Oriental medicine for tonics, anti-rheumatic effects, etc.[1] So BVT(Bee venom therapy) has been traditionally used for arthralgia and aches in joints and knees due to arthritis, or asthenia of muscles due to auto-immune disease.[4]

Currently research result is that, BV inhibited production of IL-1β and TNF-α from T cells and macrophages, has potent effects on RA. In addition, it was also investigated that the anti-inflammatory effect of the BV on an acute inflammatory process. Therefore, Bee Venom treatment(BVT) could inhibit the onset and development of arthritis and the immune responses to collagen.[3]

Although the precise medication mechanisms for the RA's bone destruction in clinically chronic stage remain unknown. It was suggested that the local conversion of sex steroid precursors to estrogens may act directly on bone formation[28].

Estrogen biosynthesis is catalyzed by the enzyme complex (aromatase-cytochrome P450), it's activity and gene expression exist in osteoblast-like cells, in adipose tissue[5]. Thus, aromatase activation stimulates skeletal modeldeling and increases bone size and mass. The osteoblastic lineage cells can express aromatase cytochrome P450 gene and the enzyme activity was markedly and transiently induced by the phorbol ester TPA or TGF-β 1.[6),7] The regulation of the human aromatase cytochrome P450 gene expressed by various agents, such as glucocorticoids, cAMP analogs, a variety of growth factors.

In this study, we examined the differential influence with BV and various agents, such as

phorbol esters which has suggestive of a control on aromatase expression, on the enzyme activity and osteoblastic cellular responses, by using human osteoblastic cells. We will gain further insight into the effectiveness of BV on arthritis by cellular responses to aromatase expression in the human osteoblastic cells.

Ⅱ. Materials and methods

1. Materials

Culture flasks and dishes were obtained from Nunc(Roskilde, Denmark). All other chemicals were purchased from Sigma(CA, USA).

Media and sera for cell culture were purchased from Jeil Biotech Services(Taegu, Korea).

[α-32β]-dCTP was from DuPont-NEN (Boston, MA).

The human β-actin cDNA insert and the ExpressHyb hybridization solution were purchased from Clontech (Palo Alto, CA).

BV kit, a saline-mixed BV was purchasd from Monmouth Pain Institute, Inc., New Jersey, USA as an injection grade for human.

Each bial contained 10㎖ of the BV. For application in cell culture, randomly selected bials were suspended in normal saline at a concentration of 5㎕/100㎕. In some case, original concentration of BV was used.

All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co.(St. Louis, MO)

2. Cell culture

1) The human leukemic cell line FLG 29.1

FLG 29.1 cells were cultured in RPMI-1640 culture medium, supplemented with 10% fetal calf serum(FCS)[10].

2) The primary first-passage osteoblastic cells(hOB cells)

For hOB cells, bone specimens were obtained from the iliac crest of a patient(man ; 35 years) undergoing corrective surgery after traumatic fracture. The patient had no signs or symptoms of bone or joint disease.

The study was approved by the Institutional Review Board of the Dongguk University, and written informed consent was obtained from patient.

We obtained RNA from the hOB cells from cultures of trabecular bone explants.[11]

These hOB cells have been shown to represent the phenotype of the mature osteoblast, were grown at 37℃, maintained in phenol red-free minimal essential medium (MEM) supplemented with 10% charcoal-stripped fetal calf serum (cs-FCS), and were cultured in serum-free MEM supplemented with 0.125% (w/v) bovine serum albumin (BSA) for 4 days prior to RNA isolation.

3.Reverse transcription-polymerase chain reaction (RT-PCR)

1) RNA isolation

Poly(A)+ RNA was isolated from FLG 29.1 cells using oligo (dT) affinity chromatography, according to Oligotex kit instructions (Quiagen Korea Co., Seoul, Korea).

Total RNA from adipose tissue specimens, obtained from women undergoing elective abdominal surgery who gave written consent, was isolated using a commercial kit (Quiagen Co. or Promega Co.).

2) cDNA synthesis

First strand cDNA synthesis and PCR amplification were performed as previously described with minor modifications.

