務形態や歯科用修復物の作製業務形態が替わりつつある。また審美に優れたポーセレン，結晶化ガラスから，強度に優れたジルコニアまで多様な歯科用セラミックスが利用できる時代になった。さらに，接着性モノマーや接着前処理法が開発され，セラミック修復物の適用が拡大している。従来のセラミック修復物は歯科技工士にとっても難易度が高く製作の手間がかかるものであった。国民が求める安全で快適な適正な価格の歯科用修復物を提供するためには，CAD/CAM システムを利用したオールセラミック修復物の導入が不可欠である。本論文で紹介したように，まだまだ改良すべき点や慎重に臨床経過を見なければいけないことはあるが，現時点での臨床術式と技工術式を遵守すれば日常臨床に安心して導入できる。

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Guidelines for Clinical Application of Ceramic Prostheses Fabricated by the Dental CAD/CAM System

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Abstract

While porcelain is accepted as an esthetically satisfactory and biologically safe material, in application, it usually requires fusion to a metal restoration due to its brittle quality. Additionally, application of conventional all-ceramic restorations has not achieved popularity, because of difficulties in processing and risk of fracture. However, densely sintered, polycrystalline materials with higher fracture toughness such as alumina and zirconia have been used recently as framework materials for all-ceramic restorations as a result of CAD/CAM technology. In this study, we investigated the potential benefits and challenges associated with the application of CAD/CAM technology for fabricating such frameworks, as well as related clinical procedures, including abutment preparations and luting restorations. We propose a set of clinical and laboratory guidelines aimed at facilitating the application of all-ceramic restorations fabricated with a CAD/CAM system in routine practice. Close observation of clinical prognoses will lead to further refinement of the guidelines herein proposed.

Key words: CAD/CAM, Ceramics, Prostheses, Zirconia, Digitizing

別添３－Ｉ－１

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（分担）研究報告書

歯科医療を取り巻く業務形態のあり方に関する研究
歯科技工士教育の現状と問題点・補綴治療の今後の動向

研究分担者　二川　浩樹・三浦　宏之

研究要旨

今日の歯科医療を取り巻く環境の変化は、歯科医師の業務のみならず、歯科衛生士、歯科技工士の業務にまで大きな影響を及ぼしている。特に、歯科技工士が担っている補綴物などの製作においては、新材料・新素材の開発やCAD/CAMシステムの普及など、その製作技法においても変革期を迎えていることは想像に難くない。しかし、日本における歯科技工の業務形態は旧態依然とした状況にあるだけでなく、超高齢化社会を迎え、技工作業そのものを行う若い労働人口の減少も顕在化している。　平成21年度の本研究において、現在の歯科技工業務における問題点を調査し、他業種における改善例などと照らし合わせた対応策を検討している。続く平成22年度には、これからの技工作業に必要な新技術や新素材に対応できる知識の整理を行い、歯科技工教育の入口の部分から転換を図り、より魅力ある仕事となるようなモデルケースを提示する。

A. 研究目的

今日の歯科保険医療の中で、その治療技術の一端を担っているはずの歯科技工士を取り巻く環境の変化はあまりにも大きい。従来ハンドメイドによって作製されてきた修復物や補綴物が、歯科用CAD/CAMシステムによる省力化、充填材料や接着材料の進歩による技工士を介さない直接修復の増加、さらには、予防中心の診療形態への移行など、これまでの歯科技工における業務形態から変化を求められている。また、厚生労働省発表の平成18年保健・衛生行政業務報告（衛生行政報告例）結果（就業医療関係者）の概況をみても、歯科技工士における20代の就業人数は20年前に比べ半分以下となっており、超高齢者社会を迎えた現在、歯科技工士の世界においても労働者の高齢化が進んでいる。一方で、こうした旧態依然の業務形態からの脱却を計ろうにも、2006年歯科技工士実態調査報告（日本歯技）にもあるように、勤務者の総人数が1～5人と少人数ですべての仕事をこなしている技工所が全体の36%もあり、日々の作業に追われてしまう現状を考えると容易ではなく、さらに、その方法の模索も十分にされていない。また、技工作業の効率化を目指して開発された歯科用CAD/CAMシステムも、本来の効率面での優位を示したものはまだ少ない。そこで本研究では、歯科だけにとらわれず、他業種で同様の課題を抱えつつも改善が図れたケースや、諸外国の先進的な事例等を参考としつつ、今後の歯科保健医療の変化、さらには社会の変化に対応した、将来における歯科技工業務形態のあり方に関してモデル的ケースを提示することを目的とする。

B．研究方法

本研究では、今後の歯科保健医療の変化、さらには社会の変化に対応した、将来における歯科技工業務形態のあり方に関してモデル的ケースを提示することを目的としている。その中でも技工士教育の問題点を克服するため、また今後の人材育成に向け、広島大学では平成17年4月に全国で初めて4年制の歯科技工士養成機関として口腔保健学科口腔保健工学講座が新設された．口腔保健工学講座は，歯工連携および歯科医療の進歩あるいは研究に対応できる人材育成と将来の歯科医療・歯科技工を切り開けるパイオニアの育成を目指し，平成21年には広島大学大学院医歯薬学総合研究科の中に，口腔健康科学専攻

（修士課程）が設置された。さらに，本年4 月には口腔健康科学専攻（博士課程後期）も設置され，口腔工学分野を新たに確立すべき教育者・研究者の人材育成を行う場が整った。近年，超高齢化社会の到来に伴い歯科医療現場では，疾病構造の変化とともに求められる歯科医療も大きく変化している。さらに医学・歯科医学の分野では日進月歩の技術革新により，いわゆる差し歯，入れ歯やブリッジという昔ながらの技工主体の治療に加えて，接着技法などのような材料の研究・進歩に基づく MI を中心とした歯科医療へと変化し，さらにインプラント治療も一般化し，生物学主体の歯科医療に変化しつつある．また，研究室では，分子生物学を基盤とした再生医療が大きく期待を集めており，間葉系幹細胞を応用した歯槽骨の再生はすでに臨床応用されている．研究室レベルでは iPS 細胞・ES 細胞を用いた研究も隆盛を極めており，歯の再生もあと数年で現実化されそうな勢いであり，まさに BioDentistry の幕開け期であるといえる。

