C-MYC PROTEIN EXPRESSION UPREGULATED BY 2-(3-ESTRONE-*N*-ETHYL PIPERAZINE-METHYL) TETRACYCLINE IN BONE

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ABSTRACT: **AIM** To study the effect of XW630 on expression of pro-oncogene *c-myc* in the long bones of fetal mice *in vitro* for postulating the mechanism by which XW630 exerts its effect on bone. **METHODS** The fetuses of pregnant mice were removed on day 16 of gestation, the long bones of the forelimbs of female fetal mice were freed of muscle and soft tissue and cultured in a specific device for 48 h in BGJb medium treated with 1×10^{-7}, 1×10^{-8} and 1×10^{-9} mol·L^{-1} XW630 in the final medium. After cultured for 48 h, the long bones were harvested and immunohistochemical analysis was performed for determination of c-Myc protein expression in epiphyseal plates. The areas of positive cells in the resting zone, proliferative zone and hypertrophic zone in epiphyseal plate were determined under image analytic system. **RESULTS** When the concentration of XW630 in the medium was 1×10^{-9} mol·L^{-1}, the area of c-Myc positive cells increased in the proliferative zone compared with 1×10^{-9} mol·L^{-1} in the estrone group, significant increase was also observed in the resting zone compared with the control group. When the concentration of XW630 in medium was 1×10^{-8} or 1×10^{-7} mol·L^{-1}, stronger expression than that in the control group and the estrone group at the same concentration was observed in each of the three zones. **CONCLUSION** The estrogenic effect of XW630 on bone was stronger than that of estrone. XW630 may promote proliferation and differentiation of chondroncytes by promoting c-Myc protein expression in chondroncytes. Thus, endochondral bone formation was enhanced.

KEY WORDS: long bone; 2-(3-estrone-*N*-ethyl piperazine-methyl) tetracycline (XW630); pro-oncogene *c-myc*; immunohistochemistry

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Estrogen is still chosen as the first agent for treating postmenopausal osteoporosis in clinic. But, estrogen may stimulate endometrial and the breast, so it may increase the risk of breast cancer and uterine cancer when used for long term. 2-(3-Estrone-*N*-ethyl piperazine-methyl) tetracycline (XW630), a novel bone-targeted estrogen, exerts beneficial effects on cultured osteoblast *in vitro* as an estrogen agonist, while its stimulating effect on uterus is only 1/1960 of estrone[1,2]. This suggests the tissue selectivity of XW630 as an estrogen-like compound. However, the mechanism by which XW630 affects the bone is still unclear. So, the effect of XW630 on expression of pro-oncogene *c-myc* in long bones of fetal mice was studied *in vitro*. The relationship between induction of c-Myc protein by XW630 and its upregulation of c-Fos was also analyzed for postulating the mechanism by which XW630 exerts its effect on bone.

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MATERIALS AND METHODS

Drugs and chemicals XW630 was synthesized in our laboratory. Estrone was purchased from Xianju Pharmaceutical Factory (Hangzhou, Zhejiang). Kunming mice were purchased from Sichuan Antibiotics Institute. All other chemicals were of analytical grade. Mouse IgG anti-*c-myc* monoclonal antibody (first antibody cat. ac-42) was purchased from Santa Cruz Corporation, California 95060, USA. Rabbit histostain™-SP kit (containing biocinylated goat anti-rabbit IgG, secondary antibody) was purchased from Zymed Corporation, South San Francisco, California 94080, USA.

Culture of long bones[3] The fetuses of pregnant Kunming mice were removed on day 16 of gestation. Under a dissecting microscope, the ulnae of the forelimbs of female fetal mice were dissected free of muscle and soft tissue and cultured in a specific device for 48 h in BGJb medium supplemented with 10% fetal calf serum (FCS). During culture, the ulnae were sealed in slowly rotating bottles filled with a mixture of 45% O_2, 5% CO_2 and 50% N_2. The gas mixture in the bottles was supplemented after culture for 24 h. Estrone and XW630

were dissolved separately in dimethyl sulfoxide (DMSO) to get solutions of 1×10^{-3} mol·L^{-1} . Then , the solutions were diluted with double-distilled water to 1×10^{-7} , 1×10^{-8} and 1×10^{-9} mol·L^{-1} in the final medium . At each concentration , 18 ulnae were cultured , 6 ulnae each time . Control cultures contained DMSO at highest concentration used in the experimental cultures .

