行政院國家科學委員會專題研究計畫　成果報告

三氧礦聚合物對骨細胞之基因表現

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-040-007-
執行期間：91 年 08 月 01 日至 92 年 07 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

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 10 月 6 日

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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中英文摘要

Mineral Trioxide aggregate 為一根尖充填材料，臨床上對於骨頭之修復表現優良。過去有研究發現其生物相容性高，對於細胞會有炎性反應出現。本研究的目的乃探討此根尖充填材料與人類股細胞朱作用後其細胞內之訊息蛋白表現狀況。本研究經由存活率試驗決定作用濃度，以西方墨點研究其 Extracellular regulated kinases (ERKs) –1 and –2 之表現，以 PD98059 抑制劑作為正對照組。結果發現 MTA 作用後之細胞生長比控制組織細胞生長好 ($p<0.05$)。 ERK 表現發現呈劑量減少之表現。結語：MTA 之生物相容性高其主要乃經由活化細胞內之 ERK 訊息傳遞蛋白脢。

關鍵字： 三養礦化物 生物相容性　訊息傳遞

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 the present study investigated the biocompatibility role, and signaling pathways of components of mineral trioxide aggregate (MTA) by culturing human osteosarcoma cell line (U2OS) in the presence of materials. Biocompatibility effects were assessed using the MTT assay for mitochondrial enzyme activity. The statistical analysis of the survival rate was performed using one- way analysis of variance (ANOVA) with $p<0.05$ shown statistical difference. The signaling pathway of MTA-treated U2OS cells were assessed by the western blotting methods. Dose-dependent and time-dependent tests were conducted. The results showed that the survival rates of the MTA extract experimental groups were higher than that of the control group ($p<0.05$). ERKs activity was dose-dependent, decreasing as the concentrations of the MTA extract decreased, and was time-dependent, decreasing as the treatment time increased. Suppression of ERK pathway by PD98059 resulted in dose-dependent and time-dependent decreases. The findings suggest that MTA is a biocompatible material to U2OS cells, and the ERK kinase pathway plays a signal transduction role in the MTA treated U2OS cells.

Key words: mineral trioxide aggregate (MTA), mitogen activated protein kinase (MAPK), Extracellular regulated kinases (ERK), western analysis, MTT assay.

計畫緣由與目的

Because root end filling materials are in contact with periradicular tissues, in addition to having good sealing ability they should also be biocompatible. A 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 [1]. When an MG63 cell line monolayer technique was used, the cells grew well in intimate contact with MTA and an ELISA assay of culture medium samples demonstrated that MTA-induced expression of interleukin 6 from cells [2]. In vivo, the response to MTA has been more favorable than that of the normal root end filling material, all MTA cases showed newly formed hard tissue over the material after one week [3].

Cell growth, division, differentiation and death are now known to be regulated in part by the mitogen activated protein kinase (MAPK) pathway [4,5]. 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.

The MTA is widely used and successful in clinic. Data are scarce about the mechanism of the cell changes after their contact with the MTA. In the present study we aimed to identify the signaling pathway(s) responsible for ERK1 and 2 activation in a human osteosarcoma cell line, U2OS, after the stimulation of the MTA-extract.

結果與討論

Biocompatibility assay

The survival rate (%) of experiment groups was shown to have increased as compared with the control ($p<0.05$). (Figure 1) The survival rates of the experimental groups are higher than that of the control group ($p<0.05$). The growth of U2OS cells is better in the experimental group. The finding is similar to that reported by the Mitchell *et al.* study, which demonstrated that the MTA had good cell growth and it was biocompatible[6]. The biocompatibility of the MTA is also indicated by the tissue reactions after subcutaneous and intraosseous implantation of the MTA, the osteogenesis occurred in association with intraosseous implants indicating that MTA was osteoconductive [7]. Clinical reports showed that the MTA was suitable for closing the

communication between the pulp chamber and the underlying periodontal tissues [8,9,10]. Thus, the present result has also demonstrated that the MTA is a biocompatibile material.

