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The role of nickel accumulation and epithelial cell proliferation in orthodontic treatment-induced gingival overgrowth

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SUMMARY The aim of this study was to investigate the role of nickel in orthodontic treatment-induced gingival hyperplasia. The nickel concentration in gingival tissues with and without overgrowth, histopathology of gingival overgrowths, and epithelial cell proliferation response to different nickel concentrations were analysed. Ten patients receiving orthodontic therapy (eight females and two males, mean age 15.4 years) were included in the study. Hyperplastic and healthy gingiva samples were collected from the same patients. The amount of nickel in the gingival tissue samples was analysed using the atomic absorption spectrometry technique. The tissues removed from hyperplastic areas during gingivectomy were also used for histological analysis. To analyse the effect of nickel on epithelial cell proliferation, four different nickel concentrations (0.5, 2, 5, and 10 μg) were incubated with keratinocyte cells for 11 days. Mann–Whitney *U*-test, analysis of variance, and Tukey's test were used in the statistical analyses.

The results did not show any difference in nickel concentration between the study and control gingiva tissue samples, but histological analysis demonstrated an increase in epithelial thickness and a significant increase ($P = 0.031$, 0.02, 0.02) in epithelial cell proliferation in response to low-dose nickel concentrations, with a toxic response to a higher dose. In the limitations of this study, it is plausible that the effect of a continuing low-dose nickel release to epithelium is the initiating factor of gingival overgrowth induced by orthodontic treatment.

Introduction

Orthodontic treatment may initiate oral clinical manifestations, such as labial desquamation (Lindsten and Kurol, 1997), multiform erythema (Starkjaer and Menné, 1990), gingivitis (Shelley, 1981), and gingival enlargement (Bishara *et al.*, 1993; Genelhu *et al.*, 2005; Kouraki *et al.*, 2005). Such manifestations are usually associated with the inflammatory response induced by the corrosion of orthodontic appliances, and major emphasis has been placed on nickel (Eliades *et al.*, 2003). Inflammatory response to nickel is considered as type IV hypersensitivity and is manifested as nickel allergic contact stomatitis but its aetiology has not yet clearly been defined (Holmstrup, 1999; Vanarsdall, 2000).

Gingival enlargement is a more common sequela of orthodontic treatment than other manifestations (Genelhu *et al.*, 2005; Kouraki *et al.*, 2005). Fibrous gingival enlargements associated with fixed orthodontic appliances seem to be transitory, and it is generally thought that enlargement resolves after orthodontic therapy (Carranza, 1996). However, there are also studies reporting that this resolution is not complete (Ramadan, 2004). Orthodontic treatment-induced gingival overgrowth shows a specific fibrous and thickened gingival appearance, different from fragile gingiva with marginal gingival redness which is seen in allergic or inflammatory gingival lesions (Zachrisson and Zachrisson, 1972; Ramadan, 2004). However, there is no clear definition on its initiation and histopathology.

Nickel may activate monocytes and epithelial cells, suppressing or promoting the expression of intracellular adhesion molecule 1 by endothelial cells, mostly depending on its concentration (Wataha *et al.*, 1997, 1999). Nickel ions can also intracellularly accumulate in human oral mucosal cells and human HaCaT keratinocytes (Ermolli *et al.*, 2001; Faccioni *et al.*, 2003). Nickel concentrations which do not significantly modify oral epithelial cell viability and inflammatory cytokines release (<1.3 mM) can stimulate apoptosis *in vitro* (Trombetta *et al.*, 2005). On the contrary, human primary cultured keratinocytes and HaCaT cells also proliferate in response to nickel ions (Jia *et al.*, 1999). Nickel-containing orthodontic wires can reduce cell viability and stimulate apoptosis in three-dimensional cell culture models (Vande Vannet *et al.*, 2006).