この一方で，CAD/CAM に代表される歯工連携も，発展しつつあり Rapid Prototyping によるデジタルデータの臨床応用や，デジタルデータからシミュレーションモデルを構築する研究も行われており，今後患者のデジタルデータを活かした検査・診断・治療・予後予測など Digital Dentistry の展開が期待される。

このように 80 年代には夢とされてきたような歯科医療が，研究の技術革新によりもうそこまできていると言っても過言ではない．そのような中，将来，再生医療などの BioDentistry を担っていける歯科技工士の育成，あるいは工学的な知識を基に技工物や培養システムをデザインしていける，すなわちデジタルデータをベースにした Digital Dentistry 実践できる人材の育成を目指し広島大学歯学部口腔工学専攻では，【高度なスキルの習得】【Digital Dentistry】【BioDentistry】という大きな3 つの枠組みによる，スキル&テクノロジー（テクノロジーには Digital Dentistry と BioDentistry が含まれている）という観点からの学生教育を行った。

C．研究結果

【１高度なスキル教育】

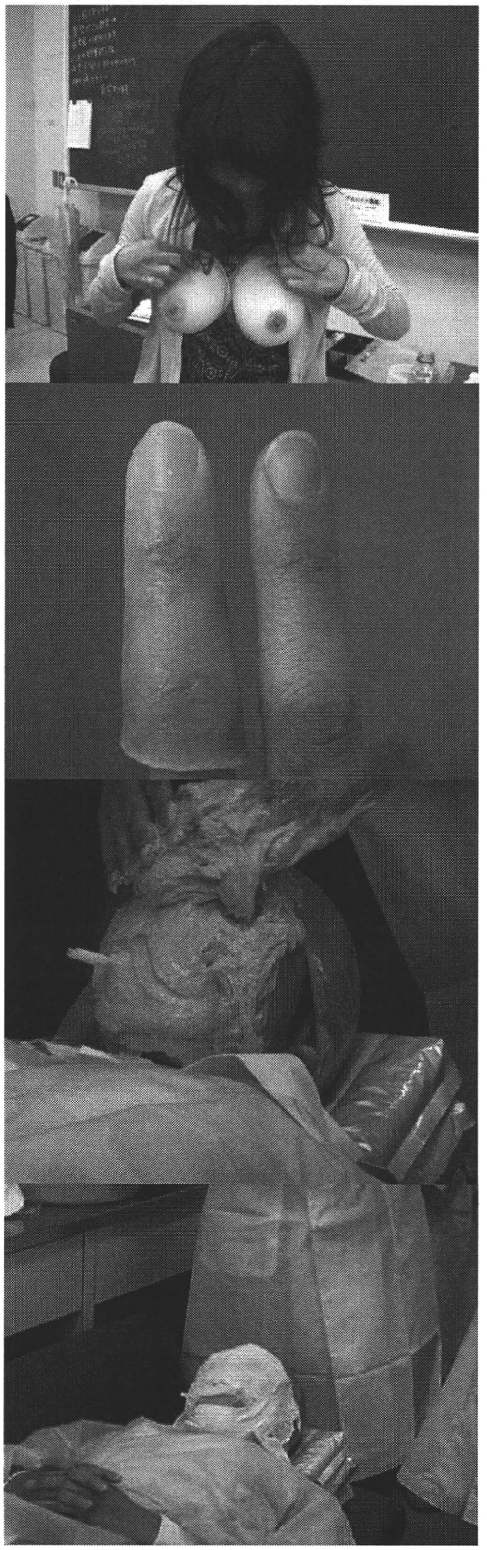
1-1 口腔インプラント実習

口腔インプラント実習では，学生はフィクスチャーの埋入から行い，インプラントの構造および生物学的な意義を含めて理解し，さらに上部構造の製作に当たって留意すべき生物学的事象と工学・材料学的事象そして技工上の事象の 3 つの観点から実践する。

1-2 人工ボディ製作実習

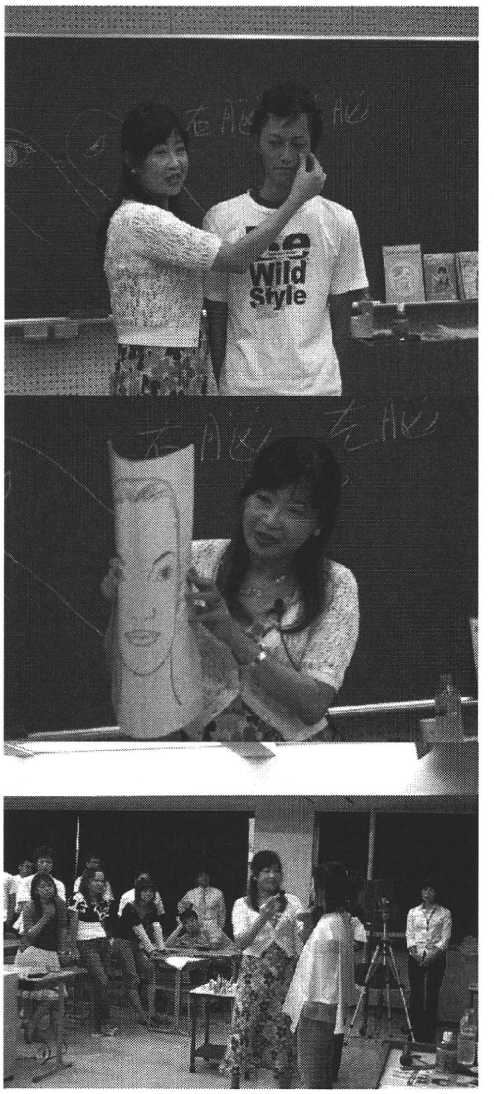
本実習では，実際に乳がんや手指切除後に使用されている人工乳房，指（小指・く

すり指の欠損は広島では他地域に比べて需要が高い)などを作製するスキルを身につける,と同時に患者の社会復帰や生活面でのQOLの向上に貢献を行うことを学ぶ.(人工乳房は,実際に患者が温泉やプールで使用しても他人に気づかれないようなクオリティをもって作製する).



