CHAPTER V

DISCUSSION

Fluocinolone acetonide is commonly used as topical medicament in the treatment of various dermatologic disorders and oral vesibuloerosive lesions (Hooley and Hohl, 1974; Lozada and Silverman, 1980; MacPhail *et al.*, 1992; Buajeeb *et al.*, 2000). It provides distinct advantages in the inhibition of inflammatory process and immunologic responses (Vogel *et al.*, 1984). Interestingly, some evidence demonstrated that a range of concentrations of fluocinolone acetonide had stimulatory effect on cell proliferation (Fisher and Maibach, 1971; Kirk and Mittwoch, 1977). An *in vitro* study in mouse fibroblasts indicated that steroids, which possessed high glucocorticoid activity, were capable of stimulation both DNA synthesis and cell division. The relative potency of these active steroids was related to their relative glucocorticoid potency (Thrash *et al.*, 1974). Fluocinolone acetonide is a synthetic glucocorticoid which has the most relative potency among various types of steroids (Berliner and Ruhmann, 1967). Thus, it may provide some advantages in the stimulation of cell proliferation and healing process.

In pulpal exposure, some odontoblasts are irreversibly damaged which may be due to dental caries, operative procedure, or traumatic injury. The main goal of regenerative pulp treatment is to induce a second generation of odontoblast-like cells

that are capable of secreting a dentin-like matrix structure to seal off the pulp wound (Goldberg *et al.*, 2003; Alliot-Licht *et al.*, 2005). The formation of hard tissue barrier may be considered as a two-part phenomenon. The first stage is the formation of organic extracellular matrix, mainly composed of type I collagen, by the progenitor cells which are differentiated into matrix synthesizing odontoblast-like cells. Finally, the newly formed tissue is subsequently calcified to form a hard tissue barrier (Mjör *et al.*, 1991; Linde and Goldberg, 1993). In this context, the human dental pulp cell cultures were conducted with respect to whether fluocinolone acetonide can stimulate both type I collagen synthesis and *in vitro* calcification.

In the present study, the effect of fluocinolone acetonide on type I collagen synthesis was examined by Western blot analysis from cell extracts. The result indicated that 1 to 10 μM fluocinolone acetonide was capable of stimulating type I collagen synthesis in human dental pulp cells. Type I collagen synthesis was maximized by the 1 μM fluocinolone acetonide when compared to the others (Fig. 2). This result was confirmed by RT-PCR. The expression of mRNAs of type I collagen in 1 μM fluocinolone acetonide groups was stronger than controls. In addition, we investigated the expression of integrin receptors which were corresponding to type I collagen. The expression of mRNAs of $\alpha 1\beta 1$ integrin receptors were also stimulated (data not shown). This result strongly supported the effect of fluocinolone acetonide in stimulation of type I collagen synthesis in human dental pulp cells in laboratory condition.

It was previously reported that fluocinolone acetonide topically applied for 7 days in mice might reduce fibroblast proliferation and collagen formation. In the wound biopsies, the extracellular space contained non-banded filamentous material and decreased mature collagen when compared with control (Berliner *et al.*, 1967). The *in vitro* effect of fluocinolone acetonide on human's skin collagen was also studied and demonstrated that formation of radioactive hydroxyproline was inhibited by all corticosteroids tested. The corticosteroid-induced inhibition of collagen biosynthesis was found to be dose-dependent. Large non-physiologic concentrations of fluocinolone acetonide inhibited the rate of collagen formation, but the lowest concentration (10 mM) of fluocinolone acetonide had no significant effect on hydroxyproline formation (Uitto *et al.*, 1972).

The result of these studies was different from our experiment which might be due to different methodology used. The earlier study (Berliner *et al.*, 1967) was conducted in mice which applied topical glucocorticoid on the skin, and microscopic observation was used to determine the appearance of collagen fibrils intra- and extracellularly. The result was descriptive and unable to compare the quantity of collagen formation. In addition, the concentration of steroid used was higher than in our study. The later experiment (Uitto *et al.*, 1972) used skin fibroblast and analyzed the radioactive hydroxyproline formation. The result indicated that the 10 mM fluocinolone acetonide had no significant effect on collagen synthesis. This concentration was also much higher than in our study. The inhibitory effect was in dose-dependent manner, thus lower concentration might be

able to stimulate collagen synthesis. In our study, human dental pulp cells were used and different cells might exhibit different response. Western blotting and RT-PCR were used in our study because they have been widely accepted as highly sensitive and reliable techniques in the detection of protein synthesis and gene expression respectively.

Several literatures, however, reported that some glucocorticoids had ability to stimulate collagen synthesis in different tissue. In fetal rat calvaria, short term treatment of some concentrations of cortisol, corticosterone and dexamethasone increased bone collagen synthesis. On the other hand, long term treatment of cortisol (96 hours) inhibited collagen synthesis (Dietrich *et al.*, 1979). After that, Canalis used the similar method and confirmed the stimulatory effect of short term treatment of these steroids in collagen synthesis and inhibitory effect of high concentrations (1-10 μM) of cortisol on type I collagen labeling after 24-hour treatment. The inhibitory effect was dose related (Canalis, 1983). In cultured bovine aortic smooth muscle cells, 0.1 μM dexamethasone produced an approximate two folds increasing in the incorporation of [^{3}H]proline into collagenase-digestible protein (Leitman *et al.*, 1984). Recently, RT-PCR was used to measure the up-regulation of type I collagen mRNA expression in tooth organ cultures. The treatment of 10 nM dexamethasone exhibited four folds greater than in the control group (Ritchie *et al.*, 2004). Hence, these studies have supported the possibility in the use of some glucocorticoids in order to stimulate type I collagen synthesis.