Briefly, 100ng of poly(A)+ RNA or 1μg of total RNA was reverse transcribed using 20 Units of moloney leukemia virus reverse transcriptase (Stratagen, La Jolla, CA, U.S.A.) in a 50μl volume.

3) PCR amplification

PCR amplification was carried out in 50μl PCR buffer (10mM Tris-HCl, pH 8.3) with 2.5mM $MgCl_2$, 200μM dNTP with 0.5Units of cloned Thermus aquaticus DNA polymerase (Amplitaq, Perkin Elmer, Korea), and 0.1μM of each primer.

One PCR cycle (30 cycles total) consisted of denaturation at 94℃ for 1 min, annealing at 60℃ or 66℃ for 1 min, and polymerization at 72℃ for 1 min.

PCR products were electrophoresed on 1~1.5% agarose gel, visualized by ethidium bromide staining and photographed under UV light.

The sequences of sense and antisense primers (Bioneer Co., Korea) were 5'GAATAT TGGAAGGATGCACAGACT, corresponding to a sequence in exon 9, and 5'GGGTAAAGATCAT TTCCAGCATGT, complementary to a sequence of exon 10 of the human aromatase gene[16], respectively; with an expected product of 293bp, previously used in humanbreast tumor tissues[13], or 5'CCGGCCTTGTTCGTATG GGTCA, corresponding to a sequence in exon 5 (sense primer) and 5'GTCTCATCTGGGTGC AAGGA, complementary to a sequence of exon 10 of the human aromatase gene (antisense primer) with an expected product of 987bp, previously used in human osteoblast-like cells[7].

The mRNA integrity and the amplification efficiency of aromatase transcripts were evaluated by the amplification of a β-actin sequence (expected size=224bp) from equivalent amounts of poly(A)+ RNA of each sample.

The sequences of sense and antisense prmers for β-actin amplification were 5'-CCC AGCACAATGAAGATCAA-3' (sense primer), corresponding to a sequence in exon 4, and 5'-TTTCTGCGCAAGTTAGGTTTTGACCAA-3', corresponding to a sequence in exon 5 (antisense primer).

PCR analyses were repeated two to four times on each sample to control whether relative differences in yield of PCR products between samples reflected differences in mRNA concentrations or random assay variability.

The transcripts were visualized by ethidium

bromide staining of agarose gel. To test the specificity of the transcripts, the RT-PCR products were electrophoresed through a 1.5% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled human aromatase cDNA probe (PCT product, Kim et al., unpublished results).

4) Aromatase activity

The aromatase activity was estimated by determining the incorporation of tritium from [^3H]androstenedione ([1b, 2b-N-3H]androst-4-ene-3,17-dione; 42 Ci/mmol; 1Ci=42GBq; New England Nuclear, UK) into [^3H] H_2O[18] in FLG 29.1 cells for 6 hours.

Cells were seeded in the appropriate growth medium with or without 5% dextran-charcoal stripped FCS (DCS-FCS) in the absence or presence of various stimuli. After 3~48 hours, cells were washed, resuspended in fresh medium with or without 5% DCS-FCS, and incubated (1×106 cells/well) in 6-well plates with [^3H]androstenedione at 0.5nM ~ 100nM concentrations for kinetic studies, or with 2nM [^3H]androstenedione for single point determination of enzymatic activity, at 37℃ in humidified atmosphere for 6 hours, unless otherwise stated.

After condensating water papor by placing the culture plates on ice for 15 min, cells were then pelleted and the incubation medium was removed.

After cells were then washed twice with phosphate-buffered saline (2.6mM KCl/1.4mM KH_2PO_4/140mM NaCl/8mM $Na_2HPO_4 \cdot 7H_2O$/0.5mM $MgCl_2 6H_2O$/1mM $CaCl_2$), protein content was determined.

The incubation medium and the combined saline dishs were pushed through a preequilibrated Sep-Pak C18 minicolumn into a counting vial.[14] The column was washed with water to remove residual [^3H] H_2O.