1-3 夏季特別実習 フェイシャルセラピストベーシック検定講座

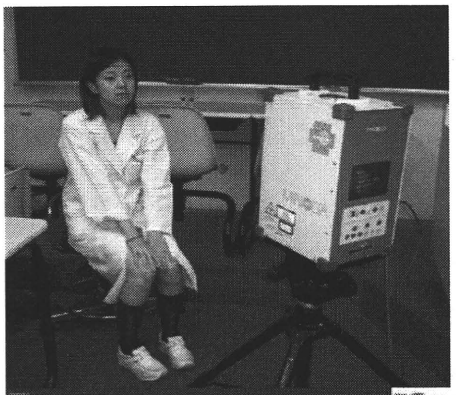
本実習では顎顔面領域の機能的・審美的な回復を工学的・生物学的知識と技術に基づき臨床を展開できるためのスキルの習得とともに,リハビリメイクを施すことで患者の心理面での社会復帰を支援するための技能を身につける.180分×12回の予定で夏休み期間に開講し,フェイシャルセラピストベーシック検定の資格を取得する.

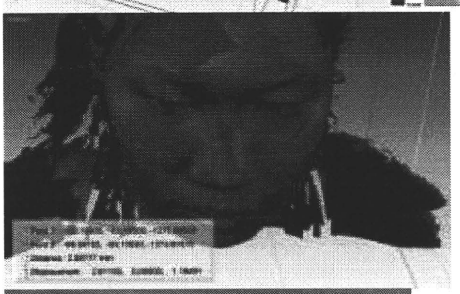


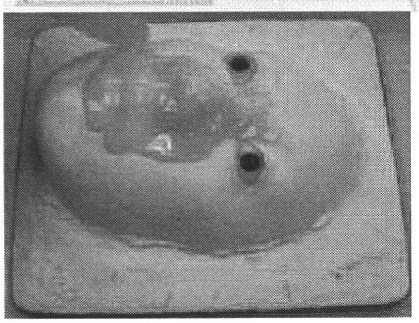
【2 Digital Dentistry】

2-1 CADシステム工学実習

デジタルデータを応用し,顎顔面補綴を行ったり,顎変形症のオペ支援システムの構築が可能となる。





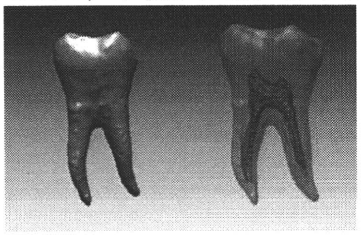


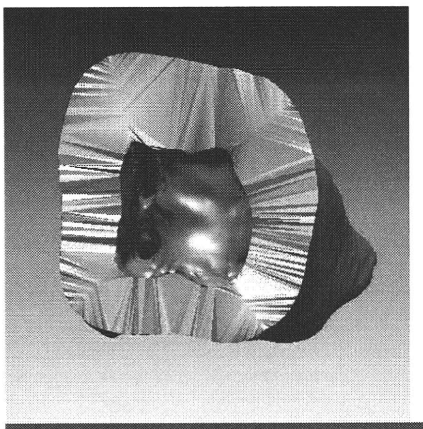
2-2 臨床実習

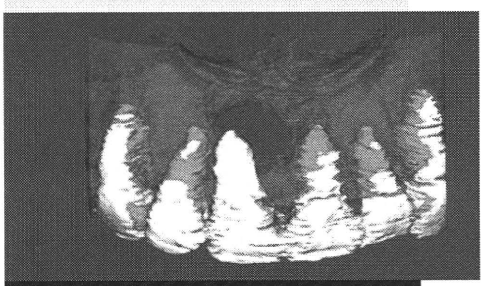
臨床実習では，各診療科のケースに対しての３次元モデルの作成を行う。

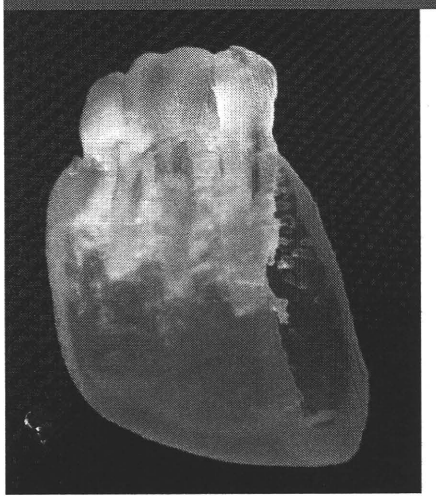
A 歯内療法

デジタルデータとして，歯根の形態，歯髄の位置だけでなく根管孔の確認や根尖病巣の状態確認も可能となり，さらに３Dモデルとして，実際の模型として確認できるため，予後が非常によくなる。