3.2. Western blot analysis

3.2.1. The U2OS cell expresses an ERK MAP kinase

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 from the regulation of cell proliferation to the cell death [11,12]. In the present study, we have assayed the all three types of kinases activity by western blotting. The activity of ERK kinase was expressed, however, the JNK and p38 kinase activities were not expressed in the present study.

One of the most important pathways for cell proliferation is extracellular signal regulated kinases pathway (ERK MAPKs). ERK MAP kinases 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). The ERK MAP kinase pathway, which contains some proto-oncogens and several factors, has been already examined in various human cancers [13,14]. However, the expression of ERK MAP kinase does not mean that the cell is going to be mutated. The different environments might affect the pathway [13]. A previous study has demonstrated that the MTA is not mutagenic as evaluated by the Ames test [15]. It is known that ERK has been shown to be involved in the proliferative response of osteoblasts to a variety of mitogens. The ERK-1 and –2 are also important in osteoblastic cell proliferation and differentiation [16,17,18]. In the present study, the western blotting assay results showed that the MTA-treated U2OS cell has a dose-dependent ERK MAP kinase activity. Whether the MTA is a mitogen needed further investigation.

4.3. The PD98059 inhibit the ERK kinase activity

The ERK inhibitor addition assay is to clear that the MTA induces the proliferation of U2OS cells by a cascade of ERK MAP kinase. The dose-dependent assay showed that at 5ë of inhibitor concentrations the ERK expression decreased (Figure 4). At 5ë of inhibitor concentrations, the 6-hour lane showed that the ERK activity was suppressed and 24-h lane showed less activity at ERK (Figure 5). At 24-h treatment without PD98059 inhibitor and the 0.1% MTA extract concentration, the ERK activity was expressed (Figure 5). The present results demonstrate that the ERK inhibitor blocks the cascade of the ERK pathway. The mechanism of the MTA-treated U2OS cells was through the ERK MAP kinase cascade.

The dominant ERK kinase activity appeared in the MTA treated U2OS cells.

This result suggests that the MTA may be a viable alternative material in certain clinical applications such as in the capping of the dental pulp tissues, root end closure, repair of root perforations as well as a root end filling material. Underlying these applications are the formidable properties of the 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.

REFERENCES

1. Torabinejad M, Hong CU, Pitt Ford, Kettering JD. Cytotoxicity of four root end filling materials. J Endodont 1995;21:489-92.
2. 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; 37:432-9.
3. Torabinejad M, Hong CU, Pitt Ford, Kettering JD. Investigation of mineral trioxide aggregate for root end filling in dogs. J Endodont 1995;21:603-8.
4. Guttman JL, Harrison JW. Surgical endodontics. Boston: Blackwell Scientific publications,1991:238-41.
5. Seger R, Krebs EG. The MAPK signaling cascade. FASEB J 1995, 9:726-35.
6. Schweikl H, Schmalz G. Toxicity parameters for cytotoxicity testing of dental materials in two different mammalian cell lines. Eur J Oral Sci 1996;104:292-9.
7. Mitchell PJC, Pitt Ford TR, Torabinejad M, McDonald F. Osteoblast biocompatibility of mineral trioxide aggregate. Biomaterials 1999;20:167-73.
8. 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;52:528-33
9. Arens DE, Torabinejad M. Repair of furcal perforations with mineral trioxide aggregate. Oral Surg Oral Med Oral Patho Oral Radiol Endod 1996;82:84-8.
10. Torabinejad M, Chivian N. Clinical applications of mineral trioxide aggregate. J Endod 1999,25:197-05.
11. Holland R, Filho JAO, deSouza V, Nery MJ, Bernabe PFE, Junior D. Mineal trioxide aggregate repair of lateral root perforations. J Endod 2001;27:281-4
12. Han J, Lee Jd, Bibbs L, Ulevich RJ. A MAP kinase targeted by endodontosin and hyperosmolarity in mammalian cells. Science 1994; 265:808-11.
13. Davis RJ. The mitogen-activated protein kinase signal transduction

pathway. J Bio Chem 1993;268:14553-6.

