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Radiation is Promotive to the Apoptosis of Differentiated Osteoclasts Induced by Silica

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Abstract

Silica is a biomedical material with excellent biological compatibility applicable in bone tissue engineering and artificial joints. The silica materials usually contact with bone tissues and stimulate the differentiation of osteoclasts. On the other hand, the differentiated osteoclasts can further decompose the silica materials resulting in corrosion or loosening of artificial bone tissues. Clinically, radiation has been found able to inhibit the activity of osteoclasts. However, the biological effect of radiation on osteoclasts is seldom investigated for the patients with bone tissues using silica materials.

Monocytes from RAW 264.7 cell line were cultured with safe dose of silica particles to investigate the biological effect of silica on monocytes in this research. Four groups including the control, silica treatment, radiation treatment (5 Gy) and a combinative treatment of silica and radiation were divided in the experiment. In this research, relative concentration of produced tartrate resistant acid phosphatase (TRAP) and the number of differentiated osteoclasts were evaluated. Osteoclasts were stained by Annexin-V-FITC kit to analyze the apoptotic cells. Silica particles were found able to stimulate the production of TRAP representing the activity of osteoclasts and increase the number of differentiated osteoclasts. However, radiation was discovered promotive to apoptosis of osteoclasts yet suppressive to the differentiation of osteoclasts. The results concluded that radiation was able to increase the apoptosis of osteoclasts induced by silica and decline the activity and amount of osteoclasts.

Keywords：Bone Tissue, Osteoclast, Silica, Radiation, Monocyte, Differentiation

Introduction

Maintaining bone mass is related to the bone formation of osteoblasts and bone resorption of osteoclasts. Over activation of osteoclasts may cause bone loss and lead bone fracture. Osteoclasts are multinucleated cells derived from monocytes/macrophages [1] and able to excrete enzymes for bone resorption, which is controlled by some physical (e.g., electric and magnetic fields) and chemical factors (e.g., cell cytokines, alcohol and caffeine).

Many inflammation-related cytokines stimulate the differentiation of osteoclasts. Therefore, many patients with chronic inflammation usually have comorbidity of

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serious bone loss due to over activation of osteoclasts (2). Several cytokines [such as IL-6(3), TGF-β(4), MCP-1(5), IFN-γ(6) and TNF-α(7)] may induce differentiation of osteoclasts adjusted by the receptor for activation of nuclear factor kappa B ligand [RANKL; also called the osteoclast differentiation factor（ODF）] excreted by stromal cells. Osteoclasts are able to excrete the TRAP (tartrate resistant acid phosphatase) for bone resorption by decomposing bone tissues. Therefore, the TRAP is usually used as an indicator for the measurement of osteoclasts.

Recently, some cancer cells, such as prostate cancer [8] and breast cancer [9], are able to stimulate excretion of ODF and result in corrosion of bone tissues [10], which further cause metastasis of cancer cells to bone tissues. Radiation is known able to inhibit the activity and differentiation of osteoclasts [11]. Artificial joints usually utilize silica as a bio-compatible material. However, silica is a potential cause of inflammation reaction in bone tissues, which may induce differentiation of osteoclasts, bone loss of surrounding regions, as well as weakness and durability decrease of artificial joints. Past researches for the biological effects of silica focused on pulmonary diseases (pneumoconiosis) [12]. Investigation about the biological effects of silica on bone tissues is rare. This study was aimed to investigate the effect of silica on the differentiation of osteoclasts as well as whether radiation is effective in reducing this effect.

Materials and Methods

Culture of monocytes

A murine macrophage cell line (RAW264.7) was maintained in RPMI-1640 medium supplemented with heat-inactivated FBS (10%) in a humidified atmosphere of 5% CO_2 at 37℃.

Preparation of silica particles

Silica particles (0.5-10 μm; Sigma-Aldrich Co. LLC.) were washed twice firstly with deionized water and then with phosphate buffer solution (PBS). After sterilized, the silica particles were dried for use.