Scintillation liquid (15㎖) was added and the radioactivity was counted after equilibration. Each experiment was conducted in triplicate and was repeated at least twice times to ensure that the results were quantitatively reproducible.

To establish the nonspecific release of [^3H]H_2O duplicate aliquots of [^3H]androstenedione-supplemented cell-free medium were incubated and the blank value, which averaged 0.8% of the added radioactivity after 6 hours incubation, was subtracted from the amount measured in the experimental incubations.

To evaluate the specificity of the assay, FLG 29.1 cells and hOB cells after 4 hours exposure to culture medium supplemented with 5㎕/㎖ BV

And it were incubated with 2nM [^3H]androstenedione in the absence or presence of 100 nM~1μM concentration of the CGS16949A which is non-steroidal aromatase inhibitor and inhibite the aromatase activity with a dose-dependent fashion reaching approximately 90% suppression of the enzyme activity.

4.Analytical methods

Protein contents were determined by a Protein assay kit of Bio-Rad Laboratories

(Richmond, CA, USA).

5. Statistics

Data were expressed as mean±SD. Statistical differences were analyzed using one way analysis of variance. Significance was adjusted for multiple comparisons of means using Bonferroni's approximation.

III. Results

1. Aromatase expression

The products of the expected size from the different amplification(987 bp) were observed in samples from FLG 29.1 cells and adipose tissue <Fig. 1>. Treatment of BV induced the expression of the aromatase with increasing activity by a dose-dependent manner(0 to 10 $\mu\ell$/mℓ). It was clearly induced by 24 hours treatment with 50nM TPA <Fig. 1>., known to affect FLG 29.1 cells differentiation.[14] The BV induction of the 987bp transcript appeared to be dose-dependent with maximal expression after 2~4 hours of treatment <Fig. 2>.

2. Aromatase activity

In all experimental conditions the rate of aromatase activity was linear for up to 24 hours incubation(data not shown).

Therefore, the enzymatic activity was e-valuated by incubating [^{3}H]androstenedione for 6 hours. BV markedly increased the enzymatic activity in a dose-dependent fashion, reaching the maximum at 5$\mu\ell$/mℓ concentration with no significant differences up to 10$\mu\ell$/mℓ concentration.

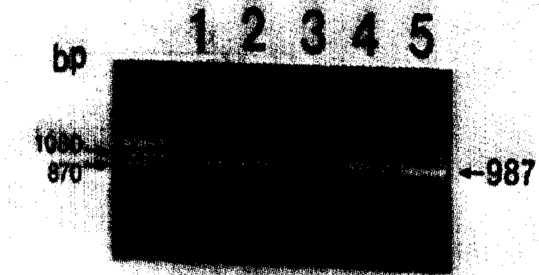

Fig. 1. RT-PCR amplification of aromatase mRNA from FLG 29.1 cells and human adipose tissue. Poly(A)+ RNA from cells treated with 10$\mu\ell$/mℓ BV (lane 1), 5$\mu\ell$/mℓ BV (lane 2) or untreated (lane 3) for 24 hours.

RNAs from 50nM TPA-treated cells(lane 5) and human adipose tissue(lane 4) were reverse transcribed using couples of primers and the amplification products were visualized by ethidium bromide staining.

The position and the size of marker fragments are reported on the left, while the position and the expected sizes of the transcripts are reported on the right(987 bp).

To evaluate the specificity of the assay, FLG 29.1 cells and hOB cells after 4 hours exposure to culture medium supplemented with 5$\mu\ell$/mℓ BV were incubated with 2nM [^{3}H]androstenedione in the absence or presence of 100nM~1μM concentration of the nonsteroidal aromatase inhibitor CGS16949A[16].

CGS16949A inhibited the aromatase activity with a dose-dependent fashion reaching approximately 90% suppression of the enzyme activity (not shown).