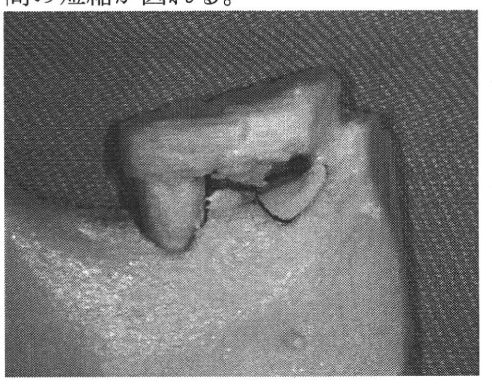


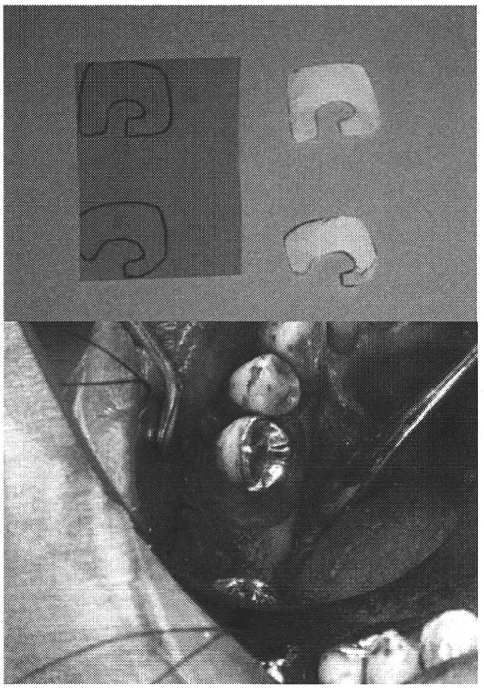




B 歯周治療

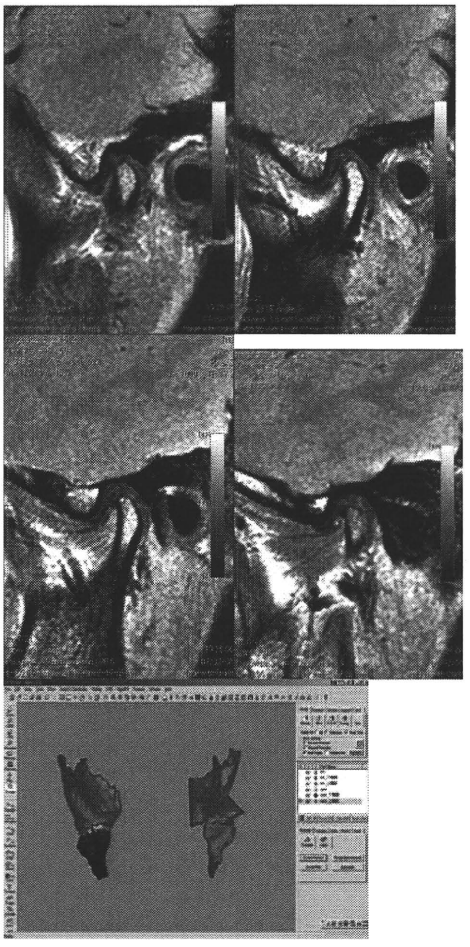
歯周オペに先立ち，オペ支援模型を作成することで，GTR や GBR に用いるメンブレンの適合が飛躍的に改善される。このオペ支援模型により，予後の向上，オペ時間の短縮が図れる。

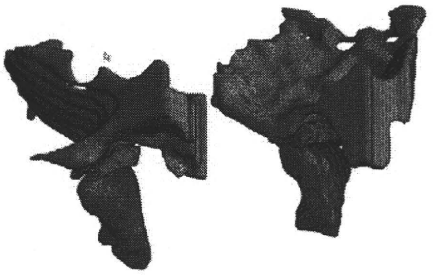




C 歯科補綴

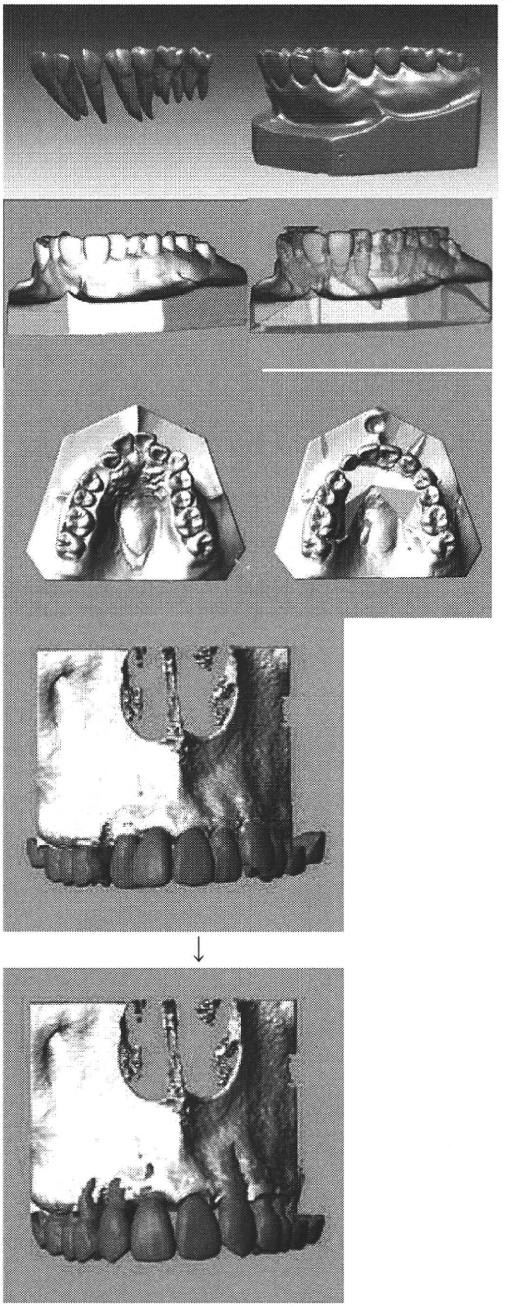
歯科補綴分野では，顎関節の MRI 画像から，ポリゴンモデルの 3 次元構築を行い，顎関節のバイオメカニクスシミュレーションモデルの構築を行っている。





D 歯科矯正学

歯科矯正学では，患者の診断用模型から取り込んだデータと，CBCT の歯根も含めた歯牙データを重ね合わせた後，矯正治療のシミュレーションを行うことで治療におけるリスクの提示を可能にしている。