Measurement of cytotoxicity of silica for monocytes

Sterilized silica particles (0, 50, 100, 500, 1000 or 2000 ppm) were added to monocytes (1×10^4 cell/cm^2) and incubated for 1 day. The cytotoxicity to monocytes was evaluated by the absorbance at 570 nm for living-cell number according to the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay. In the mitochondrion of living cells, MTT can be reduced by lactate dehydrogenase and react with cytochrome C to form formazan (blue colored). The relative number of living cells can be estimated by measuring the absorbance of formzan dimethyl sulfoxide (DMSO).

Measurement of monocyte proliferation

The monocytes (1×10^4 cell/cm^2) were incubated with sterilized silica particles (0, 50, 100, 500, 1000 or 2000 ppm) for 3 days and then analyzed by MTT assay to determine the proliferation rate.

Time course of osteoclast differentiation

The monocytes (5×10^4 cell/cm^2) were cultured in 24-well plates with RANKL (2 ng/mL). Differentiated osteoclasts were counted on day 0, 3, 5, 10 and 15 respectively.

Cell treatment

Four groups, including the control, silica treatment (50 ppm), radiation treatment (X-ray; 5 Gy) and a combinative treatment of silica (50 ppm) plus radiation (X-ray; 5 Gy), were divided in the experiment.

Osteoclast formation assay

Monocytes (1×10^4/mL) cultured in 48-well plates containing the M-CSF (50 ng/mL) and RANKL (2 ng/mL) were stained with the TRAP Staining Kit (Sigma-Aldrich Co. LLC.) after 10 days. The positively stained of multinucleated (≧3 nuclei) cells were pinky and indicated as osteoclasts.

TRAP activity assay

After 10 days of culture, the TRAP enzyme activity of the monocytes [originally 1×10^4 cell/cm^2 in M-CSF (50 ng/mL) and RANKL (2 ng/mL)] was determined using a TRAP assay kit (Sigma-Aldrich Co. LLC.). The absorbance was measured at 405 nm using a microplate reader.

Cell apoptosis assay

After 10 days of culture, the medium was removed, and the attached cells [originally 1×10^4 cell/cm^2 in M-CSF (50 ng/mL) and RANKL (2 ng/mL)] were washed with PBS, fixed with 70% of ethanol and stained with an Annexin V-FITC kit (Strong Biotech Corporation) for apoptosis assay. Apoptotic cells were identified by double staining with Annexin V-FITC (2 μL/mL) plus propidium iodide (PI) (2 μL/mL). The apoptotic rate of cells was represented as apoptotic number per 100 osteoclasts.

Statistical analysis

The statistically processed data were represented as mean value ± standard error and analyzed by Student's t-test. Statistical significance was set at the level of $P<0.05$.

Results

Cytotoxicity of silica for monocytes

No apparent cytotoxicity was observed at silica concentration lower than 500 ppm ($p>0.05$). The viability of monocytes is about 50% at 1000 ppm as compared to the control group and decrease with the silica concentration higher than 1000 ppm (Fig. 1).

Effect of silica on the proliferation of monocytes

The proliferation rate of monocytes apparently decreases (<50%) when the concentration of silica is higher than 100 ppm (Fig. 2). At 500 ppm, the proliferation rate of monocytes is only 16.67±4.16 %. To evaluate the biological effect of silica, the concentration was accordingly set as 50 ppm, which was the maximum dose with disapparent cytotoxicity.

Effect of silica on the time course of osteoclast differentiation

The number of osteoclasts formed is increasing with culturing time, and reached a maximum value on the 10th day yet decreasing from the 15th day (Fig. 3).

