行政院國家科學委員會專題研究計畫　成果報告

根尖充填劑於活體之細胞素表現研究

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-040-018-
執行期間：92 年 08 月 01 日至 93 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
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報告類型：精簡報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 93 年 9 月 30 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

細胞素影響組織之生長發育與分化功能。根尖充填材料須讓根尖組織具有正常的癒合反應，而且不會造成其他副作用。本研究目的為研究根尖充填劑於細胞之免疫反應，採取三種根尖修復材料 Resin (Super EBA), Calcium base (Life) 和 Calcium trioxide (MTA)作用於 U2OS 細胞株觀察其 IL2, IL4, IL10 之表現。實驗經 enzyme-linked immunosorbent assay 分析結果發現 MTA 材料與細胞附著程度最佳，IL4, IL10 於 MTA 有明顯表現，IL10 則於三組表現均較控制組多，本研究之根尖修復材料均會造成免疫學上之改變。

關鍵字：細胞素 根尖充填材料 免疫反應

Cytokines are critical controllers of cell and tissue growth, development and differentiation. Root-end filling materials often contact with existing periapical tissue, and they need to be biocompatible with remnant periapical tissue. The aim of this study was to evaluate the immune effect of root-end filling materials and to attempt to quantify filling material-elicited cytokine expression. Calcium hydroxide-based (Life), zinc oxide eugenol-based (Super EBA) and mineral trioxide aggregate-based (MTA) root-end filling materials were used to investigate their effect upon a human osteosarcoma cell line (U2OS). The cell attachment assay was observed microscopically and the expression of interleukin-2, -4 and -10 were quantified by enzyme-linked immunosorbent assay. Any resultant difference between the root end filling material was analyzed statistically by one-way analysis of variance. The results demonstrated that the best cell attachment to root-end filling material occurred with MTA. Circulating IL-4 and IL-10 levels were greater for the MTA group, whilst IL-2 expression for the three kinds of root-end filling materials was similar. All materials were able to induce an immune response.

Key words: Biocompatibility cytokine expression, interleukine

計畫緣由與目的

Chemicals to be used for human treatment should be screened to determine if they are potentially immunotoxic. Compounds that may adversely affect the human immune system are found amongst various drugs, pesticides, solvents, halogenated and aromatic hydrocarbons and metals, and ultraviolet radiation can also be immunotoxic.[1]

Dental materials are placed inside the tooth and may be resorbed from the oral cavity, and, as a consequence, they may be able to induce an immune reaction.The primary objective of root-end surgery is to introduce the appropriate placement of a seal between the root canal system and the periodontium .[2] An ideal root-end filling material should not only seal the root-end cavity hermetically, but it should also be biocompatible, non-toxic, insoluble in tissue fluids, non-resorbable, dimensionally stable , capable of inducing osteogenesis and cementogenesis, easy to prepare and to use, sterilizable , radio-opaque, inexpensive, and not be susceptible to denaturing in the presence of moisture,[2,3]

Over time, several materials have been suggested as potential root-end filling materials including mercury amalgam, a zinc oxide eugenol-based cement (Super-EBA), mineral trioxide aggregate (MTA) and myriad other restorative materials.[4] When inserted, such materials must remain in contact with the periodontal tissue including periodontium and bone tissue. In cases of periapical lesions, alveolar bone is typically destructed, and over the years, many different attempts to rebuild lost bone have been made, commonly used materials for such a purpose including autografts, hydroxyapatite and tricalcium phosphate.[5-7] Bioactive bioglass or biogran exhibit the ability to induce some level of hard-tissue formation.[8,9] Hydroxyapatite is an inorganic constituent of bone, and it may be used as synthetic bone-graft material.[10]. Calcium disalicylate demonstrates sustained hydroxyl-ion release and appears to exhibit a favorable dental-pulp tissue response.[10-12]