Immunohistochemistry of c-Myc After cultured for 48 h , the long bones were fixed in 4 % paraformaldehyde (pH 7.2) , decalcified in 7 % EDTA (pH 7.2 , 4 ℃) and embedded in paraffin . Longitudinal long bone sections of 5 μm thick , starting from the medial part of the bone , were prepared .

The immunohistochemical stain for c-Myc protein was carried out as follows : after dewaxing in xylene and rehydrating in accordance to routine steps , the sections were incubated in 3.0 % H_2O_2 for 30 min in order to block endogenous peroxidases followed by several rinses in phosphate buffer solution (PBS) . The sections were allowed to react successively with 0.4 % pepsin and 2 % bovine serum albumen (BSA) at 37 ℃ for 15 min and 30 min and followed by 2 h incubation with mouse IgG anti-*c-myc* monoclonal antibody diluted 1∶200 at 37 ℃ . After thorough washing in PBS , the sections were incubated for a further 1 h at 37 ℃ in peroxidase-labelled goat anti-rabbit IgG diluted 1∶200 with PBS . After washing in PBS , the sections were incubated in peroxidase substrate (6 mg diaminobenzidine in 10 mL PBS and 10 μL H_2O_2) for 4 min and rinsed with PBS and treated with DAB chromogen . The sections were finally washed , dehydrated and cleared .

Qantification The area of positive cells in the resting zone , proliferative zone and hypertrophic zone in epiphyseal plate was determined under image analytic system . All sections analyzed were enlarged 40 times and the area of each section determined was limited to 100 μm^2 .

Statistical analysis Results were expressed as $\bar{x} \pm s$ and F test and Q test were performed by statistical system .

RESULTS

Under microscope , a few c-Myc protein stained cells were observed in the resting zone , proliferative zone and hypertrophic zone in the control group . The positive substance located in the nuclei , appeared as brown grains . The area of c-Myc positive cells in the estrone group at 1×10^{-9} mol·L^{-1} increased compared with the control group . When the concentration of estrone was 1×10^{-8} mol·L^{-1} , besides the proliferative zone , a significant increase in the area of c-Myc positive cells was also observed in the resting zone . When the concentration of estrone in the medium was increased to 1×10^{-7} mol·L^{-1} , significant increase was observed in each of the three zones . After treatment with XW630 , the location of c-Myc protein stained positive substances did not change , but the level of expression changed . When the concentration of XW630 in medium was 1×10^{-9} mol·L^{-1} , the area of c-Myc positive cells increased in the proliferative zone compared with 1×10^{-9} mol·L^{-1} in the estrone group . The increase were also observed in the resting zone compared with the control group . When the concentration of XW630 in medium was 1×10^{-8} mol·L^{-1} , the area of c-Myc positive cells increased in each of the three zones compared with the estrone group at the same concentration and with the control group , whereas the increase was not observed in the hypertrophic zone in the estrone group compared with the control group . When the concentration of XW630 in medium was increased to 1×10^{-7} mol·L^{-1} , the area of c-Myc positive cells increased in each of the three zones compared with the estrone group at the same concentration and with the control group (Figure 1 ~ 3) .

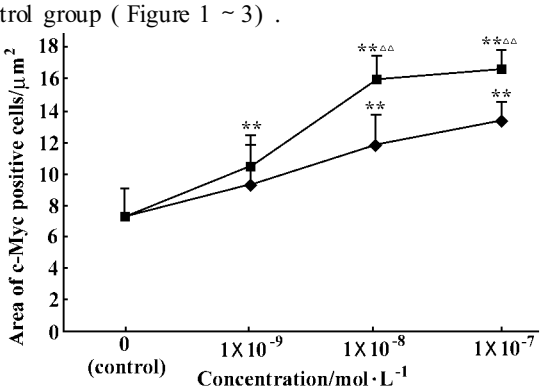

Figure 1 Effect of 2-(3-estrone-*N*-ethyl piperazine-methyl) tetracycline (XW630) on the area of c-Myc positive cells in the resting zone