The *in vitro* calcification of human dental pulp cells was studied by long term cultures for up to 28 days. The results in all groups demonstrated small amount of calcified nodules formed in a variety of size and time (Fig. 6). Hence, the quantification of nodules and statistical analysis was not possible. However, the formation of mineralized nodules observed in the experimental groups was not different from the control group. It might be explained that fluocinolone acetonide might lack of the ability to enhance the differentiation of dental pulp cells. In another way, the cells treated by fluocinolone acetonide might need more time to exhibit the calcification process. Therefore, further investigations are needed with regard to the markers of cellular differentiation into hard tissue forming cells.

However, the formation of calcified nodules in this study was consistent to the previous study (Tsukamoto *et al.*, 1992). Tsukamoto and colleagues (1992) reported the alterations of cell morphology and mineralized nodule formation in human dental pulp fibroblasts in cultures. The nodules were formed after 10-15 days and their characteristics were similar to our study.

In the development of mineralized nodules by rat periodontal cells, four distinct stages based on the appearance of cultures under the phase contrast microscope were identified. The first stage was the formation of confluent monolayer of cells which generally exhibit spindle or polygonal morphology. When treated with steroids, ascorbic acid and β-glycerophosphate, cell proliferation began to occur in certain areas with the formation of several cell layers appearing like clusters of cells referred to as the

multilayer formation stage. The third stage was associated with the formation of nodules formed by deposition of matrix in the clusters of cells. Finally, mineralization of the matrix occurred and was referred to as mineralized nodule stage. The mineralized nodules showed densely stained mineral deposits with globular shape that appeared to increase in size and fuse towards the lower cell layers of the nodules. As mineralization progressed, the deposits increased in size and fused together, forming a contiguous mineral deposit in the intercellular space (Ramakrishnan *et al.*, 1995). In contrast, the observation of calcified nodules in our study demonstrated that the nodules were rarely fused together. The isolated nodules were frequently found which was different from the periodontal cell cultures. These results might be because of the different cell type in experiment.

Glucocorticoids demonstrated some adverse effects when used in human. The major adverse effects of corticosteroids particularly applicable to dental usage are inhibition of fibroblastic proliferation which favors dissemination of microorganisms, and inhibition of cellular response (both vascular and local tissue) to an irritant (Sinkford and Harris, 1964). Thus, living noxious irritants are able to multiply and disseminate into systemic circulation at an increased rate. The increased susceptibility to infection resulting from corticosteroid administration appears to be secondary to the suppressed protective inflammatory response (Sinkford and Harris, 1964). Klotz and co-workers suggested that bacteremia might occur by topical prednisolone treatment in infected monkey pulps, especially in young permanent teeth with wide apexes (Klotz *et al.*,

1965). In rat models, prednisolone and triamcinolone acetonide produced poor responses when applied to the mechanically exposed pulps. Bacteremia was observed to be able to distribute widely throughout the necrotic pulp tissues with the presence of inflammation in the periapical tissues (Watts and Paterson, 1988).

Abbott discussed about the release of triamcinolone acetonide from Ledermix® which applied onto the dental pulp. It was initially rapid in the first day, then drastically diluted, and reduced once it entered the circulation. The released concentration appeared to be an extremely low possibility of producing systemic side-effects from intra-dental use of corticosteroids as pulp capping and cavity lining materials (Abbott, 1992). In the present study, the low concentrations of fluocinolone acetonide which were probably too low to induce any serious adverse effect were used. No evidence has been reported that fluocinolone acetonide enhances the spread of infection when used topically. However, *in vivo* study is needed to examine the side-effect of this material. In addition, the results from this study showed that fluocinolone acetonide stimulated dental pulp cell proliferation and type I collagen synthesis, which were involved in healing process. In vital pulp therapy, case selection is a crucial step for predictable outcome. An ideal case should be a mechanical exposure from operative procedure or, otherwise, an infected pulp has to be eradicated before application of pulp capping agent. In addition, sterilization has to be strictly controlled by using rubber dam and aseptic technique (Cotton, 1974; Cox *et al.*, 1985; Stanley, 1989). With proper clinical conditions, the problem of using topical fluocinolone acetonide would be minimized.

There were also some limitations in the present study. It was an *in vitro* experiment using human cultured pulp cells. The results obtained from this trial can only assess the efficacy of fluocinolone acetonide in the enhancement of pulpal healing process. The whole mechanisms of pulpal healing *in vivo* are more complex, involved both cellular and extracellular events. Due to the nature of experiment, the number of samples was limited. Thus, the results cannot be completely judged to total populations. The data supported that application of fluocinolone acetonide to a dental pulp might promote increased type I collagen synthesis, the major component of extracellular matrix in dentin-like structure. It may be efficient to promote early stage of pulpal healing and may be beneficial in short term pulpal treatment. Thus, it is probable that this substance may be developed as a new commercial effective pulp capping agent in the future. However, the induction of pulpal cell differentiation in this study is unclear. Other markers in hard tissue formation (such as alkaline phosphatase activity, osteopontin, osteocalcin) are needed to investigate for this purpose (Arceo *et al.*, 1991). Moreover, further *in vivo* investigations are needed before this material can be recommended for routine use in clinical practice.

In conclusion, fluocinolone acetonide could promote type I collagen synthesis of human dental pulp cells. In addition, the ability to form *in vitro* calcification in the presence of fluocinolone acetonide is comparable to the control. Regulating the collagen synthesis of pulp cells by fluocinolone acetonide could be crucial in the repair and regeneration of human dental pulp.