Effect of silica on osteoclast formatiom

According to the time course of osteoclast differentiation, the test for the formation of osteoclasts

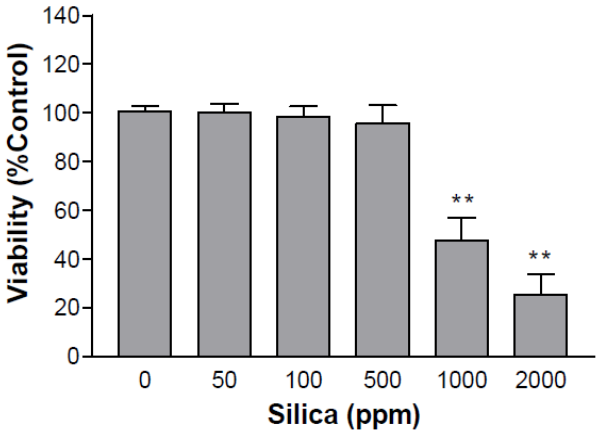

Fig 1. Cell toxicity of silica for monocytes. The monocytes were cultured with silica and the cell toxicity was determined by the MTT assay on the next day. The results indicated that silica particles were nontoxic at the concentration less than 500 ppm. Histogram here is represented as mean± standard error (n=3) and the significance is indicated (**) when $P<0.01$.

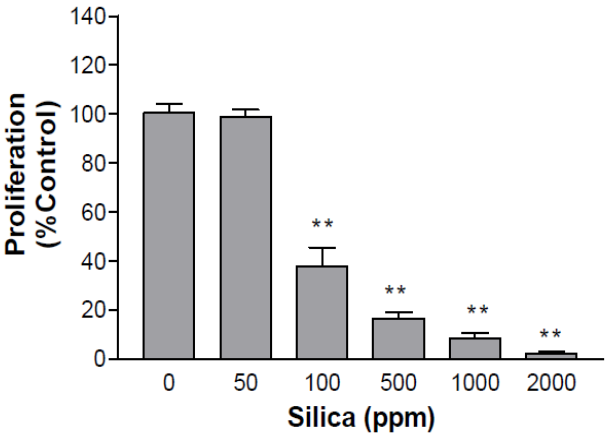

Fig 2. Effect of silica on the proliferation of monocytes. The proliferation of monocytes cultured with silica for 3 days were analyzed by the MTT assay. No apparent effect was found on the proliferation of monocytes when the silica concentration was less than 50 ppm. Obvious inhibition was observed on the proliferation of monocytes while the silica was higher than 100 ppm. Histogram here is represented as mean±standard error (n=3) and the significance is indicated (**) when $P<0.01$.

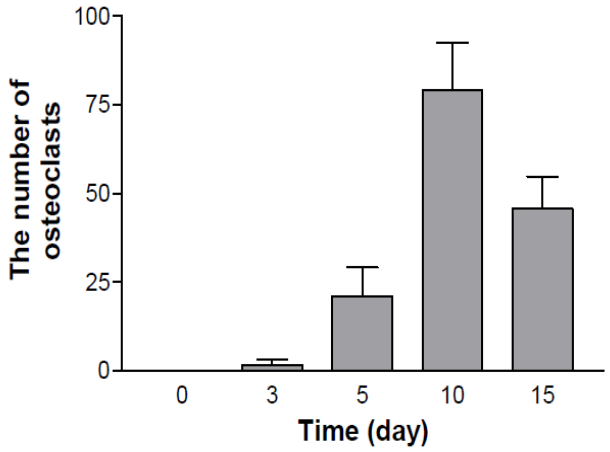

Fig 3. Evolution of the differentiation of osteoclasts with time. The number of differentiated osteoclasts derived from the monocytes [cultured in M-CSF (50 ng/mL) RANKL (2 ng/mL)] were counted after a period of time. The mean (n=3) and standard error are represented.