The relative biocompatibility of alternative root-end filling materials upon human periodontal ligament fibroblasts was previously reported as being classified in the following order of toxicity: amalgam > super EBA > MTA in a freshly mixed state.[13] The expression of high levels of interleukin-6 (IL-6) and interleukin-8 (IL-8) in the presence of MTA may promote healing of bone but this may not necessarily be the case for the other root-end materials,[14] although to the best of our knowledge, little has been published regarding the relative toxicity to the immune system elicited by various root-end filling materials. The

tools of and theoretical concepts embodied in immunotoxicology are increasingly being used in novel ways, such as using toxic reagents to attempt to understand immune-system function.[1] One of the most potentially useful of these new tools is the assessment of the role of cytokines, such molecules being responsible for regulating a variety of processes including immunity, inflammation, apoptosis and hematopoiesis.[15] Cytokines are a group of communicating molecules, originally identified in the leukocyte population, that are produced by both macrophages and osteoblasts.[16]

The purpose of the present study was to evaluate the immunotoxic effect of root-end filling materials and to attempt to quantify filling material-elicited cytokine expression. The study was performed using a human osteosarcoma cell line in the presence of various root-end filling materials.

結果與討論

Morphology analysis

The U2OS cells were confluent for the control group (Fig. 1a), whilst the cell density and number increased gradually during the experimental period for the MTA group.(Fig. 1b) Proliferating U2OS cells did attach to the MTA root-end filling material, although by contrast, the cell density and number of U2OS cells decreased to almost zero for both the Life and Super EBA groups. (Fig. 1c and d) An examination of associated cell morphology revealed the loss of intercellular connection and the appearance of the contraction of the cell body. The cultured U2OS cells were not able to attach to either the Life or the Super EBA root-end filling materials.

Expression of the cytokines

The IL-2 cytokine levels expressed by the cells in contact with these three root-end filling materials (MTA: 0.035 ± 0.004243ng/ml, Super EBA: 0.0325± 0.000707ng/ml, and Life: 0.032 ± 0.001414ng/ml) were found to be significantly greater ($P<0.01$) than the corresponding value for the control (0.0215 ± 0.001233ng/ml; Fig. 2)

The cells in contact with the MTA root-end filling material exhibited the most substantial IL-4 cytokine levels (0.824 ± 0.396ng/ml) when compared with other root-end filling materials (Super EBA: 0.107 ± 0.001273ng/ml and Life: 0.008 ± 0.01131ng/ml), difference being significant ($P<0.01$; Fig. 3).

The cells in contact with MTA revealed the most substantial IL-10 cytokine levels (2.063 ± 1.244ng/ml) when compared with other root-end filling materials (Super EBA: 0.076 ± 0.01414ng/ml and Life: 0.00605 ± 0.000707ng/ml), difference. again, being significant ($P<0.01$; Fig. 4).

Cells in contact with cements based on MTA-type root-end filling material expressed greater levels of cytokines (IL-4 and IL-10) than did the Life and Super EBA($P<0.01$). When individual

cytokine levels were evaluated, cells in contact with MTA were found to produce the most substantial concentration of IL-4 (0.842 ± 0.396ng/ml) and IL-10 (2.063 ± 1.244ng/ml).

