行政院國家科學委員會補助專題研究計畫成果報告

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（計畫名稱）根尖充填劑對骨細胞之系列研究究

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計畫編號：NSC-2314-B-040-014-;

執行期間：90年　8月　1日至91年　7月 31日

執行單位：中山醫學大學牙醫學系

計畫主持人：黃翠賢

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一、 中文摘要

根尖手術在根管治療中已經有百年之歷史，作為一個優良的根管修復材料，它必須具備下列三種特性：一、具有抵抗邊緣滲漏的能力。二、讓組織具有正常的癒合反應。三、臨床上易於操作。針對讓根尖組織具有正常的癒合反應，此材料必須是具生物相容性。本研究目的探討根尖充填劑與骨細胞作用後，骨細胞之訊息傳遞途徑為何。即比較其它之根尖充填劑對骨細胞 MAPK 訊息蛋白之時間影響。研究方法將以 Western assay 作細胞內之機制研究。結果發現 MTA 材料與骨細胞株作用後，有促進細胞之生長、對訊息傳遞因子 MAPK 家族中之 ERK 蛋白有明顯的表現，但對於 p38 或 JNK 蛋白則不見表現。MTA 材料於生物學上是屬於生物相容性之材料。

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關鍵詞：
根尖充填劑、三氧礦化合物、訊息蛋白

Abstract

Extracellular regulated kinases (ERKs) –1 and –2 are members of the MAPK family of protein kinases involved in the proliferation, differentiation and apoptosis of bone cells. The purpose of present study investigated the biocompatibility and the role of components of signaling pathways of mineral trioxide aggregate (MTA), by culturing human osteosarcoma cell line (U2OS) in the presence of materials. Cytotoxic effects were assessed using the MTT assay for mitochondrial enzyme activity. The statistical analysis of survival rate was performed using one way analysis of variance (ANOVA) with $p<0.05$ shown statistical difference. Signaling pathway of MTA treated U2OS cells were assessed by the western blotting methods. Dose-dependent and time-dependent tests were evaluated. The results showed the survival rates of the MTA extract treated groups are higher than that of the control group. ($p<0.05$) ERKs activity were dose dependent decreased as the concentrations of the MTA extract decreased and were time dependent decrease as the treatment time increased. Suppression of ERK pathway by PD98059 resulted in a dose dependent decreased and time dependent decreased. Our findings suggest that MTA is a biocompatible material and the ERK kinase pathway plays a role in the proliferation of U2OS cells.

二、緣由與目的

The ideal characteristic required for root end filling materials should adhere and seal the root canal system in three dimensions, be nontoxic and be well tolerated by peri-radicular tissue. It needs dimensionally stable, nonabsorbable, and not affected by the

presence of moisture. In addition, it should not corrode or be electrochemically active, be easy to manipulate, and be radiopaque.(1)

Numerous materials have been suggested as root end filling materials, including gutta-percha, zinc oxide eugenol, glass ionomers and gold foil etc.(2) A new cement, mineral trioxide aggregate (MTA), has recently been developed. The material consists primarily of tricalcium silicate, tricalcium oxide and silicate oxide. (3) Besides the trioxides, there are small amounts of other oxides responsible for its chemical and physical properties. The powder consists of thin particles that are hydrophilic, and thus sets in the presence of water.

Because root end filling material are in contact with periradicular tissues, in addition to having good sealing ability they should also be biocompatible. Previous study found that MTA was significantly less toxic than other root end filling materials when freshly mixed, and toxicity was negligible when fully set at 24h.(4) When an MG63 cell line monolayer technique was used, the cells grew well in intimate contact with MTA and assay of culture medium samples demonstrated that MTA-induced expression of interleukin 6 from cells.(5) In vivo, the response to MTA has been more favourable than that to the normal root end filling material, all MTA cases showed newly formed hard tissue over the material after 1 weeks.(6).