Therefore, the aims of this study were to determine (1) the amount of nickel accumulation in gingival tissues with or without gingival overgrowth within the same group of orthodontic patients, (2) the histological pattern of orthodontic treatment-induced gingival overgrowth, and (3) the dose- and time-dependent effect of nickel on human keratinocyte cell proliferation *in vitro*.

Subjects and methods

Analysing nickel accumulation in gingival tissues of orthodontic patients with or without gingival hyperplasia in vivo

A detailed study plan was given to the patients and Cumhuriyet University ethical committee approval was obtained.

The subject group consisted of eight females and two males (mean age 15.4 years; range 13–19 years) who received fixed orthodontic therapy for a period of 2–4 years (mean 36.3 months). All had excellent oral hygiene. For each patient, orthodontic therapy consisted of maxillary molar bands (Convertible triple tube, 0.018 × 0.025 GAC International Inc., Bohemia, New York, USA) with edgewise triple buccal tubes and second and first premolar, canine, and lateral and central incisor direct-bonded brackets (Roth Generous brackets, GAC). In the mandible, the first molar bands had edgewise double-rectangular buccal tubes (Roncone tube, 0.018 × 0.025 GAC) with vertical hooks and first and second premolar, canine, and lateral and central incisor direct-bonded brackets (Roth Generous brackets, GAC). The maxillary teeth were levelled with continuous archwires, starting with 0.012-inch nickel–titanium and working up to 0.016 × 0.022-inch stainless steel (Sentalloy and stainless steel archwires, GAC).

The hyperplastic and healthy gingiva samples were collected from the same patients. Fibrous gingival enlargements [covering one-third to two-thirds of the clinical crown, gingival index <1 (Löe, 1967)] neighbouring teeth with orthodontic appliances with meticulous oral hygiene [plaque index <1 (Löe, 1967)] formed the study group. Gingiva covering the partially erupted mandibular third molars without inflammatory or fibrous enlargement formed the control group. Both study and control samples were collected using the gingivectomy technique. During sample collection, only gingiva from the hyperplastic area was initially collected, then gingivectomy was continued and gingivoplasty was performed to maintain an ideal gingival structure.

The tissue samples were stored at −80°C before being analysed. The atomic absorption spectrometry (AAS) technique was chosen as it permits analysis of metals in biological samples without any separation of the metal from its biological matrix (Kocadereli *et al.*, 2000). Before analysis the tissue samples were scaled; 1.5 ml HNO_3 + 0.25 ml H_2O_2 were added and acid digestion was performed. A final 2.5 ml volume was reached by the addition of distilled water. The amount of nickel in the gingival tissue samples was analysed with the electrothermal atomization technique (Stojanovic *et al.*, 2004).

Histological analysis of orthodontic treatment-related gingival hyperplasia

The tissues removed from the hyperplastic areas during gingivectomy were fixed in formalin and also used for histological analysis. Tissue samples embedded in paraffin blocks and tissue sections stained with haematoxylin and eosin were analysed under a light microscope. Histological analysis was performed at the Institute of Dentistry, Helsinki University, Finland.

The effect of different nickel concentrations on epithelial cell proliferation

HaCaT non-malignant human skin keratinocytes (provided by Hubert Fusenig, German Cancer Center, Heidelberg, Germany) were cultured in 96-well plates to 60–70 per cent confluence in Dulbecco's modified Eagle's medium containing 10 per cent foetal calf serum, 1 ml L-glutamine, and 100 μg penicillin G, in 5 per cent CO_2 at 37°C. The wells were rinsed twice with phosphate-buffered saline and fresh medium [containing four different Nickel (II) chloride hexahydrate (Sigma, Munich, Germany) concentrations (0.5, 2, 5, and 10 μg)] was placed in the wells. Cultures without nickel served as the control. For each different nickel concentration and the control group, four wells were prepared. Proliferation of the HaCaT cells was analysed on days 1, 3, 5, 7, 9, and 11 using Cell Titer 96 assay (Promega Corporation, Wisconsin, USA) with an ELISA plate reader at 490 OD.