Discussion

Root-end filling materials placed in contact with periodontal tissue should possess good biocompatibility, it having been previously suggested that cell adhesion and spreading on root-end filling materials could be used as a specific criterion for the evaluation of the relative acceptability for use of various root-end filling materials.[17] Adhesion and the spreading of the cells on a material surface are the initial phase of cellular function, the key point events in this phase being the attachment of the cell to the substratum, radial growth of the filopodia, cytoplasmic webbing and the resultant flattening of the cell.[17] The morphological observation of the cellular attachment of U2OS cells to the root-end filling material was conducted using the MTA material. Both the Life and Super EBA groups revealing that cellular growth in the culture plate ceased at a greater distance from these root-end materials than was the case for the MTA material. Such results suggest that the U2OS cells did not survive as well in close proximity to the Life and Super EBA materials as they did for the MTA root-end filling material. One possible reason for the relatively low survival results for U2OS cells in close proximity to the Life material might be that the pH of the extracts was too high for good cell growth. Further, the Super EBA material has been previously shown to be cytotoxic to human periodontal ligament fibroblasts, [13] this root-end filling material consisting of a powder containing zinc oxide, alumina, and resin and a liquid containing ortho-ethoxy-benzoic acid and eugenol. As regards the subcutaneous and intraosseous implantation adjacent to tested filling materials in a previous rat study, the Super EBA material reflected a predominantly moderate inflammatory response.[18] Further, it has been previously proposed that ortho-ethoxy-benzoic acid, which has been shown to be released from freshly mixed eugenol material[19], is another component of EBA cement that may be irritating to the tissue.[20]

A number of chemical-induced immunotoxic effects of cellular exposure to certain root-end filling materials have been reported previously, such effects having been suggested to have been organ specific, cell specific immune-function specific, or alternatively they may have been, essentially, secondary effects following prior toxic effects to other organs, or they could simply be non-specific effects .[21-27] Virtually all situations where significant cell damage occurs as a result of chemical exposure to

biological tissue, such cell damage will give rise to inflammation, cell migration and aggregation to the site of the damage and it is believed that the controlling molecules for these reactions are cytokines.[28] Hence, monitoring cytokine concentrations at the site of damage appears to represent the potential for monitoring the extent of cytotoxicity elicited at the site by a specific agent, and possibly relating, in a more specific way, to the actual type of cytotoxicity occurring.[28]

The enzyme-linked immunosorbent assay used to assess cytokine expression is more sophisticated. The assaying of cytokines under such experimental conditions would appear to provide good quantification of the relative level of biocompatibility at biochemical level of the tested filling materials. Immunotoxicity data provide information relating to health hazards likely to arise from subchronic exposure to a test chemical, this usually being accomplished subsequent to dosing by the oral route.[28] Tests are selected in an attempt to provide as much qualitative and quantitative data pertaining to the capacity of a chemical to adversely affect components of antibody-mediated and specific and non-specific cell-mediated immunity as is possible. T lymphocytes involved in dermal sensitization secrete type-1 cytokines, as produced by type-1 helper (Th1) cells, whilst those cells that are involved in respiratory sensitization have previously been shown to be type-2 helper cells (Th2) secreting type-3 cytokines. Type-1 cytokines include IL-2, interferon gamma, IL-12 and tumor necrosis factor beta, whilst type-2 cytokines include IL-4, IL-5, IL-6 , IL-10 and IL-13.[29]

IL-2 was originally identified as a T-cell growth factor subsequent to IL-2 having been found to be obligatory for T-cell activation and expansion.[30] It is now known that IL-2 plays a central facilitative role in immune response, promoting the proliferation of most subsets of lymphocytes.[31] Previous study showed that root-end filling materials existed various cell toxicity.[12,13] The present study has found that the IL-2 level of cells adjacent to abutting root-end filling material was greater than the corresponding level for the control.(Fig. 2) The interaction between the root-end filling material and adjacent cells observed herein has revealed that a notable inflammatory response was triggered by the presence of the filling material.

Interleukin 4 (IL-4), also called B-cell growth factor or B-cell stimulating factor, could be an important mediator of the T cell-dependent activation of B cells.[32] The IL-4 is the upstream of the Ras kinase and P13K pathways[33,34], and it is possible that the Ras kinase signal-regulation pathway could affect the growth and survival gene after external stress of the cells. Ras kinase has been previously reported to regulate MAP kinase activation [34],

and the signal pathway of cell proliferation was through the MAPK kinase pathway. The P13K pathway has been reported to be involved in regulating PIP2 and the pathway's activation may result in cell apoptosis [36]. Our previous study found that the signaling pathway for MTA root-end filling material-treated U2OS cells proceeds through extracellular signal regulated kinase (ERK) expression.[35] The present results suggest that IL-4 expression was substantially induced by MTA and not by the Life or Super EBA filling materials. (Fig. 3) From the results of the assay of the attachment of the root-end filling materials to U2OS cells, we found that most U2OS cells had actually died. (Fig. 1c and d) It is proposed here, that the toxicity mechanism underlying the root-end filling materials such as was evidenced by the Life- and Super EBA-treated groups involved the P13K pathway, whereas the corresponding mechanism for the MTA-treated group may have been via the Ras pathway.

