Abstract: Magnetic attachments are commonly used for overdentures. The deleterious effects of exposure to magnetic flux on human health have not been substantiated so far; nevertheless, there is a need to understand the extent of magnetic field exposure in the oral area resulting from the use of magnetic attachments. The purpose of this study was to investigate the influence of a magnetic field on oral squamous cell carcinoma. Tumor cells cultured on a magnetic plate were compared with those not cultured on a magnetic plate (controls). The cells were seeded at a density of $1 \times 10^5$ cells/well and cultured for 6 days. The influence of the magnetic field on cytokine production was examined by cytokine array analysis. Secretion of platelet-derived growth factor-AA (PDGF-AA) was measured by enzyme-linked immunosorbent assay and Western blotting. The expression of PDGF-AA messenger RNA was examined by real-time polymerase chain reaction, whereas nuclear factor-kappa B activity was measured by luciferase assay. The results indicated that the magnetic field inhibited the secretion of PDGF-AA, thereby inhibiting PDGF-AA-induced expression, thus reducing the risk of cancer recurrence.

Keywords: platelet-derived growth factor-AA (PDGF-AA); nuclear factor-kappa B (NF-κB); magnetic attachments.

Introduction

In recent years, health appliances that utilize magnetic fields have become widespread; moreover, the areas of application of magnetic fields in living organisms are expanding (1). Magnetic fields produced by permanent magnets are classified into two types: static magnetic fields that are oriented in a certain direction; and variable magnetic fields, where the strength and direction change periodically, similar to an electromagnetic field.

Dental magnetic attachments are retentive devices that exert retentive force on a denture using a permanent magnet. Attempts to utilize magnets as retentive devices began in the 1950s. Samarium-cobalt (Sm-Co) magnets, which use rare earth elements, were invented in 1966, resulting in a dramatic improvement in the performance of magnets. Furthermore, from a structural viewpoint, rainproof measure and magnetic circuitry have been applied, along with the development of a magnetic shield that covers the periphery of the magnet with a magnetic stainless alloy in order to increase the magnetic force, thus...
forming a special structure called the magnet assembly. In addition to these improvements, neodymium-iron-boron (Nd-Fe-B) magnets, the most powerful of the rare earth magnets, are now used instead of Sm-Co magnets, with a magnetic attachment that was adopted as the international standard (ISO 13017) in 2012 (2).

It has been reported that the attraction of the magnetic assembly to the keeper of the magnetic attachment reduces external magnetic flux leakage to a large degree (3,4). However, some medical devices use permanent magnets to promote blood circulation and aid wound healing.

The effects of various magnetic fields on living organisms have been widely reported. However, few detailed studies have investigated the influence of magnetic fields on humans (5,6). Previous studies have evaluated the effects of magnetic fields on physiological phenomena, in the context of the growth and behavior of small animals. A statistical method to predict and infer the effects of these interactions has also been developed (7-9). Bassett et al. reported that a magnetic field was effective for promoting the healing of fractures, especially refractory fractures, and this had led to the clinical application of low-frequency magnetic fields in orthopedic surgery (10). In addition, Luben et al. have reported that a magnetic field enhances the proliferation and differentiation-promoting activities of osteoblasts. Subsequently, many studies have examined the effects of magnetic fields on osteoblasts (11). Nevertheless, the biological changes that occur when a magnetic field is applied to a living body have not been elucidated at the cellular level so far. Moreover, reports on cells other than osteoblasts have been limited. Therefore, the present study was conducted to investigate the effects of permanent magnets on oral squamous cell carcinoma (OSCC).

Materials and Methods

Cell culture

HSC-3 cells (an OSCC-derived cell line) were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 50 μg/mL streptomycin and 50 U/mL penicillin (10% FCS-RPMI1640).