Cell growth, division, differentiation and death are now known to be regulated in part by mitogen activated protein kinase (MAPK) pathway. (7,8) Cellular signal transduction is a two step process: First, a signaling molecule is sensed by a receptor at a target cell and then the receptor is activated. When the receptor sensing the signal is a catalyst, a kinase, the response is amplified. At least three parallel MAPK pathways have been identified; these are frequently referred to as ERK (extracellular signal regulated kinase), SAPK (stress activated protein kinase; also known as JNK for c-jun-N-terminal kinase), and p38/MAPK.

In the present study we aimed to identify the signaling pathway(s) responsible for ERK1/2 activation in human osteosarcoma cell line, U2OS cell line, after a extract of mineral trioxide aggregate stimulation.

三、結果與討論

The MTA is a biocompatible material

The MTA is a hydrophilic substance likely to release ionic components, it would be more apt to interfere with intracellular enzyme activities than influence membrane permeability.(13) Therefore, the MTT assay was chosen for the present study. The selected 24h of MTA treatment time is according to the Karl et al. study. (14)

The survival rates of the experimental groups are higher than that of the control group. It is similar with the Mitchell et al. study, that he demonstrated MTA has good cell growth, and it is biocompatible. (14) The MTA is a biocompatible material can also be seen by that tissue reactions after subcutaneous and intraosseous implantation of MTA. The study showed that osteogenesis occurred in association with intraosseous implants indicating that MTA is osteoconductive. (15) In clinical reports that MTA is suitable for closing the communication between the pulp chamber and the underlying periodontal tissues.(16,17,18) Thus, the present results is similar with above study that MTA is a biocompatibile material.

The expression of ERK MAPKs activity

Mammalian MAPKs consist of three major subfamilies: ERK MAPKs, JNK MAPKs/SAPKs, and the p38 MAPKs. Each MAPK has specific substrates and functions, which range form regulation

of cell proliferation to death. (19,20) In present study, we have assayed the all three type kinases activity by western blotting. The JNK and p38 kinase activities were not expressed in present study

One of the most important pathways for cell proliferation is extracellular signal regulated kinases pathway (ERK MAPKs). ERK MAP kinased are located at central position of mitogenic signaling which is a cascade of phosphorylation reactions involving cell surface receptor, Ras, Raf, and MEK or protein kinase C (PKC). ERK MAP kinase pathway, which contains some proto-oncogens and several factors, has been already examined in various human cancers.(21,22) But the expression of ERK MAP kinase does not mean that the cell is going to be mutation. The different environments might affect the pathway. The investigation of mutagencity of mineral trioxide aggregate demonstrated that MTA is not mutagenic as measured by the Ames test.(23) It is known that ERK has been shown to be involved in the proliferative response of osteoblasts to a variety of mitogens. ERK-1 and –2 are also important in osteoblastic cell proliferation and differentiation. (24,25,26) In present study, the western blotting results showed that the MTA treated U2OS cell has dose depend ERK MAP kinase activity. Whether the MTA is a mitogen or not needed for further investigated.

The PD98059 inhibit the ERK kinase activity

Whether MTA induce the proliferation of U2OS cells is by cascade of ERK MAP kinase or not. It is assayed by ERK inhibitor addition. The dose dependent shown that the at 5ë of inhibitor concentrations the ERK expression is decreased. At 5ë inhibitor concentrations, the 6h lane showed that the ERK activity was suppressed and 24h lane showed less activity of ERK. At 24 h treatment and the 0.1% MTA extract concentration, the ERK activity is expressed.(Figure3) The result showed the ERK inhibitor block the cascade of the ERK pathway. It is proved that MTA treated U2OS cells was through ERK MAP kinase cascade.

Since the dominant ERK activity is appeared in the MTA treated U2OS cells. The result is suggested with the MTA may be a viable alternative material in certain clinical applications such as in capping of the dental pulp tissues, root end closure, repair of root perforations as well as a root end filling material. Underlying these application are the formidable properties of MTA: its biocompatibility, good sealing ability and the ability to promote regeneration of original tissue when placed in direct contact with dental pulp and periradicular tissues.

Clinical implication

Endodontic root end filling material such as zinc oxide eugenol cement and resin composite have been used in the past to repair root defects, but their use resulted in the formation of fibrous connective tissue adjacent to the bone. The western blotting assay of the MTA treated cells demonstrated that ERK kinase has been activated. It allows the overgrowth of cementum and periodontal ligament, MTA may be an ideal material for certain endodontic procedures.