Statistical analysis

All analyses were performed using version 13.0 of the Statistical Package for Social Sciences (SPSS Inc., Chicago, Illinois, USA). Nickel concentrations in gingival samples were compared between groups using the Mann–Whitney *U*-test. The number of viable epithelial cells for each nickel concentration and control group was compared using analysis of variance and differences were identified using Tukey's test. P values of <0.05 were accepted as statistically significant.

Results

Orthodontic treatment-induced gingival overgrowth showed firm and pink tissue enlargements with no tendency to bleeding. Histological examination of the gingiva showed a well-structured and thickened epithelium, with elongated very thick papillae inserted in fibrous connective tissues. The connective tissue showed thick collagen fibre bundles. Inflammatory cell infiltration was negligible (Figure 1). According to the AAS analysis, differences in gingival nickel amount were higher in the control (1.81 ± 1.20 μg/g) than in the study group (1.32 ± 1.47 μg/g), but there was no statistical difference ($P = 0.28$).

Four different nickel concentrations (0.5, 2, 5, and 10 μg) tested in the *in vitro* part of this study had varying effects on epithelial cell growth. A remarkable decrease in the number of living epithelial cells, starting after 24 hours and continuing during the whole experiment, was observed when 10 μg of nickel was added to the wells. The addition of 0.5 μg nickel

to each well did not affect the epithelial cell proliferation significantly on any day, while the addition of 2 µg nickel to each well resulted in slight changes in the number of viable epithelial cells, on days 1, 3, 7, and 11, but a significant increase on day 5 ($P = 0.031$). However, 5 µg of nickel added to each well decreased cell viability up to day 3 ($P = 0.033$), but thereafter the epithelial cell number increased remarkably until day 11 ($P = 0.00$). This increase was statistically significant on days 5 and 9 ($P = 0.00$; Figure 2).

Discussion

The aim of this study was to investigate the role of nickel in gingival hyperplasia induced by orthodontic treatment. The nickel accumulation in gingival tissues was analysed, the histopathology of gingival overgrowth was examined, and the epithelial cell proliferation in response to different nickel concentrations, *in vitro,* was studied.

Nickel, the most common metal used in orthodontic appliances, has been stated to be corrosive in the oral cavity (Jia *et al.*, 1999). An average release of 40 µg nickel per day from a stimulated full-mouth fixed appliance has been reported (Park and Shearer, 1983) and also nickel accumulation was found to be higher in dental plaque samples of patients receiving orthodontic therapy in comparison with untreated subjects (Fors and Persson, 2006). However, it is also suggested that the release of nickel is not necessarily proportional to the nickel content of the alloy (Grimsdottir *et al.*, 1992). Even though nickel is related to allergic response seen in orthodontic therapy (Holmstrup, 1999; Vanarsdall, 2000), its role in gingival overgrowth has not been studied in detail.

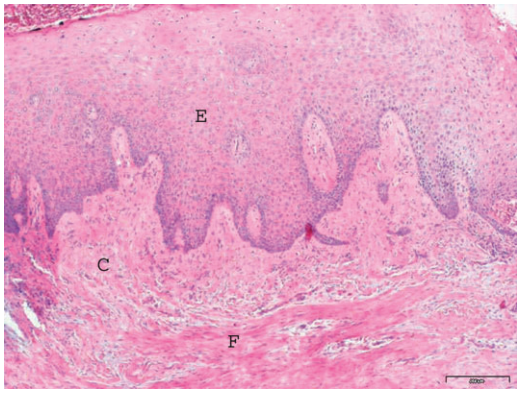

Figure 1 Histological view of the gingival overgrowth (magnification ×4). E = epithelium, C = connective tissue, F = fibre bundles. Scale bar = 200 µm.