Cell stimulation

The cells were plated at a density of 1 × 10⁵/35-mm dish and cultured for 24 h in a 5% CO₂ incubator. The cells were washed twice with 10% FCS-RPMI1640 and cultured on a magnetic plate (8 × 12 cm, Super Magnet Plate, Oz Bioscience, Marseille, France) having a static magnetic field strength of about 150 mT. Cells not cultured on a magnetic plate were used as controls.

Cytokine arrays

Culture supernatant was collected from the cells that had been cultured on the magnetic plate for 24 h. After centrifugation, the supernatant was subjected to cytokine array analysis using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA).

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed on culture supernatants from stimulated or unstimulated cells in order to estimate the concentration of platelet-derived growth factor-AA (PDGF-AA) using the PDGF-AA ELISA kit (R&D Systems). The absorbance was measured using a microplate reader model 3550 (Bio-Rad, Tokyo, Japan).

Western blotting

The cells were cultured in the magnetic field for 4 days, then washed twice with phosphate-buffered saline (PBS) and lysed in cell lysis buffer (500 μL; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.5% TritonX-100). The samples were centrifuged at 14,000 × g for 5 min and the supernatants were transferred to new tubes. Protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad); 100 μg of total protein was subjected to 10% SDS-PAGE. The separated protein was then transferred to an Immobilon membrane (Millipore, Tokyo, Japan). Rabbit anti-human PDGF-AA antibody (N-30, Santa Cruz, Dallas, TX, USA) and rabbit anti-human GAPDH antibody (FL-335, Santa Cruz) were diluted to 1000-fold with 1% BSA-PBST (0.1% Tween-20/PBS) and used as the primary antibodies. HRP-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research, West Grove, PA, USA) diluted to 10,000-fold with 1% BSA-PBST was used as the secondary antibody. The band was detected using Clarity Western ECL Substrate (Bio-Rad), and analyzed with ChemiDoc XRS (Bio-Rad).

RNA extraction and real-time polymerase chain reaction (PCR)

After culture with or without a magnetic field, the cells were washed once with PBS and total RNA was extracted using the RNeasy mini kit (Qiagen, Tokyo, Japan). RNA concentrations were measured using the Nano Drop ND1000 Spectrophotometer (Thermo Fisher Scientific, Yokohama, Japan). Real-time PCR was performed as described previously (12). The primers used in this study are listed in Table 1.
For NF-κB inhibition experiments, the cells were incubated with 25 mM L-1-4'-tosylamino-phenylethyl-chloro-methyl ketone (TPCK, Sigma-Aldrich, St. Louis, MO, USA) for 1 h in a CO₂ incubator. After preincubation, the cells were washed and further cultured for 24 h. The culture supernatants were then harvested and subjected to PDGF-AA ELISA.

**Luciferase assay**

NF-κB reporter plasmids (pNF-κB -Luc) were purchased from Agilent Technologies (Tokyo, Japan). The HSC-3 cells were plated in 6-well culture plates at a density of 5 × 10⁵ cells/well, washed twice with OPTI-MEM (Life Technologies, Carlsbad, CA, USA), and transfected with 500 ng of pNF-κB-Luc using the Lipofectamine transfection method (Thermo Fisher Scientific). After 5 h of transfection, the cells were washed with 10% FCS-RPMI and further cultured on a Super Magnet Plate for 24 h. After stimulation, the cells were lysed with 1 × passive lysis buffer (Promega, Tokyo, Japan) and the cell lysates were collected. Transfection efficiency was normalized to Renilla luciferase activity by co-transfection with the pRL/CMV vector (Promega). Both firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega).

**Table 1 PCR primers used in the experiments**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers</th>
<th>Genbank acc.No.</th>
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| β-actin   | F; 5'-GGAGCAAGTATCTTGATCTTC-3'  
R; 5'-CCTTCTCGCGATGAGTCCTG-3'  
NM_007493 |
| PDGF A    | F; 5'-GGCCTAAGCTGCGGGGAGGGGAAAATCCA-3'  
R;5'-CGGATGCAAGCTGCGGCTCAGGCGCT-3'  
NC_018918.2 |

**Fig. 1** Effect of exposure to a magnetic field on cell culture. Graph demonstrating the number of cells in the exposed and non-exposed (control) groups. Cells were seeded at a concentration of 1×10⁵ cells/well and cultured for 6 days, after which the number of cells was counted.