計劃成果自評

日本計劃之執行，對根尖充填劑提出有意義之參考訊息，並作為日後研究類似材料之一模式，和作為改良根尖充填劑之一參考資料

Reference

1. Gartner AH, Dorn So. Advances in endodontic surgery. Dent Clin North Am 1992;36:357-379.
2. Guttman JL, Harrison JW. Surgical endodontics. Boston: Blackwell Scientific publications,1991:238-241.

3. Torabinejad M, Hong CU, McDonald F, Pitt Ford TR. Physical and chemical properties of a new root-end filling material. J endodont 1995;21:349-353.
4. Torabinejad M, Hong CU, Pitt Ford, Kettering JD. Cytotoxicity of four root end filling materials. J Endodont 1995;21:489-492.
5. Koh ET, Torabinehad M, Pitt Ford TR, Brady K, McDonald F. Mineral trioxide aggregate stimulates a biological response in human osteoblasts. J Biomed Mat Res 1997; 37:432-439.
6. Torabinejad M, Hong CU, Pitt Ford, Kettering JD.Investigation of mineral trioxide aggregate for root end filling in dogs. J Endodont 1995;21:603-608.
7. Seger R, Krebs EG. The MAPK signaling cascade. FASEB J 1995, 9:726-735.
8. Neary JT. MAPK cascades in cell growth and death. News in Physiol Sci., 1997, 12:286-293.
9. Keiser K, Johnson CC, Tipton DA. Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. 2000;26:288-291.
10. Schweikl H, Schmalz G, Toxicity parameters for cytotoxicity testing of dental materials in two different mammalian cell lines. Eur J Oral Sci 1996;104:292-299.
11. Wataha JC, Craig RG, Hanks CT. Precision and new methods for testing in vitro alloy cytotoxicity. Dent Mater 1992;8:65-71.
12. Gardner AM, Xu FH, Fady C, Jacoby FJ, Duffey DC, Tu Y, Lichtenstein A. Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxoide. Free Radic Bio Med;1997, 22:73-83.
13. Schweikl H, Schmalz G. Toxicity parameters for cytotoxicity testing of dental materials in two different mammalian cell lines. Eur J Oral Sci 1996;104:292-299.
14. Mitchell PJC, Pitt Ford TR, Torabinejad M, McDonald F. Osteoblast biocompatibility of mineral trioxide aggregate. Biomaterials 1999;20:167-173.
15. Moretton TR, Brown CE, Legan JJ, Kafrawy. Tissue reactions after subcutaneous and intraosseous implantation of mineral trioxide aggregate adnethoxybenzoic acid cement. J Biomed Mater Res 2000;52:528-533.
16. Arens DE, Torabinejad M. Repair of furcal perforations with mineral trioxide aggregate. Oral Surg Oral Med Oral Patho Oral Radiol Endod 1996;82:84-88.
17. .Torabinejad M, Chivian N. Clinical applications of mineral trioxide aggregate. J Endod 1999,25:197-205.
18. Holland R, Filho JAO, deSouza V, Nery MJ, Bernabe PFE, Junior D. Mineal trioxide aggregate repair of lateral root perforations. J Endod 2001;27:281-284
19. Han J, Lee Jd, Bibbs L, Ulevich RJ. A MAP kinse targeted by endodontosin and hyperosmolarity in mammalian cells. Science 1994; 265:808-811.
20. Davis RJ. The mitogen-activated protein kinase signall transductin pathway. J Bio Chem 1993;268:14553-14556.
21. Marshall CJ. MAP knase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev 1994;4:82-89.
22. Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Bio Chem 1995; 270:14843-14846.
23. Kettering JD, Torabinejad M. Investigation of mutagenicity of mineral trioxide aggregate and other commonly used root end filling materials. J Endodon 1995; 21:537-539.
24. Lai CF, Chaudhary L, Fausto A, Halstead LR, Ory DS, Avioli LV,

Cheng SL. ERK is essential for growth, differentiation, intergrin expression and cell function in human osteoblastic cells. J Bio Chem 2001, 276; 14443-14450.