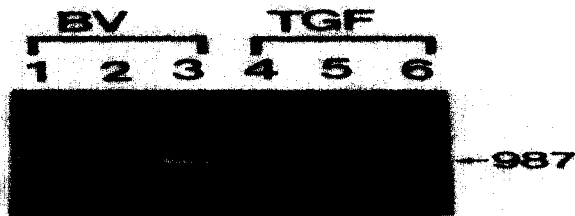

Fig. 2. Time-dependent and dose-depen- dent effect of BV and TGF-β1 on aromatase RT-PCR products. FLG 29.1 cells were treated with 5㎕/㎖ BV for 0 (lane 1), 2 hours (lane 2) and 4 hours (lane 3).

Also, cells were treated with 0.01ng/㎖ TGF-β1 (lane 4), 0.1ng/㎖ TGF-β1 (lane 5) and 1.0ng/㎖ TGF-β1 (lane 6) for 24 hours and the aromatase mRNA expression was evaluated by RT-PCR analysis.

The size and the position of the expected transcript of 987 bp is reported. Equivalent amounts of poly(A)+ mRNA of each sample were analyzed for the amplification of a human β-actin sequence (not shown).

Similarly, TGF-β1 induced aromatase expression by dose-dependent manner after 3h treatment, although the expression level was much lower than those of BV or TPA.

The same products of the expected size as that of FLG 29.1 cells were also observed in samples from hOB cells from cultures of trabecular bone explants (Fig. 3). Treatment of BV induced the expression of the aromatase with increasing activity by a dose-dependent manner (0 to 10㎕/㎖). Treatments for 50nM TPA or TGF-β1 induced the aromatase expression. However, the expression level by TGF-β1 was almost same level of BV or TPA, as compared with the FLG 29.1 cells.

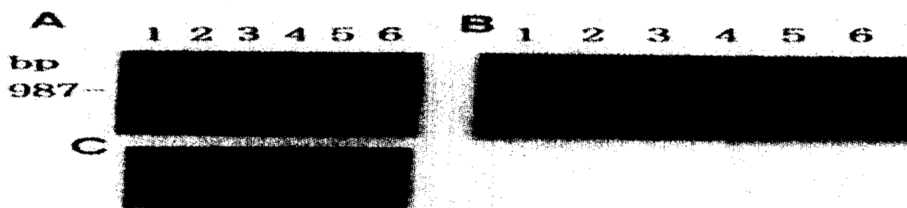

Fig 3. RT-PCR amplification of aromatase mRNA from the hOB cells and Southern blot of RT-PCR products from the hOB cells with a ^{32}P-labeled human aromatase cDNA.

A) Poly(A)+ RNA from cells treated with 50nM TPA (lane 1), 1.0ng/㎖ TGF-β1 (lane 2), 5.0㎕/㎖ BV (lane 3), 2.0㎕/㎖ BV (lane 4), 0.5㎕/㎖ BV (lane 5) for 24 hours or untreated (lane 6) were reverse transcribed using couples of primers and the amplification products were visualized by ethidium bromide staining.

B) Southern blot analysis of panel A. The expected sizes of the transcripts are reported on the right.

C) Equivalent amounts of poly(A)+ mRNA of each sample were analyzed for the amplification of a human β-actin sequence.

Table Ⅰ. Effect of BV, TPA, TGF-β1, Bt2cAMP and dexamethasone on aromatase activity in FLG 29.1 cells and hOB cells.

A) Aromatase activity (fmol/mg/protein/2h) in FLG 29.1 cells as Time of treatment

Agents \ Time	2 hours	12 hours	24 hours
None	16.7±2.4 (n=6)	11.4±1.6 (n=5)	10.5±3.5 (n=4)
+ 5μℓ/mℓ BV	87.3±14.3 (n=4)**	76.8±4.5 (n=5)**	33.3±5.3 (n=6)*
+ 50nM TPA	43.3±4.3 (n=4)*	41.5±6.2 (n=3)*	21.3±2.5 (n=5)*
+ TGF-β1 (0.1ng/mℓ)	45.3±5.2 (n=5)*	36.5±4.3 (n=4)*	25.2±3.5 (n=4)*
+ 0.5mM Bt2c-AMP	14.3±1.6 (n=3)	10.5±2.2 (n=3)	9.4±1.1 (n=4)
+ 50nM dexamethasone	12.2±3.1 (n=4)	12.1±2.1 (n=3)	9.6±2.3 (n=3)