Interleukin-10 (IL-10) functions as an anti-inflammatory cytokine that inhibits the synthesis and release of IL-1, IL-6, IL-8, TNF- α , and certain colony-stimulating factors[36] The present result has shown that IL-10 was induced by the MTA-treated group but not so for the Life and Super EBA groups. (Fig. 4) The MTA group has revealed a more desirable anti-inflammatory response to its application than was the case for the Life and Super EBA groups, it having been suggested previously that the less substantial inflammatory response triggered by MTA as compared to other filling materials has resulted in MTA being deemed to be less toxic to human periodontal ligament cells than other tested root-end filling materials.[13]

The use of cytokines and their tissue levels as markers of cytotoxicity for the purposes of monitoring toxicity under certain conditions would appear to be dependent upon the investigator making the correct choice of cytokine to study. In the present study we chose type-I (IL-2) and type-II(IL-4 and IL-10) cytokines to use as specific markers of cytotoxicity or apoptosis, we believing that their presence may constitute a rather sensitive indicator of the interaction of a tested drug/chemical with biological tissue. The present study has demonstrated that certain root-end filling materials may be antigenic to the cells that come in contact with the material. Clearly thus, and along such lines, one of the most-important aspects of treating dental patients is for the dental practitioner to attempt to prevent the emergence of any patient hypersensitivity reaction as a consequence of the use of certain dental materials, such an aspect warranting much further research.

計畫成果自評

The present study was the primary result of this serial project. From this result we may decided to investigating

the next step on the study. It is planning to perform the animal study and detect the immune reaction after the implantation the root end filling material into animal. It is hope that the experiment can provide some information to the clinical doctor in selecting the material and provide researcher good concept on the material improvement.

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Table 1. The composition of the root end filling materials.

Product		Composition	Reference no.
Super EBA (Bosworth Co., Durham, England)	powder	Zinc oxide 60% Alumina 34% Natural Resin 6%	0921007
	liquid	Ortho Ethoxy Benzoic acid 62.5% Eugenol 37.5%	
Life (Kerr Co. Romulus, MI, USA)	base	Calcium hydroxide 6.01g Zinc Oxide 1.65g	2-1032
	catalyst	Barium Sulphate 4.32g Polymethylensalicylate Resin 3.98g Methylsalicyate 1.81g Barium Sulphate 4.32g Polymethylensalicylate Resin 3.98g Methylsalicyate 1.81g	
MTA (Proroot, Tlusa Dental, Tulsa, OK, USA)	powder	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, calcium sulfate, bismuth oxide	A040500000100
	liquid	Distilled water	

Figure 1. The root-end filling materials tested with cultured U2OS cells. 1a. the control group. 1b. the MTA-treated group. 1c. the Life-treated group. 1d. the Super EBA-treated group.