【メディカルデザイン工学】

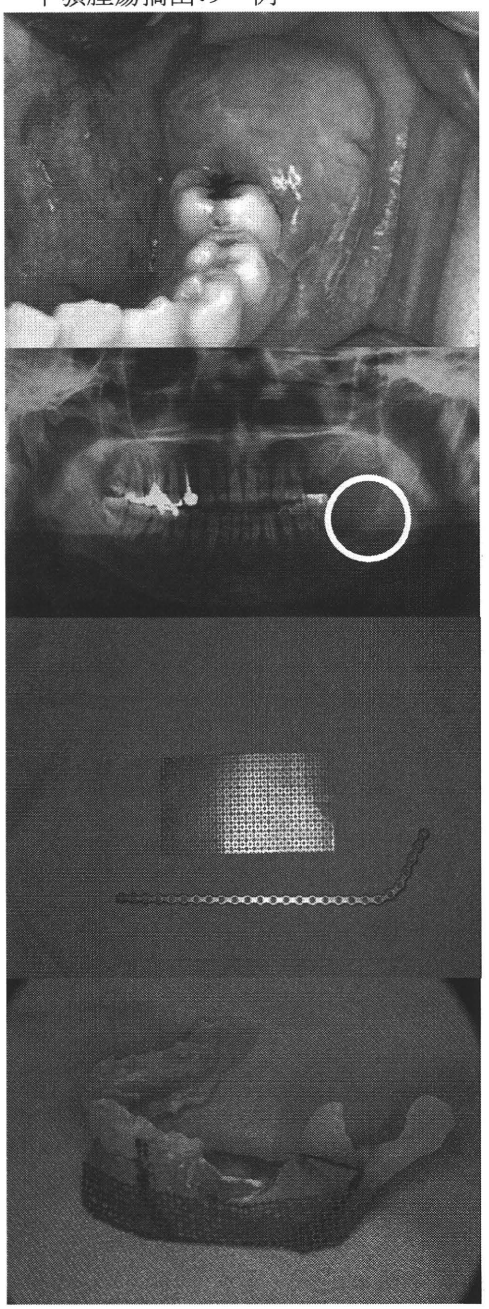
クリーンベンチ5基を使用し，日本組織培養学会の組織培養士の認定資格のための実習と微生物培養実習．

前者の資格の取得は 4 年次のキャリア活動に有利に働くだけでなく，培養のスキルを用いて様々な研究を展開できるメリットがある．

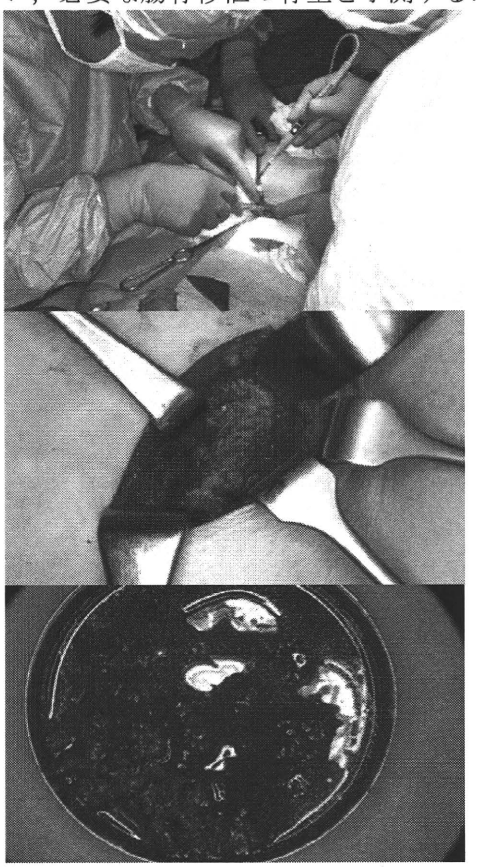
E 口腔外科における Ope 支援モデル

口腔外科のオペ前にオペ支援モデルの作成およびチタンプテートなどの屈曲を行うことで，より高い予後とオペ時間の短縮による患者の負担軽減を実現している。

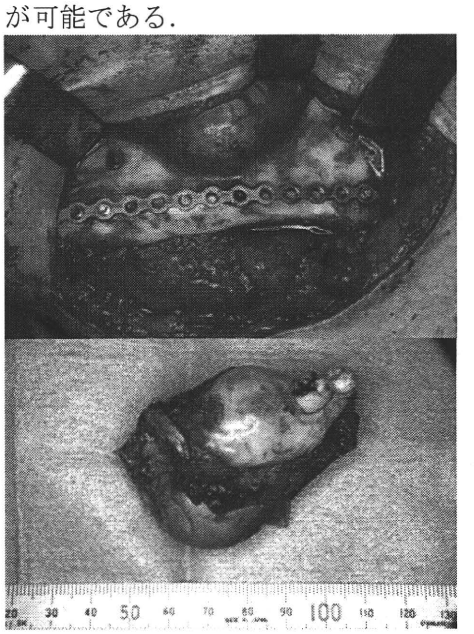
下顎腫瘍摘出の一例



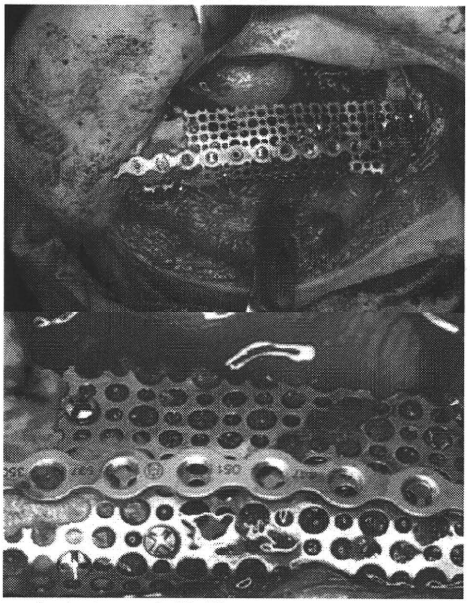
患者の CT データから 3 次元構築し，STL データとして Rapid Prototyping により作成した Ope 支援モデル上でチタンプレートとチタンメッシュを理想的な形態にあらかじめ屈曲し，Ope に備える．また，RP モデル上でワックスアップを行い，必要な腸骨移植の骨量を予測する．



