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Protein kinase C inhibitor H-7 blocks effects of tumor necrosis factor on bone cells[1]

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AIM: To study the effects of tumor necrosis factor (TNF) on the neonatal mouse osteoblast-enriched calvarial cells and effects of protein kinase C (PK C) inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on the TNF actions. **METHODS**: [^{3}H] TdR uptake by the osteoblasts was used to measure cell proliferation. Cellular alkaline phosphatase (AlP) and tartrate resistant acid phosphatase (trAcP) activities were determined spectrophotometrically. **RESULTS**: TNF (1—100 kU · L^{-1}) inhibited both proliferation and expression of AlP activity, but stimulated trAcP activity. These TNF-induced actions were blocked by simultaneous addition of H-7 (5—20 μmol · L^{-1}). **CONCLUSION**: TNF has potent effects on the osteoblasts, and the blockade of TNF actions by H-7 suggests that TNF exert its effects through PK C.

KEY WORDS tumor necrosis factor; protein kinase C; osteoblasts; cultured cells; piperazines; H-7

Tumor necrosis factor (TNF) stimulates osteoclastic bone resorption and inhibits osteoblastic bone formation *in vitro* and *in vivo*[1-3]. TNF elicited responses in target cells by binding to cell surface receptors, and protein kinase C (PK C) activation may play a major role in TNF signal transduction in some, but not all target cells[4,5]. These findings prompted us to examine whether PK C inhibitor could block the actions of TNF on osteoblasts. In this paper, effects of TNF on the neonatal mouse osteoblast-enriched calvaria l cells and the blockade of TNF actions by a selective potent PK C inhibitor[6,7], 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H-7) were studied.

MATERIALS AND METHODS

Reagents Recombinant human TNF (specific activity 3×10^9 U · g^{-1}) was kindly provided by Dr Y Sohmura (Dainippon Pharmaceutical Co, Japan). H-7 ($C_{14}H_{17}N_3O_2S$) was synthesized by Dr ZHAO Jing-Pu (Chemistry Department of this Lab), and the dose of its dihydrochlorides (H-7 · 2HCl) was used in the following experiments. [^{3}H]methylthymidine ([^{3}H]TdR) was from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences, and the remainder of the reagents were purchased from Sigma (USA).

Cell isolation and cultivation Osteoblast-enriched cells were prepared from neonatal ICR mouse calvaria (Animal centre of this University), by a modification of the method[3]. Briefly, 100 calvariae were diggested in isotonic saline containing 0.2 % collagenase and 0.1 % trypsin. The cells were centrifuged and incubated with minimal essential medium containing 10 % fetal calf serum at 37 C in humid 5 % CO_2 for 1 wk. The culture medium was replaced with fresh media until cell confluence. These cells appeared morphologically to be osteoblasts which responded to parathyroid hormone to increase intracytoplasmic cAMP and expressed inducible high alkaline phosphatase (AlP) activity.

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Cell proliferation The cells were treated with 0.1 % trypsin for 5 min, then 1×10^4 cells were placed in 96-well microplates for 24 h and incubated with TNF or H-7 for 24 h. To test effect of PK C inhibitor on TNF action, cells were incubated with TNF (100 kU · L^{-1}) in the presence of H-7. To each culture well, [^{3}H]TdR 7.4 kBq was added 8 h before termination of culture.

The cells were harvested onto glass fibers and its [^{3}H]TdR was counted in a liquid scintillation counter.

Alkaline phosphatase (AlP) and tartrate resistant acid phosphatase (trAcP) activity Cells (1×10^4/well) were seeded in 96-well microplates for 24 h, and then incubated with TNF or H-7 for 48 h or 10 d to induce AlP and trAcP activity, respectively. To test effect of PK C inhibitor on TNF action, cells were incubated with TNF (100 kU · L^{-1}) in the presence of H-7. After incubation, cell layers were washed twice with phosphate buffer saline and solubilized by the incubation with 10 μL of 0.1 % Triton X-100 in saline.

Cellular AlP and trAcP activity were determined by a modification of methods[2,3] using *p*-nitrophenyl phosphate 27 mmol · L^{-1} in monoethanolamine 0.1 mol · L^{-1} (pH 10.5) and in sodium tartrate 27 mmol · L^{-1} (pH 3.3) as substrates respectively. In brief, the substrate (100 μL) was added to the resultant cell lysates, which was then incubated at 37 C for 30 min. The reaction was stopped by adding 100 μL NaOH 1 mol · L^{-1} and the absorbance at 410 nm was measured in a microplate reader using *p*-nitrophenol as a standard reference. AlP and trAcP activity was expressed as units (μmol · min^{-1} by 1×10^4 cells).