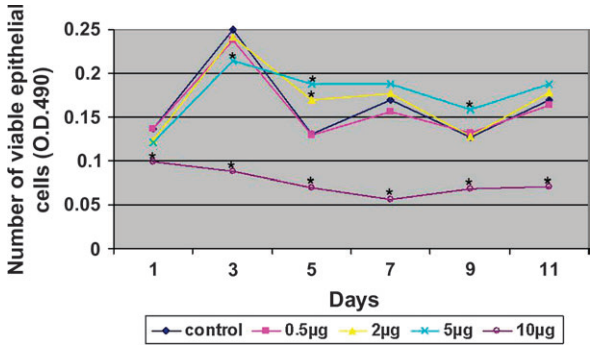

Figure 2 Effect of different nickel concentrations on the ability of keratinocyte cells to proliferate after 1–11 days of exposure. The values are the mean results of four separate measurements for each concentration ($P < 0.05$). Asterisks indicate a statistical difference with the control group.

The *in vivo* results of the present study did not show any significant difference in nickel accumulation between samples with or without gingival overgrowth. In contrast, Faccioni *et al.* (2003) reported relatively high nickel levels in the mucosa of patients undergoing orthodontic therapy. It could be possible that nickel accumulates in epithelium rather than connective tissue. The accumulation of nickel to human HaCaT keratinocytes has been reported previously, which supports this theory (Trombetta *et al.*, 2005). However, in the present study, as the analysed samples consisted of both epithelia and connective tissue, it is not possible to discuss the precise nickel accumulation in epithelial and connective tissues separately.

Histological analyses of the gingival samples of the patients with overgrowth showed a thickened epithelium with elongated very thick papillae inserted into fibrous connective tissue. All layers of the epithelium were thickened. Fibre bundles were also thickened in connective tissue. This shows that orthodontic therapy-induced gingival overgrowth includes a prominent epithelial proliferation response, supported by increased collagen fibre bundles, which is similar to several overgrowth types such as drug-related gingival enlargement (Carranza, 1996). Histological analysis of gingival overgrowth showed differences from chronic inflammatory gingival enlargements associated with plaque accumulation around orthodontic appliances, with a lack of exudative and proliferative characteristics of chronic inflammation, such as a preponderance of inflammatory cells and fluid with vascular engorgement, and new capillary formation (Carranza, 1996). Also, instead of clinically deep red, soft, easily bleeding lesions, which are common in inflammatory enlargements, orthodontic therapy-related gingival hyperplasia was thick and pink and showed no tendency to bleeding (Carranza, 1996).

To understand the effect of nickel on epithelial cells, an *in vitro* study incubating epithelial cells together with different nickel concentrations for a culture period of 11 days was also performed. The results showed that epithelial cells give different responses to different nickel concentrations. A nickel concentration of 10 µg showed a toxic effect on HaCaT cells, confirming the results of other studies reporting cytotoxicity of nickel on human cells (Ermolli *et al.*, 2001; Trombetta *et al.*, 2005). In contrast,

lower nickel concentrations increased epithelial cell proliferation. Nickel has the capability of upregulating the synthesis of several key stress proteins and keratinocyte growth factor expression in keratinocytes (Carroll and Wood, 2000; Marchese *et al.*, 2003). Marchese *et al.* (2003) showed that nickel ions induce an upregulation of the expression of keratinocyte growth factor receptors and, hence, proliferation of keratinocytes in culture. The increase in epithelial cell growth factors and their receptors may be an important factor for the increase in epithelial thickness during orthodontic treatment. The present study demonstrated that even the early epithelial cell response to nickel was a decrease in living cell numbers; later low doses of nickel initiated a proliferation in epithelial cell numbers, which proves that the epithelial cell response to nickel changes over time and in a dose-dependent manner.

Conclusions

The *in vivo* and *in vitro* results of this study suggest that low-dose continuing nickel release from orthodontic appliances might be the initiating factor for gingival overgrowth, as it has the capability of increasing epithelial cell proliferation.

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