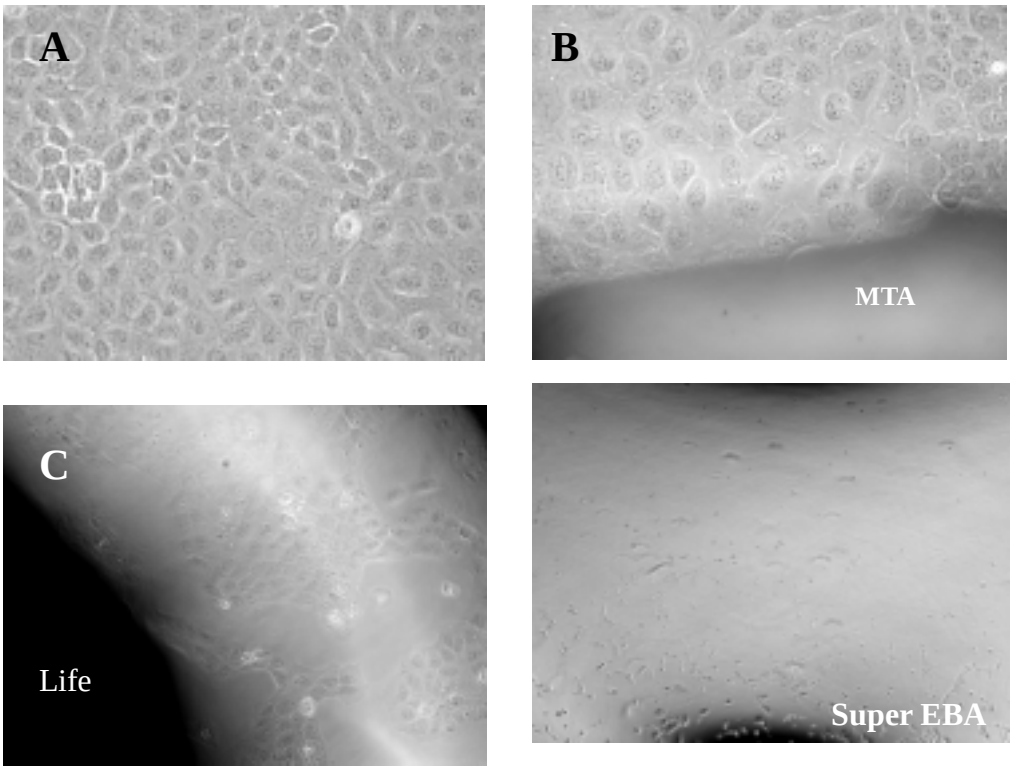

Figure 2. The cytokine expression of IL-2 as elicited by the interaction between certain root-end filling materials and cultured U2OS cells.

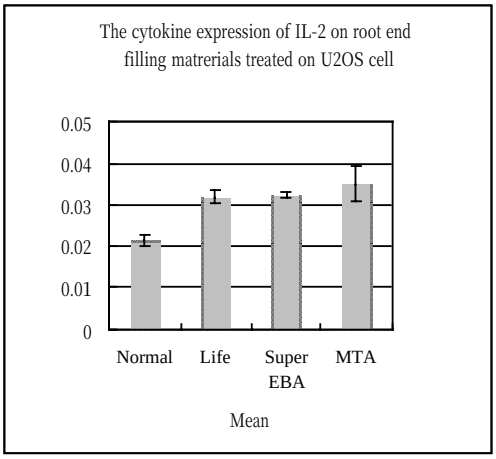

Figure 3. The cytokine expression of IL-4 as elicited by the interaction between certain root-end filling materials and cultured U2OS cells.

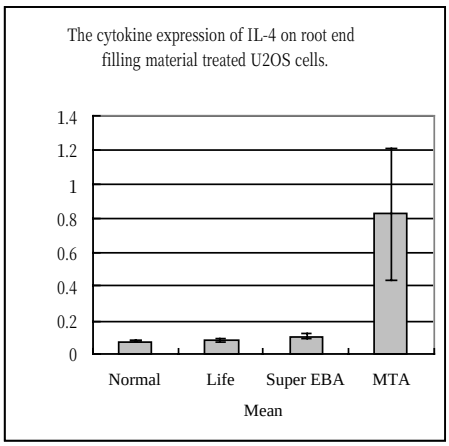

Figure 4. The cytokine expression of IL-10 as elicited by the interaction between certain root-end filling materials and cultured U2OS cells.