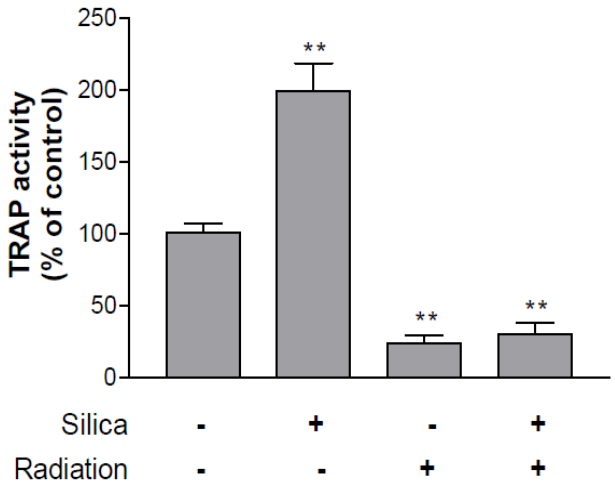

Fig 5. Analysis of TRAP activity. The monocytes were treated with silica (50 ppm), radiation (5 Gy) or silica combined with radiation. After 10 days of culture, the medium was removed and the activity of TRAP was determined. The results show that silica is able to increase TRAP activity, which can be inhibited by radiation. The mean (n=3) and standard error are represented, and the significance is indicated as ** for $P < 0.01$.

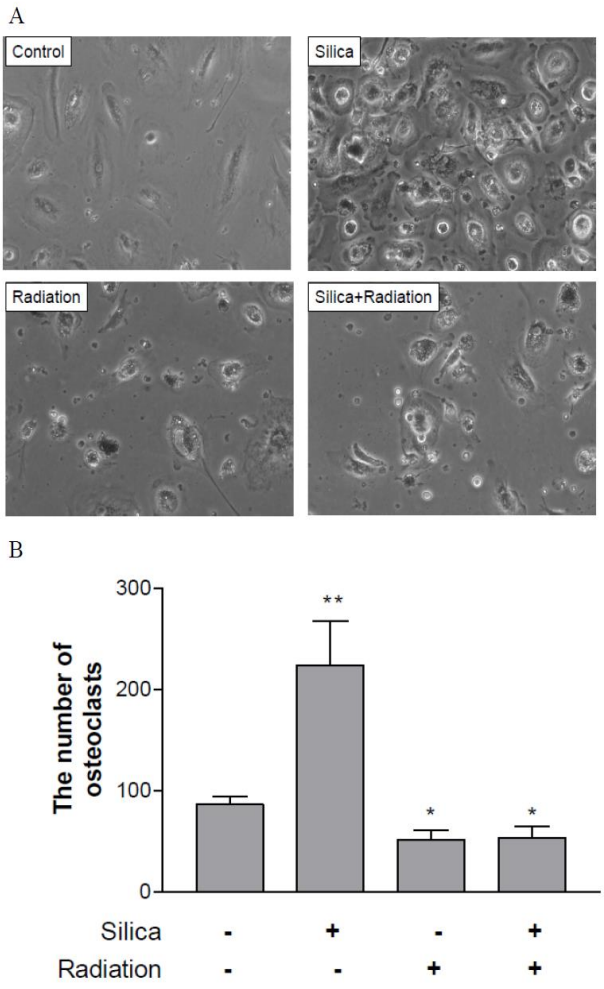

Fig 4. Effect of silica and radiation on the differentiation of osteoclasts. The monocytes were treated with silica (50 ppm), radiation (5 Gy) or silica combined with radiation. After 10 days of culture, the osteoclasts were stained with TRAP staining kit, and the TRAP-positive cells with more than three nuclei were counted as osteoclasts. Diagram (A) illustrates the differentiation of osteoclasts after different treatments. Diagram (B) represents the number of differentiated osteoclasts. Silica particles were found able to stimulate the differentiation of osteoclasts, which can be inhibited by radiation. The mean (n=3) and standard error are represented, and the significance is indicated as * for $P < 0.05$ and ** for $P < 0.01$.