Statistics Statistical analyses were carried out using *t* test.

RESULTS

Cell proliferation Proliferation of calvarial cells was inhibited by TNF in a concentration-dependent manner. The inhibition induced by TNF was blocked by H-7 (5 — 20 μmol · L^{-1}) concentration-dependently. These concentrations of H-7 did not inhibit the growth of the cells, as [^{3}H]TdR incorporation in all of the treated groups was within 10 % of that in the control cultures (Tab 1).

AlP and trAcP activity TNF caused a concentration-dependent inhibition of AlP activity, whereas trAcP activity was increased. The AlP and trAcP activities were not affected by H-7. However, H-7 blocked both the decrease in AlP activity and the increase in trAcP activity induced by TNF in concentration-dependent manner. Treatment with H-7 20 μmol · L^{-1} completely prevented the actions of TNF (Tab 1).

Tab 1. Effects of tumor necrosis factor (TNF) and H-7 on proliferation, alkaline phosphatase (AlP) and tartrate resistant acid phosphatase (trAcP) activities in neonatal mouse calvarial cell cultures. $n = 3$ **homogenates (each was pooled from 100 mice and assayed in triplicate).** $\bar{x} \pm s$.
[a] $P > 0.05$, [b] $P < 0.05$, [c] $P < 0.01$ *vs* control.
[d] $P < 0.05$, [e] $P < 0.01$ *vs* TNF (100 kU · L^{-1}).

TNF/ kU · L^{-1}	H-7/ μmol · L^{-1}	[^{3}H]TdR uptake/ dpm	AlP activity/ mU	trAcP activity/ mU
0	0	976±91	2585±55	89±5
1	0	763±81[b]	1663±27[c]	97±8[a]
10	0	450±63[c]	1522±40[c]	117±10[b]
100	0	362±32[c]	1200±64[c]	142±6[c]
0	5	972±50[a]	2523±81[a]	91±12[a]
0	10	880±93[a]	2461±51[a]	91±8[a]
0	20	872±102[a]	2585±68[a]	92±7[a]
100	5	502±47[d]	2100±70[d]	129±4[d]
100	10	748±39[e]	2253±72[e]	112±11[e]
100	20	907±184[e]	2558±107[e]	94±6[e]

DISCUSSION

Our results indicated that TNF inhibited osteoblastic cells and enhanced osteoclastic cells. These observations support the previous studies[2,3]. The role of PK C in TNF actions on osteoblasts remains unclear. We have utilized PK C inhibitor H-7 to demonstrate the role of PK C. H-7 has been demonstrated to be a more potent inhibitor of PK C with a K_i of 6 μmol · L^{-1} [6]. Our results that H-7 blocked TNF-induced proliferation and expression of AlP and trAcP activity concentration-dependently in neonatal mouse calvari-

al cell cultures suggest that effect of TNF on osteoblasts may be contributed to the activation of PK C. However, other intracellular signals cannot be ruled out because the actions of TNF were completely prevented by H-7 at concentration above the K_i for PK C.

In summary, this study showed the modulation of TNF actions on osteoblasts by PK C inhibitor, providing a theoretical basis for the treatment of inflammatory diseases because cytokines such as TNF have been showed to play a potential role in the bone resorption associated with inflammatory diseases[10].

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蛋白激酶 C 抑制剂 H-7 抑制肿瘤坏死因子对骨细胞的作用[1]

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R965.2

目的：用富含成骨细胞的小鼠头盖骨细胞测试肿瘤坏死因子（TNF）对骨细胞的作用以及蛋白激酶 C（PK C）抑制剂 H-7 对 TNF 的影响. **方法**：[^{3}H]TdR 参入法观察细胞增殖. 以对-硝基苯酚磷酸酯为底物用微量分光光度法测定细胞内碱性磷酸酶和耐酒石酸酸性磷酸酶活性. **结果**：TNF（1-100 kU·L^{-1}）以剂量依赖方式抑制骨细胞增殖和碱性磷酸酶活性，但增强耐酒石酸酸性磷酸酶活性. H-7（5-20 μmol·L^{-1}）对 TNF 上述作用具有显著抑制作用且呈剂量依赖. **结论**：TNF 对骨细胞的作用与 PK C 有关.

关键词 肿瘤坏死因子；蛋白激酶 C；成骨细胞；培养的细胞；哌嚓；H-7