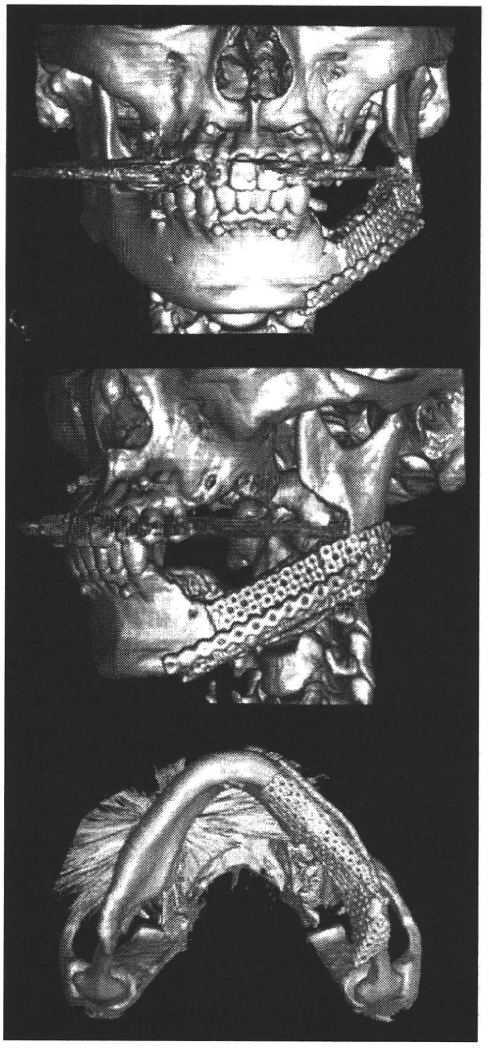
腸骨採取時には，あらかじめ採取量が予測されているので，過剰な侵襲を防ぐことが可能である．



次にチタンプレート・メッシュを試適後に，腫瘍の摘出を行う．



あらかじめ準備しておいたチタンメッシュをチタンプレートにて顎骨にねじ止めを行う．

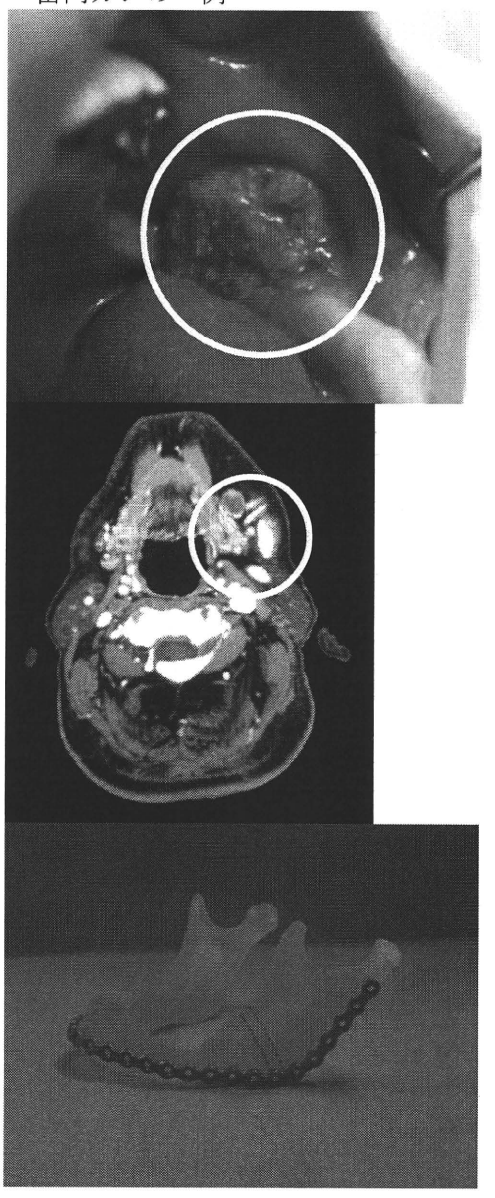


RP による Ope 支援モデルは，口腔外科医より CT データを預かって口腔工学学生が 3 次元構築→RP を行い，チタンプレートおよびメッシュの屈曲は主治医の指示の下で学生が，ディスカッションしながら製作を行う．

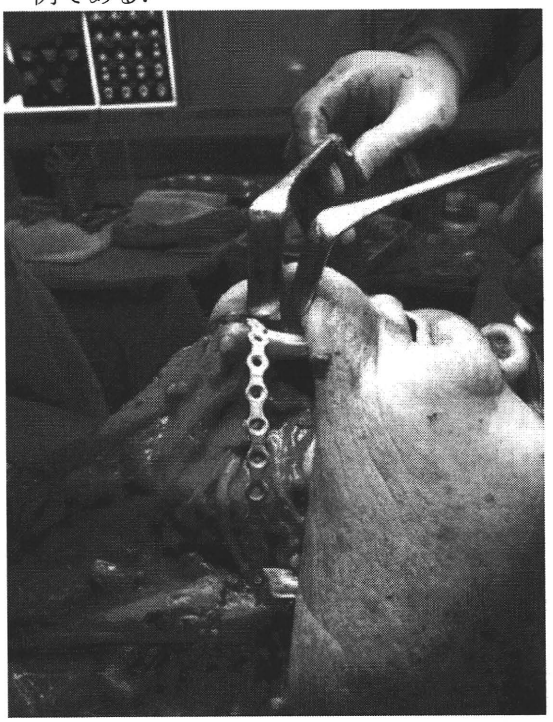
このような Ope 支援システムによって，口腔外科の施術時間は平均で 2 時間短縮できており，患者にとっても Doctor にとっても，非常に大きなメリットがあり，今後，国民医療には欠くことができないのではないかと考えている．