The long bones of female fetal mice of 16 days were cultured for 48 h in BGJb medium treated with XW630 or estrone and the same volume vehicle solution were added to the control group . The long bones were then decalcified in EDTA and embedded in paraffin wax . Midlongitudinal 5 μm thick sections were used for immunohistochemical stain for analysis of c-Myc protein . The total area of positive cells of c-Myc protein in epiphyseal plates was determined . $n = 18$, $\bar{x} \pm s$. ** $P < 0.01$ *vs* the control group; △△ $P < 0.01$ *vs* the estrone group at the same concentration . ◆—◆ Estrone ; ■—■ XW630

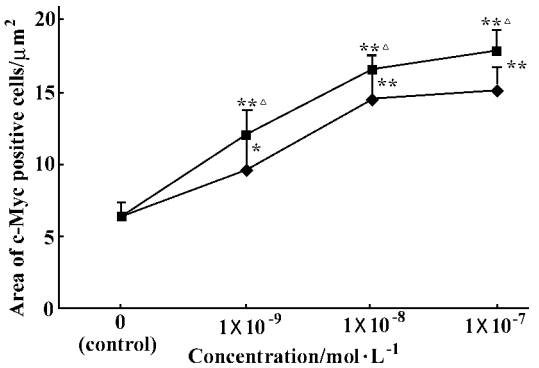

Figure 2 Effect of XW630 on the area of c-Myc positive cells in the proliferative zone

The long bones of female fetal mice of 16 days were cultured for 48 h in BGJb medium treated with XW630 or estrone and the same volume vehicle solution were added to the control group. The long bones were then decalcified in EDTA and embedded in paraffin wax. Midlongitudinal 5 μm thick sections were used for immunohistochemical stain for analysis of c-Myc protein. The total area of positive cells of c-Myc protein in epiphyseal plates was determined. $n = 18$, $\bar{x} \pm s$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the control group; $^{\triangle}P < 0.05$ *vs* the estrone group at the same concentration. ◆—◆ Estrone; ■—■ XW630

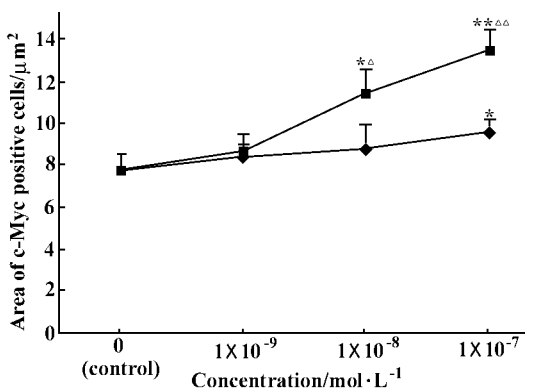

Figure 3 Effect of XW630 on the area of c-Myc positive cells in the hypertrophic zone

The long bones of female fetal mice of 16 days were cultured for 48 h in BGJb medium treated with XW630 or estrone and the same volume vehicle solution were added to the control group. The long bones were then decalcified in EDTA and embedded in paraffin wax. Midlongitudinal 5 μm thick sections were used for immunohistochemical stain for analysis of c-Myc protein. The total area of positive cells of c-Myc protein in epiphyseal plates was determined. $n = 18$, $\bar{x} \pm s$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the control group; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ *vs* the estrone group at the same concentration. ◆—◆ Estrone; ■—■ XW630

DISCUSSION

Strong evidence exists for the involvement of the *c-myc* oncogene in the regulation of cell proliferation and differentiation. During cell differentiation, c-Myc expression is down-regulated. The majority of studies have indicated that terminal differentiation is generally accompanied by an abrupt reduction in c-Myc expression[4]. Furthermore, it has been shown that the differentiation of some cell lines is not only accompanied by a simple decrease in c-Myc expression. A transient elevation of c-Myc mRNA levels has been observed in the differentiation of rat PC12 pheochromocytoma cells or mouse erythroleukemia cells[5]. The studies on cardiac muscle and skeletal muscle also suggest that c-Myc protein expression is observed in hypertrophic cardiac muscle cells and skeletal muscle cells[6]. Hypertrophy for chondroncytes means differentiation. All of these suggest that c-Myc plays a complex role during proliferation and differentiation of cells.