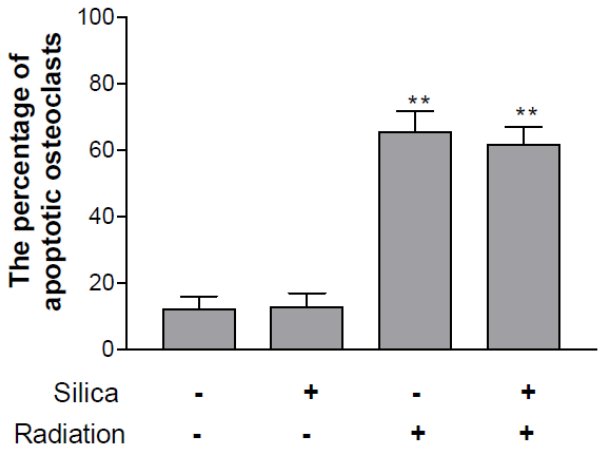

Fig 6. Analysis for the apoptosis of osteoclasts. The monocytes were treated with silica (50 ppm), radiation (5 Gy) or silica combined with radiation. Annexin-V staining was used for the apoptosis of osteoclasts after 10 days of culture. The results show that radiation is able to increase the number of apoptotic osteoclasts whether combined by the treatment of silica or not. The mean (n=3) and standard error are represented, and the significance is indicated as ** for $P < 0.01$.

was carried out after ten days of culture. The number of osteoclasts increased in the silica treated group is ~200% as compared to the control group (Fig. 4). In the radiation treated group and the combinative treatment group of silica plus radiation, the number of osteoclasts decreases to ~60%. The results indicate that X-ray is found able to decrease the number of osteoclasts and inhibit the formation of osteoclast induced by silica.

Effect of silica on the TRAP activity

The TRAP activity in silica treated group is about twice as that in the control group (Fig. 5), which infers that the bone resorption is higher after treated with silica. On the other hand, in the radiation treated group as well as the combinative treatment group of silica plus radiation, this effect is suppressed to < 30%, indicating that radiation is able to decrease bone resorption whether silica is combinatively treated.

Effect of silica on the apoptosis of osteoclasts

At the initial stage of apoptosis of osteoclasts, the exposed phosphatidyl serine on cell membrane has high affinity for Annexin-FITC and emits green fluorescent light under microscope. At the late stage of apoptosis of osteoclasts, orange colored apoptotic bodies appear when the propidium iodide (PI) stain binds with chromatins in the cells. The percentage of apoptotic cells is highest in radiation treated group, while that between the silica treated group and the control one represents no significant difference (Fig. 6). Nevertheless, the percentage of apoptotic osteoclasts in the radiation treated group as well as in the combinative treatment group of silica plus radiation is apparently higher (> 60%) than the silica treated group.

Discussion

No immediate toxicity was found for monocytes cultured in medium with silica ≤500 ppm after one day. Cell toxicity was apparent when the concentration of silica was higher than 1000 ppm. However, apparent cell damage was observed for monocytes after 3 days of culture in the medium with ≥100 ppm of silica. The macrophaged silica by monocytes was found increasing with time in microscope investigation, which is similar to the phenomenon observed in pulmonary study. Macrophage cell is derived from monocytes and both of them possess powerful ability of phagocytosis. Therefore, the cytotoxicity of silica for monocytes may be ascribed to the amount of silica phagocytosed by monocytes and the excretion of inflammation-related cytokines [13-14]. Osteoclast apoptosis was not found in the silica treated group (Fig. 6). However, accumulation of silica in cells may lead to apoptosis of monocytes. In addition, the breakdown of apoptotic cells may release the phagocytosed silica particles, which can further be phagocytosed by other monocytes and causes serial apoptosis of cells. Therefore, monocytes are more sensitive in apoptosis than osteoclasts toward silica treatment. This result is similar to the development of pneumoconiosis.

Osteoclasts are multinuclear cells (generally 10-20

nuclei) fused by monocytes, of which the size is about 50-100 μm in diameter. The number of osteoclasts increases with culture time and reaches the maximum on the 10^{th} day, which decreases later (Fig. 3). Although the number of osteoclasts decreases on the 15^{th} day, the average diameter of cells is larger than that on the 10^{th} day. Moreover, the number of nucleus is also higher. This phenomenon can be ascribed to cell fusion among the osteoclasts [15], which makes the cell number decreasing yet the nucleus number as well as the cell size increasing. In order to investigate the number of osteoclasts, the cell fusion should be avoided. Therefore, the counting of osteoclasts in this study was carried out on the 10^{th} day after culture.