しかしながら，歯科診療では CT は保険では認められておらず，RP モデルの作製は，無論算定すらできないのが，現状である．

歯肉ガンの一例



同様に，あらかじめ下顎の歯肉ガンの患者に対して，Ope 支援モデルを活用した一例である．



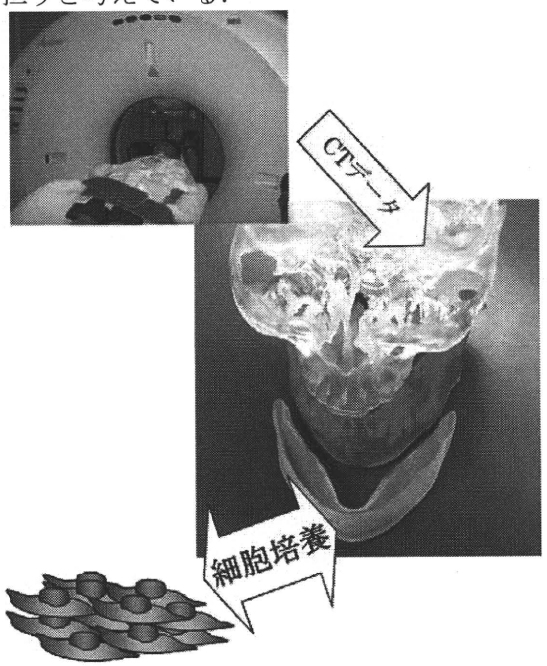
【リハビリメイクと再生医療の必要性とその将来】

2 例目の症例のように，口腔ガンの摘出は可能である。しかしながら，現在の歯科医療では顔貌の回復は望めず，また，義歯などによる咀嚼の改善にも限界がある。女性患者の多くは Ope 後に自分の顔を見て「どうして殺してくれなかったのか」と泣き崩れることも経験する。現在の歯科医療のシステムでは，このような患者の社会復帰の支援に関するものは含まれていないのである．

口腔工学では，リハビリメイクの実習を取り入れており，Ope 後の患者のリハビリメイクによる支援に加えて，心理的なリハビリにも取り組めるような歯科技工士の育成を行っている．

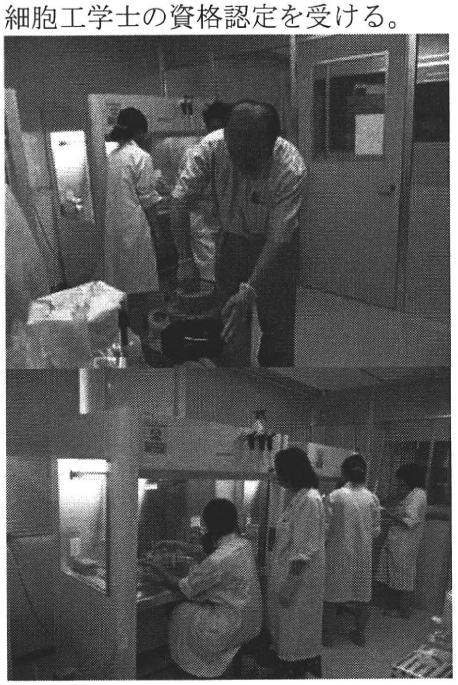
さらに，将来的には（おそらく 15~20 年先には），CT データから得られた，患者固有の顎骨の形態と同じ，スキャフォールドを RP によって構築し，歯科医師が患者から採取した幹細胞を，歯科技工士が増殖させ，さらに骨への分化を行い，その骨組織を技工物のように歯科医院に納入し，歯科医師が患者に移植することが歯科医療の将来像として望まれる．

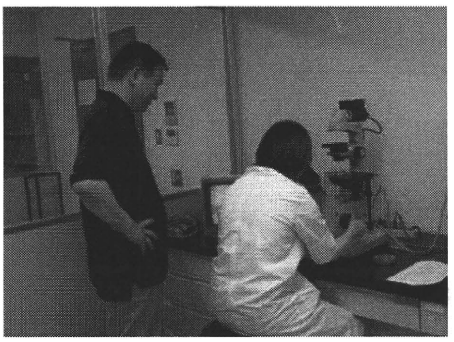
口腔工学では，学生のほとんどが，日本組織培養学会の認定資格である細胞工学士の資格を取得しているが，将来の再生医療における細胞の品質管理と組織再生を担うと考えている．



【３BioDentistry】

組織培養実習は，夏期特別実習として開講するが，細胞工学士コースⅠの修了証が与えられる。その後，細胞培養士を目指すものは，卒業研究のテーマに細胞培養を手技として用いる研究テーマを選び，日本組織培養学会の会員となりコースⅡおよびコースⅢを受講し，審査を受けることで，細胞工学士の資格認定を受ける。



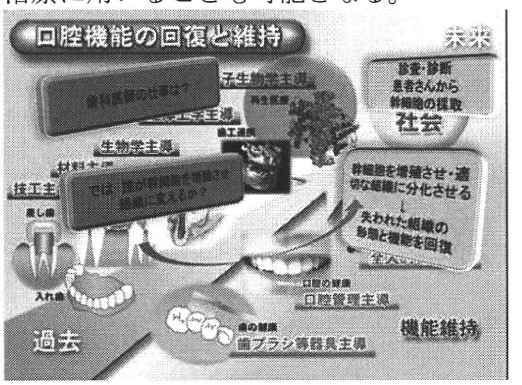


D．考察

2008 年に日本口腔検査学会が設立され，また，2009 年 3 月には日本歯科 CAD/CAM 学会が設立された。今後，歯科治療に際しての検査・診断に関しては，①シミュレーションを用いた形態学的な・機能学的な面からの予後予測，②微生物学的な側面からの予後予測，③血液生化学検査などからの全身的因子にかかわる予後予測，④個体としての遺伝子多型（SNPS）や表現形（Phenotype）からの診断および予後予測など多くの検査を基盤としてより予知性の高い歯科臨床システムの開発を行うことで，本当の意味でのオーダーメイド医療が提供できると考えている。