B) Aromatase activity (fmol/mg/protein/2h) in hOB cells as Time of treatment

Agents \ Time	2 hours	12 hours	24 hours
None	16.5±2.4 (n=3)	13.6±2.2 (n=4)	12.1±1.2 (n=3)
+ 5μℓ/mℓ BV	95.4±21.2 (n=5)**	68.6±7.2 (n=4)**	43.3±4.6 (n=3)*
+ 50nM TPA	56.7±5.7 (n=3)*	47.3±5.4 (n=3)*	42.2±4.4 (n=5)*
+ TGF-β1 (0.1ng/mℓ)	62.3±5.6 (n=3)**	42.4±6.4 (n=5)*	34.3±6.4 (n=3)*
+ 0.5mM Bt2c-AMP	16.3±2.3 (n=4)	14.3±3.2 (n=4)	12.2±4.3 (n=4)
+ 50nM dexamethasone	13.4±5.2 (n=3)	12.3±3.6 (n=4)	10.4±2.2 (n=4)

*. P<0.05, significantly different from each nomal control as expressed to "None".
**. P<0.01, significantly different from each nomal control as expressed to "None"

A similar pattern of induction was shown by TPA and TGF-β1 in bothe cell types. After 24 hours incubation, although the mean values of aromatase activity in both cells treated with the different agents were higher than in untreated cells, only those under BV and TPA treatment were significantly different from the control group <Table 1>. On the contrary, either 0.5mM Bt2-cAMP or 50nM dexamethasone did not significantly affect the basal rate of the enzyme activity <Table 1>.

Ⅳ. Discussion

RA is a chronic inflammatory autoimmune disease in which the body attacks its own tissues, synovial membranes, the cartilage and become inflamed. So the result was joint linings and bone destruction. RA is referred to the category of severe and migratory arthral-

gia(歷節風, 白虎歷節風, 痛風) in oriental medicine.

BV, which has bitter, pungent tastes and mild temperature, main effects are related to tonics, tranquilizers, relieving cough and asthma, anti-Rheumatic, anti-inflammatory analgesic, immunity and anti-cancer, its indications are acute or chronic arthritis, neurologic pain, rheumatoid, rheumatic arthritis, etc.[4]

According to the records, there are two proscriptions of BV in the ancient books of Oriental medicine that were buried in the tomb of Mawangdui, BC 168. In those prescriptions, bee venom was applied to enhance the vitality or to treat masculine sexual importance. It is considered those two prescriptions could be considered as a prototype of bee venom therapy and are evidence that bee venom therapy was studied and practiced at least 2000 years ago as a part of Oriental medicine.[1]

In Oriental medicine, BV has been widely used in the treatment of some immune-related diseases, especially RA as an immunosuppressive agents, and satisfactory results are obtained[1].

In 19 century, BV has become related to RA. Desjardins(1858 French) and Austrian, Russian Doctors began the first clinical studies with BVT. After this study, many studies ware reported that related to BV's immunity, toxicity, anti-inflammatory and analgesic effects, clinical investigation, anti-cancer effects[2]. Still unknown, how about the mode of action of this toxical medication on RA.

And it was suggested that BVT could inhibit the onset and development of arthritis and the immune responses to collagen, but that BV can not change the severity when the disease was established.[2]

Until present time it became known, BV inhibited production of IL-1β and TNF-α from macrophages in response to *in vivo* stimulation with bacterial lipopolysaccharides(LPS) when it was administered into mice.[10] And suggesting that the BV, administered into the patients, inhibit cytokine production from both T cells and macrophages and potent effects on RA. In addition, it was also investigated that the anti-inflammatory effect of the BV on an acute inflammatory process using the carrageenan-induced edema test[3].

There is general consensus that CD4+ T cells act as initiators of RA, by migrating to the affected joints, recognizing peptides derived from processed antigens, and releasing several types of cytokines[17,18]. Such cytokines enhance the function of other cells, expecially macrophages to produce pro-inflammatory cytokines including IL-1β and TNF-α[19].