According to Fig. 4, the differentiations of osteoclasts are significantly promoted by silica particles. In addition, radiation is effective to down regulate the differentiation of osteoclasts whether silica is combined in the treatment. In the study of pneumoconiosis, silica was found able to stimulate the excretion of monocyte chemotactic proteins (MCP) by monocytes or macrophages [14], which can induce the differentiation of osteoclasts [5]. Metastasis is common for prostate and breast cancer cells to bone tissues and further causes differentiation of osteoclasts. This is thought to be related to the excretion of RANKL, TNF-α and INF-γ [9] by cancer cells. Moreover, the differentiation of osteoclasts was found mainly regulated by NF-κB, which may activate considerable differentiation of osteoclasts in patients with chronic inflammation [16-18]. Therefore, the effects of silica and radiation are likely working via the regulation of cytokines, such as MCP, RANKL, TNF-α, INF-γ, and NF-κB.

In addition to stimulating the differentiation of osteoclasts, the NF-κB is responsible for promoting the activity of osteoclasts. Moreover, some drugs have been used to decrease apoptosis of osteoclasts by suppressing the excretion of NF-κB. Similarly, radiation is usually applied to inhibit the over-activation of osteoclasts. It has been discovered that radiation is able to enhance apoptosis of osteoclasts by reducing the production of RANKL and further inhibiting the excretion of NF-κB [19]. The effect of radiation on osteoclasts is therefore suggested via inhibiting the differentiation of osteoclasts (Fig. 4) and the formation of TRAP (Fig. 5) by suppressing the excretion of NF-κB.

Conclusion

Cytotoxicity was observed at silica concentration higher than 1000 ppm. The proliferation rate of monocytes apparently decreases when silica concentration of was higher than 100 ppm. The differentiated osteoclasts increased with culturing time and reached a maximum on the 10^{th} day. Radiation (X-ray) was found able to decrease the differentiation of osteoclasts (and thus the bone resorption) as well as the TRAP activity whether the monocytes were treated by silica. The effects of silica and radiation were suggested via the regulation of MCP, RANKL, TNF-α, INF-γ, and NF-κB.

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References

1. Fujikawa Y, Quinn JM, Sabokbar A, McGee JO, Athanasou NA. The human osteoclast precursor circulates in the monocyte fraction. Endocrinology 1996; 137(9): 4058-60
2. Boyce BF, Schwarz EM, Xing L. Osteoclast precursors: cytokine-stimulated immunomodulators of inflammatory bone disease. Curr Opin Rheumatol 2006; 18(4): 427-32
3. Udagawa N, Takahashi N, Katagiri T, Tamura T, Wada S, Findlay DM, et al. Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors. J Exp Med 1995; 182(5): 1461-8