14. Marshall CJ. MAP knase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev 1994;4:82-9.
15. Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Bio Chem 1995; 270:14843-6.
16. Kettering JD, Torabinejad M. Investigation of mutagenicity of mineral trioxide aggregate and other commonly used root end filling materials. J Endodon 1995; 21:537-9.
17. 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; 14443-50.
18. Lou J, Tu Y, Li S, Manske PR. Involvement of ERK in BMP2 induced osteoblastic differentiation of mesenchymal progenitor cell line C3H10T1/2. Biochem Biophys Res Commun 2000,268:757-62.
19. 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;276:7586-92.

Fig.1. The survival rate of MTA cement
treated on U2OS cells by MTT assay.
C: control group without MTA addition.
*: statistical difference at $p<0.05$.

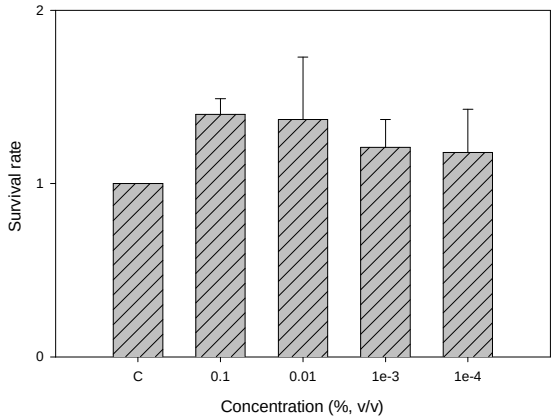


Fig. 2. The dose dependent test. The immuno blot of ERK in U2OS cells. Following SDS-PAGE and immunoblotting, U2OS cells were extracted and probed with ERK antibodies.

0.1% 0.01% 0.001% 0.0001%

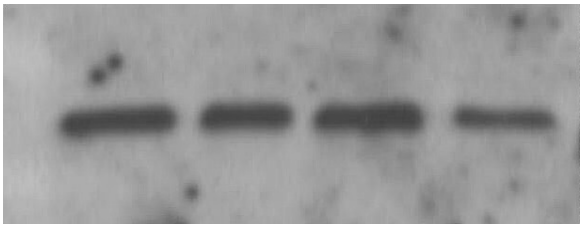
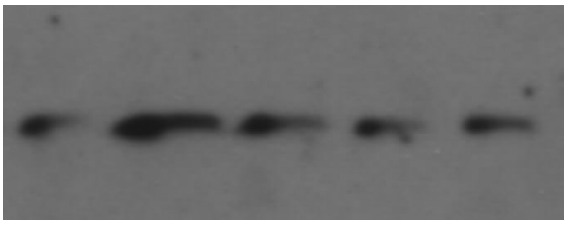


Fig. 3. Time dependent test. The ERK expression at different time intervals under 0.1% MTA extract concentrations.

44 kD
42KD



C 0.1% 0.01% 0.001% 0.0001%

Fig. 4. The dose dependence of the ERK inhibitor addition on the MTA treated U2OS cells. The MTA extract concentration was 0.1%(v/v), and then the overnight culture was performed.

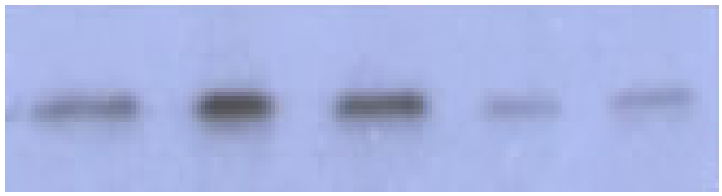


Fig. 5. The time dependent test of ERK inhibitor adding on the MTA treated U2OS cells. 5 λ of the inhibitor was added and the time course were ranged from3h to 24 h.