On the other hand, BVT which began concurrently with a booster injection, also significantly suppressed the development of arthritis and immune responses to collagen. The precise mechanisms accounting for these phenomena are not clear, but similar observations were made by Asano et. al.[20], who showed that delayed traditional Chinese extract treatment could suppress development of arthritis and of immunity to collagen.

It is observed that BV is able to suppress clonal expansion of helper T cells, when it is administered intraperitoneally into rat. Therefore, although the mechanism(s) by which BV exerts suppressive effects on clonal T cell expansion is not well understood, this regimen might theoretically lead to specific clonal depletion and result in inhibition of development of the diseases. An alternative explanation is that the time of a booster injection may still be within the induction phase of arthritis.

Since the clinical treatment with immuno suppressice agents such as cyclosporin A and FK-506 had a beneficial effect in patients with refractory RA[21),22)], BV might be a useful tool for the treatment of RA. However, biochemical and metabolic analysis of the constituents of BV have to be analysed in further delineating its mechanisms of action in arthritis.

But, still unknown about the mode of medication on arthritis's bone destruction in clinically chronic stage. Therefore, in this study, we examined the influence of BV on osteoblastic cellular responses by using human osteoblastic cells.

It was suggested that the local conversion of sex steroid precursors to estrogens could be involved in the pathogenesis of postmenopausal osteoporosis. Estrogen biosynthesis is catalyzed by the enzyme complex (aromatase - cytochrome P450) mainly in the adipose tissue with androstenedione as the principal precursor[28)].

The enzyme complex termed aromatase cytochrome P450 is implicated in bone metabolism and suggested that locally synthesized estrogen may act directly on bone formation. Thus, aromatase activation stimulates skeletal modeling and increases bone size and mass in growing rats.[15)] The regulation of the human aromatase cytochrome P450 gene expression by various agents, such as glucocorticoids which known stimulators of estrogen formation in adipose tissue[5)], cAMP analogs, a variety of growth factors and phorbol esters[23),24)], is suggestive of a control of aromatase expression[6),7)] in osteoblast-like cells[7)].

The cells of osteoblastic lineage can express aromatase cytochrome P450 gene and the enzyme activity was markedly and transiently induced by the phorbol ester TPA or TGF-β1. In FLG 29.1 and hOB cell, aromatase activities are not induced by cAMP analogs and dexamethasone. This is in contrast to other systems[23),25),28)]. However, the stimulation by TGF-β1 is inducive. Bone matrix is one of the major storage sites for TGF-β1 and TGF-β1 isoforms are directly involved in bone remodeling via an autocrine and/or paracrine mechanism of action[26)].

Moreover, it was recently demonstrated that FLG 29.1 and hOB cells produce TGF-β1 and bear functional receptors for the growth factor[9)]. Interestingly, both TGF-β1 and phorbol ester appear to induce aromatase mRNA expression in a transient fashion, however, enzyme activity although time-dependently modulated by the different agents, are detectable. However, cAMP was ineffective in stimulating

aromatase activity in both cells of FLG 29.1 cells and hOB cells.

These findings confirm the *in vitro* estrogen production by bone-derived cells, as also demonstrated in cells of the osteoblastic lineage[6),7),26),27)].

This result can explain that the effectiveness of BV on arthritis by cellular responses to aromatase expression in the human osteoblastic cells. Further long-term clinical studies are needed to evaluate the effect of BV about arthritis's bone destruction.

V. Conclusion

1. BV induced the expression of the aromatase with increasing activity by a dose-dependent manner(0 to 10㎕/㎖) more then the treatments of TPA, TGF-β1 in osteoblastic cells.
2. In all experimental conditions the rate of aromatase activity was linear for up to 24 hours incubation. BV, reaching the maximum at 5㎕/㎖ concentration, markedly increased the enzymatic activity in a dose-dependent fashion, then TPA and TGF-β1.
3. As aromatase activity was markedly increased by BV treatment. These findings confirm that BV is effective for the treatment of RA's bone destruction.

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