4. Fox SW, Haque SJ, Lovibond AC, Chambers TJ. The possible role of TGF-beta-induced suppressors of cytokine signaling expression in osteoclast/ macrophage lineage commitment in vitro. J Immunol 2003; 170(7): 3679-87
5. Kim MS, Day CJ, Morrison NA. MCP-1 is induced by receptor activator of nuclear factor-{kappa}B ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. J Biol Chem 2005; 280(16): 16163-9
6. Gao Y, Grassi F, Ryan MR, Terauchi M, Page K, Yang X, et al. IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. J Clin Invest 2007; 117(1): 122-32
7. Kudo O, Fujikawa Y, Itonaga I, Sabokbar A, Torisu T, Athanasou NA. Proinflammatory cytokine (TNFalpha/IL-1alpha) induction of human osteoclast formation. J Pathol 2002; 198(2): 220-7
8. Wakchoure S, Swain TM, Hentunen TA, Bauskin AR, Brown DA, Breit SN, et al. Expression of macrophage inhibitory cytokine-1 in prostate cancer bone metastases induces osteoclast activation and weight loss. Prostate 2009;69(6):652-61
9. Lau YS, Danks L, Sun SG, Fox S, Sabokbar A, Harris A, et al. RANKL-dependent and RANKL-independent mechanisms of macrophage-osteoclast differentiation in breast cancer. Breast Cancer Res Treat 2007; 105(1): 7-16
10. Ohshiba T, Miyaura C, Inada M, Ito A. Role of RANKL-induced osteoclast formation and MMP-dependent matrix degradation in bone destruction by breast cancer metastasis. Br J Cancer 2003; 88(8): 1318-26
11. Scheven BA, Wassenaar AM, Kawilarang-de Haas EW, Nijweide PJ. Comparison of direct and indirect radiation effects on osteoclast formation from progenitor cells derived from different hemopoietic sources. Radiat Res 1987; 111(1): 107-18
12. Liou SH, Shih WY, Chen YP, Lee CC. Pneumoconiosis and pulmonary function defects in silica-exposed fire brick workers. Arch Environ Health 1996; 51(3): 227-33
13. Gozal E, Ortiz LA, Zou X, Burow ME, Lasky JA, Friedman M. Silica-induced apoptosis in murine macrophage: involvement of tumor necrosis factor-alpha and nuclear factor-kappaB activation. Am J Respir Cell Mol Biol 2002; 27(1): 91-8
14. Fogelmark B, Sjostrand M, Bergstrom R, Rylander R. Pulmonary macrophage phagocytosis and enzyme production after in vivo exposure to silica dust. Toxicol Appl Pharmacol 1983; 68(1): 152-9
15. Ishii M, Saeki Y. Osteoclast cell fusion: mechanisms and molecules. Mod Rheumatol 2008; 18(3): 220-7
16. Soysa NS, Alles N, Shimokawa H, Jimi E, Aoki K, Ohya K. Inhibition of the classical NF-kappaB pathway prevents osteoclast bone-resorbing activity. J Bone Miner Metab 2009; 27(2): 131-9
17. Yoshitake F, Itoh S, Narita H, Ishihara K, Ebisu S. Interleukin-6 directly inhibits osteoclast differentiation by suppressing receptor activator of NF-kappaB signaling pathways. J Biol Chem 2008; 283(17): 11535-40
18. Kim MH, Kim BT, Min YK, Kim SH. Profiling signalling pathways of the receptor activator of NF-kappaB ligand-induced osteoclast formation in mouse monocyte cells, RAW264.7. Amino Acids 2008; 34(3): 497-506
19. Sawajiri M, Nomura Y, Bhawal UK, Nishikiori R, Okazaki M, Mizoe J, et al. Different effects of carbon ion and gamma-irradiation on expression of receptor activator of NF-kB ligand in MC3T3-E1 osteoblast cells. Bull Exp Biol Med 2006; 142(5): 618-24

輻射可促進二氧化矽誘發分化之破骨細胞凋亡

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摘要

二氧化矽(silica)為具有良好生物相容性之生物醫學材料，可應用於骨組織工程與人工關節，這些材質常與骨組織接觸進而刺激破骨細胞分化，而破骨細胞會分解二氧化矽材料導致人工骨組織腐蝕或鬆動，臨床上已發現游離輻射有抑制破骨細胞活性的作用，然而使用二氧化矽為骨組織材料之病患接受輻射治療時，其破骨細胞之生物效應則甚少研究。

本研究取齧齒類單核球細胞株(Raw 264.7)與安全劑量範圍之二氧化矽顆粒共同培養，觀察二氧化矽顆粒之生物效應。實驗中，細胞分成對照組、二氧化矽處理組、輻射(5 Gy)處理組及二氧化矽合併輻射處理組等四組。本實驗驗分析破骨細胞活性酵素抗酒石酸酸性磷酸酶(tartrate resistant acid phosphatase; TRAP)之相對產量與破骨細胞之分化數目等相關參數，並以細胞凋亡染劑(Annexin-V-FITC)評估破骨細胞凋亡百分比。結果顯示，二氧化矽顆粒可促進代表破骨細胞活性之 TRAP 產量與破骨細胞之分化數目，而輻射照射可促進破骨細胞凋亡，進而抑制二氧化矽顆粒所誘發之破骨細胞分化。結論：輻射可促進二氧化矽誘發分化的破骨細胞凋亡而降低破骨細胞之活性與數目。

關鍵字: 骨組織，破骨細胞，二氧化矽，輻射，單核球，分化