It has been shown that estrogen receptor exists in chondrocytes, this means that estrogen can directly exert its effects on chondroncytes. The results in this study showed an increased c-Myc expression in the resting zone, proliferative zone and hypertrophic zone at 1×10^{-7} mol·L^{-1} estrone and a decreased expression followed by decrease of estrone concentration. This provided another evidence of the direct effect of estrogen on chondroncytes.

XW630 was shown to have stronger effect than estrone on chondroncytes. After long bones were treated with 1×10^{-7} or 1×10^{-8} mol·L^{-1} XW630, upregulation of c-Myc protein expression was observed in the resting zone, proliferative zone and hypertrophic zone, compared with the control group and the estrone group. Whereas no evidence showed that c-Myc protein expression was upregulated in the hypertrophic zone at 1×10^{-8} mol·L^{-1} estrone and in the resting zone at 1×10^{-9} mol·L^{-1} estrone, compared with the control group. The results indicate that the estrogenic effect of XW630 on bone was stronger than that of estrone, especially on the resting and differentiating chondrocytes. This may be resulted from the difference of configuration between XW630 and estrone or from the bone targeted action of tetracycline which may make estrone concentrated in the long bone. A significant increase was observed in both total bone length and diaphyseal length compared with the control group after the long bones were treated with 1×10^{-7} mol·L^{-1} XW630[7]. As the chondroncytes move into the hypertrophic zones they showed various signs of

differentiation such as production of alkaline phosphatase (ALP) and type X collagen which are both associated with matrix calcification and ultimately endochondral ossification. The activity of ALP in the long bone increased after treatment with XW630 also. All of these suggest that XW630 may promote proliferation and differentiation of chondroncytes by promoting c-Myc protein expression in chondroncytes, thus, endochondral bone formation was enhanced.

Rolf Müller and his coworkers[7] showed that stimulation of fibroblasts with serum or purified factors leads to a dramatic induction of expression of both *c-fos* mRNA and protein within a few minutes, followed by activation of *c-myc*. It was also observed that the level of c-Fos protein was regularly upregulated by XW630 in dose dependent manner in each zone and the regularity of change in c-Fos protein expression was similar to that in c-Myc protein expression[8]. This suggests that induction of *c-fos* gene and protein by XW630 might precede activation of *c-myc*. It will be necessary for proving this to observe the temporal pattern of changes of the *c-fos* expression and *c-myc* expression in the long bone after treatment with XW630.

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四环素-哌嗪雌酚酮上调骨中 c-Myc 蛋白表达水平

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摘要: 目的 研究四环素-哌嗪雌酚酮(XW630)对长骨原癌基因 *c-myc* 蛋白表达的影响,探讨其对骨的作用机制。方法 取 16 d 孕龄的雌性胚胎小鼠,剥取前肢尺骨,在特制的培养装置上于 BGbJ 培养基中培养 48 h,培养基中 XW630 终浓度分别为 1×10^{-7},1×10^{-8}和 1×10^{-9} mol·L^{-1}。用免疫组织化学方法测定长骨骺板 c-Myc 蛋白的表达。图象分析系统下测定静止区、增殖区、肥大区 c-Myc 蛋白免疫反应阳性细胞面积。结果 当培养基中 XW630 浓度为 1×10^{-9} mol·L^{-1}时,增殖区阳性细胞面积与同浓度雌酚酮组相比增加,静止区阳性细胞面积与对照组相比也增加,此浓度的雌酚酮组静止区阳性细胞面积与对照组无差异。XW630 浓度为 1×10^{-8}和 1×10^{-7} mol·L^{-1}时,各区阳性细胞面积均比对照组及同浓度雌酚酮组增加。结论 XW630 对骨的雌激素活性强于雌酚酮。XW630 可能通过促进骺板软骨细胞表达 c-Myc 蛋白而促进了软骨细胞的增殖和分化,从而促进了软骨内成骨。

关键词: 长骨; 四环素-哌嗪雌酚酮(XW630); 原癌基因 *c-myc*; 免疫组织化学