**Statistical processing**

Results were presented as mean ± standard deviation (SD) using the SPSS software package (v22; IBM Corp., Tokyo, Japan). Statistical significance was assessed using Mann-Whitney *U* test.

**Results**

**Effect of exposure to a magnetic field on cell proliferation**

HSC-3 cells were cultured while exposed to a magnetic field, or under normal conditions, and the proliferation rates were compared. The cells were seeded at a concentration of 1 × 10⁵ cells/well and cultured for 6 days. No significant difference was observed between the control group and the group exposed to the magnetic field (Fig. 1).

**Effects of the magnetic field on cytokine production**

The influence of the magnetic field on the cytokine production profile was examined using a cytokine array assay. Among the 36 cytokines examined, the secretion levels of PDGF-AA and suppression of tumorigenicity 2 (ST2) demonstrated significant reductions (Fig. 2). To
confirm these findings, PDGF-AA levels in the culture supernatants were measured by ELISA. No changes in PDGF-AA levels in the control and magnetic field groups were noted until day 3 of culture; however, on day 4, the levels were significantly reduced in the magnetic field group (758 pg/mL) relative to the control group (1,553 pg/mL), indicating approximately 50% inhibition (Fig. 3). Western blotting further confirmed the reduction in PDGF-AA (Fig. 4A). The band intensities in both groups were compared using National Institutes of Health (NIH) Image, and a 40% reduction was noted in the magnetic field group (Fig. 4B).

These results indicated that the production of PDGF-AA in the culture supernatant and cell lysate was inhibited by exposure to the magnetic field.

**Comparison of PDGF-AA gene expression by real-time PCR**

To examine whether the reductions in PDGF-AA production and secretion were attributable to transcriptional control of the gene, real-time PCR was performed. About 25% of the expression was inhibited in the group exposed to the magnetic field relative to the control group (Fig. 5).

**Effects of specific inhibitors of NF-κB on PDGF-AA production**

As expression of PDGF-AA mRNA is partly dependent on regulation of NF-κB, the cells were incubated in the presence or absence of its specific inhibitor, TPCK. The concentration of PDGF-AA in the culture supernatants was compared by ELISA. The cells cultured without TPCK showed a significant decrease in PDGF-AA levels compared to the control group (Fig. 6).
TPCK secreted 96 pg/mL PDGF-AA, whereas the TPCK-treated cells secreted only 54 pg/mL PDGF-AA, indicating a reduction of ~50% in the control group (Fig. 6).

Influence of exposure to a magnetic field on NF-κB transcriptional activity

A 30% reduction in NF-κB activity was noted in the group exposed to the magnetic field, relative to the control group (Fig. 7).

Discussion

An overdenture with a magnetic attachment has many advantages, such as resistance of the denture to detachment, high esthetics, and a low burden on the teeth. Magnetic attachments are useful not only in prosthodontics but also for maxillofacial prosthetics (3,13-19). Despite several case reports on the use of magnetic attachments, the influence of permanent magnets on living cells remains unclear.

In the present study, the effect of a magnetic field on cells was observed by exposing them to the Super Magnet Plate, a permanent magnet with a static field strength similar that of the largest GIGAUSS D1000 magnet assembly (GC, Tokyo, Japan) in the magnetic attachment GIGAUSS series. When the magnet assembly attaches to the keeper in the oral region, it creates a closed magnetic circuit, reducing any leakage of magnetic flux into the surroundings (20). The present experiments were conducted assuming that the maximum magnetic effect would be produced while using only the magnet assembly for the magnetic attachment. The induction of PDGF-AA at both the mRNA and protein levels was suppressed in the group exposed to the magnetic field.