25. Lou J, Tu Y, Li S, Manske PR. Involvement of ERK in BMP2 induced osteoblastic differentiation of meschymal progenitor cell line C3H10T1/2. Biochem Biophys Res Commun 2000,268:757-762.
26. Swarthout JT, Doggett TA, Lemker JL, Partridge NC. Stimulation of extracellular signal regulated knases and proliferation in rat osteoblastic cells by parathyroid hormone is protein kinase C-depedndent. J Bio Chem 2001;276:7586-7592.

Figure 1. The survival rate of the MTA cement treated on the U2OS cells by MTT assay.

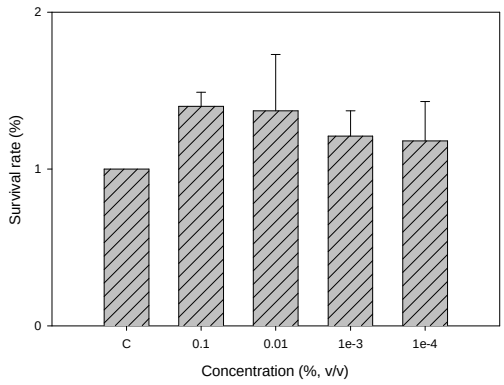


Figure 3. Time dependent test. The ERK expression at different time interval under 10^{-1}% MTA extract concentrations.

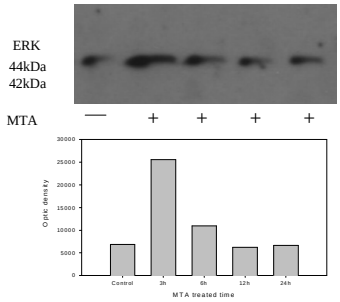


Figure 4. The dose dependent of the ERK inhibitor addition on the MTA treated U2OS cells. The MTA extract concentration is 0.1%(v/v), and the overnight culture is performed.

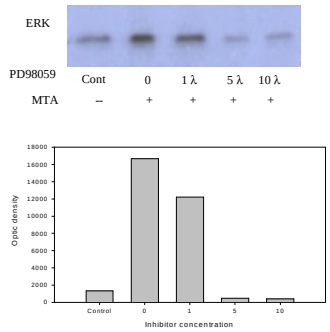
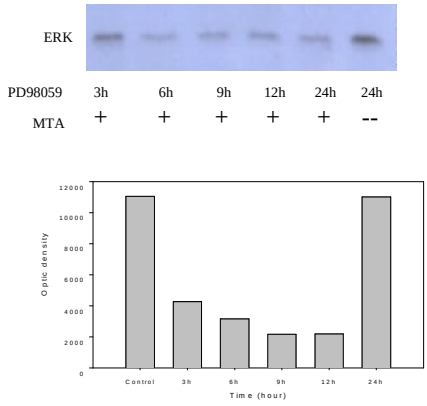


Figure 5. The time dependent test of ERK inhibitor Added on the MTA treated U2OS cells. 5 λ of the inhibitor added and the time course were ranged from3h to 24 h.



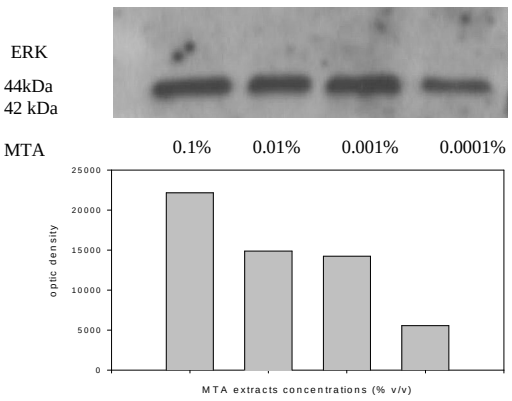


Figure 2. The dose dependent test. The immuno blot of Erk in the U2OS cells. Following SDS-PAGE and immunoblotting, U2OS cell extracted were probed with ERK antibodies.