According to Raylman et al., the cell proliferation rate is slowed in the presence of a magnetic field (21); on the other hand, Hiraoka et al. demonstrated no effects of magnetic field exposure on cell proliferation (22). In the present study, the time course of cell proliferation was confirmed from day 1 to day 6, and no difference in proliferation rates was observed between the exposed and non-exposed groups. In subsequent experiments, the culture conditions and numbers of seeded cells were maintained at the same levels in both groups.

Next, differences in the induction of various cytokines in the group exposed to the magnetic field and the non-exposed control group were evaluated by cytokine array analysis. Among the 36 cytokines examined, PDGF-AA and ST2 demonstrated significant reductions in secretion levels. PDGF-AA is a growth factor secreted by platelets and smooth muscle cells. Shikada et al. reported that despite being a growth factor, PDGF-AA exerts mild effects on the proliferation and migration of smooth muscle cells; alternatively, it controls the expression of the angiogenic factor, vascular endothelial growth factor (VEGF) (23). On the other hand, ST2 is the transmembrane receptor of interleukin-33; however, due to limited information about its mechanism of action, we focused on PDGF-AA, which has been studied extensively in the past.

PDGF-AA levels were quantified and compared using ELISA and Western blotting, respectively. The production of PDGF-AA was decreased in the exposed group relative to the control group. Further analysis performed by real-time PCR revealed a similar pattern, indicating that the induction of PDGF-AA is regulated at the transcriptional level. Liburdy et al. reported that the cell membrane ion channel system was affected by a magnetic field, causing a change in the movement of ions (24). Furthermore, Nakamura et al. demonstrated that DNA polymerase performs a nucleotide transfer reaction through a mechanism that requires two Mg$^{2+}$ ions. A change in ion balance caused by a magnetic field may thus affect DNA synthesis (25). Exposure of gastric cancer to a static magnetic field increased superoxide dismutase (SOD) activity in the cells (26). Similarly, the proliferation of fibroblasts in bleomycin-induced pulmonary fibrosis is thought to be due to increased release of PDGF-A from alveolar macrophages. In a study by Tamagawa et al., decreased development of pulmonary fibrosis in mice treated with SOD was attributed to suppressed expression of PDGF-A mRNA (27).
Therefore, in the present study, exposure to a magnetic field may have increased SOD activity within the cells and suppressed the production of PDGF-A. The fact that PDGF-A was not formed indicates that PDGF-AA was suppressed due to lack of dimer formation.

Based on these results, we investigated the presence of inhibitory sites in order to ascertain the effect of a magnetic field on signal transduction pathways. Shionome et al. reported that OSCC constantly activates the transcription factor NF-κB (28). Although PDGF-AA does not have an NF-κB binding site in the regulatory region, the action of PDGF-AA was observed by suppression of NF-κB using TPCK, a specific NF-κB-inhibitor. The inhibitor assay was performed in the absence of a magnetic field, following which PDGF-AA protein production was found to be suppressed. According to Aizawa et al., the NF-κB p50 subunit cooperatively activates the PDGF-A chain promoter via protein-protein interaction with Krüppel-like factor 5 (KLF5), suggesting that NF-κB is involved in the production of PDGF-AA (29). Based on this assumption, the cells were transfected with NF-κB reporter plasmids, and the two groups in the present study were compared using the luciferase assay. Transcription activity was decreased in the group exposed to the magnetic field, suggesting that the latter inhibited NF-κB, thereby inhibiting PDGF-AA production.

Magnetic attachments are often used in patients with maxillofacial prostheses. PDGF-AA is an autocrine regulator of VEGF involved in tumor angiogenesis and metastasis, and is reported to be a switch for angiogenesis in malignant tumors. In other words, the suppression of PDGF-AA by a magnetic field demonstrated in the present study may represent a promising avenue for prevention of tumor recurrence.

Conflict of interest
The authors have no conflict of interest to declare.

References