加療・処置に関しては，事前の CT/MRI データに基づく Ope あるいは加療支援システム，光学印象による感染リスクの低い歯科診療，さらには CAD/CAM による技工システムの拡充が望まれる。特に，修復物を入れる以前に，例えば国民全員が 20 歳で自分の顎口腔系の CT データを撮影し，データベースを構築しておくことによって，仮に 20 年先に歯科治療を受け，ブリッジを入れることになっても，20 歳の頃の歯列データをもとに理想的なブリッジは製作可能であり，歯科医師も現場で，20 年間の咬耗量だけを調整すればよい補綴物が作製可能になり，補綴治療の失敗による咬合崩壊を防ぐことができる。さらには，このようなデータベースの構築は，法歯学や犯罪捜査の観点からも有用なデータベースとなる。

再生医療に関しては，例えば腸骨移植や歯槽骨再建，サイナスリフトなどに対して，患者の口腔粘膜を綿棒で擦過し，得られた頬粘膜細胞を iPS 細胞へと変換することで，非常に低侵襲での再生医療のための幹細胞採取が可能となる。この細胞を増殖させ，必要な組織に分化させた後に，Ope を行う。また，患者個々の iPS 細胞をバンクとして保存しておけば，多くの疾病の治療に用いることも可能となる。



特に，医療として再生医療が具現化する時には，歯科医師の仕事は，おそらく「患者さんから細胞を採取する」ということになるであろう。では，「誰が，その細胞を増殖させ，適切な組織に分化させる」のであろうか？工学部や理学部出身者ではなく，医療人として口腔生物学や，歯の形態，顎機能学など必要な歯科医学を学び，そしてさらに患者さんに対する倫理を学んだ人材がその任に当たるべきではないのだろうか？と考えている。そのための人材育成である。

E．結論

このように，IT および分子生物学の両者，すなわち Digital Dentistry と BioDentistry の両方を理解し，使いこなすことで，より精度の高い検査・診断システム，より予知性が高く，より予後の良い歯科医療を提供できると考えている。

これらを踏まえて，歯科技工士の業務範囲の見直しや歯科技工士法の見直し，あるいは新たな歯科医療職種などが必要ではないかと考える。

F．健康危険情報

なし

G．研究発表

1．　論文発表

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2) Makihira S, Mine Y, Nikawa H, Shuto T, Iwata S, Hosokawa R,

Kamoi K, Okazaki S, Yamaguchi Y. Titanium ion induces necrosis and sensitivity to lipopolysaccharide in gingival epithelial-like cells. Toxicology in Vitro 2010; 24: 1905–1910.
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4) Makihira S, Mine Y, Nikawa H, Shuto T, Kosaka E, Sugiyama M, Hosokawa R. Immobilized-OPG-Fc on a tita-nium surface inhibits RANKL-dependent osteoclast differentiation in vitro. J Mater Sci: Mater Med 2010; 21: 647–653.
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1) 二川浩樹. 歯科技工士教育の新しい展望. 日本歯科チタン学会学術大会公開市民講座（鶴見）2011 年 2 月

H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

資料１

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Titanium ion induces necrosis and sensitivity to lipopolysaccharide in gingival epithelial-like cells

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ABSTRACT

Gingival epithelial-like cells (GE-1) were cultured and used to examine the cellular responses of gingival tissues to varying concentrations of titanium (Ti) ions. Titanium ions at concentrations of more than 13 ppm significantly decreased the viability of GE-1 cells and increased LDH release from the cells into the supernatant, but had no significant effect on their caspase 3 activity. These data suggest that a high concentration of Ti ions induced necrosis of the GE-1 cells. Titanium ions at a concentration of 5 ppm significantly increased the level of CCL2 mRNA expression in GE-1 cells exposed to lipopolysaccharide derived from *Porphyromonas gingivalis* in a synergistic manner. Moreover, the mRNA expression levels of TLR-4 and ICAM-1 in GE-1 cells loaded with Ti ions at 9 ppm were significantly enhanced as compared with those in GE-1 cells without Ti stimulation. We suggest that Ti ions are in part responsible for monocyte infiltration in the oral cavity by elevating the sensitivity of gingival epithelial cells to microorganisms. Taken together, these data indicate that Ti ions may be involved in cytotoxicity and inflammation at the interfaces of dental implants and gingival tissue.

1. Introduction

Titanium (Ti) is extensively utilized for numerous medical applications because of its excellent corrosion resistance, mechanical properties and biocompatibility (Wataha, 1996; Long and Rack, 1998). However, Ti ions have been detected in the fibrous membranes encapsulating implants and in synovial fluid (Dorr et al., 1990). The facilitation of osteoclast differentiation by Ti particles has also been reported (Bi et al., 2001). Moreover, clinical studies have shown that Ti particles released during the aseptic loss of orthopedic implants accumulate in the tissues (Jacobs et al., 1998; Gallo et al., 2002). Recently, we revealed that Ti ions had biological and/or adverse effects on the expressions of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) in osteoblastic cells (Mine et al., 2010). It is well known that the RANKL–RANK pathway and OPG, an antagonist of RANKL function, are essential for the differentiation and development of osteoclasts, which cause pathologic bone resorption (Koide et al., 2003). The corrosion resistance of Ti is decreased under low dissolved-oxygen conditions such as in the oral cavity, particularly in the presence of small amounts of fluoride (Nakagawa et al., 2002). Taken together, these findings imply that released Ti ions play a pivotal role in bone resorption at the interface of bone and dental implants (Bi et al., 2001; Dorr et al., 1990; Gallo et al., 2002; Jacobs et al., 1998; Mine et al., 2010).

Peri-implantitis accompanies inflamed gingival tissues and is often followed by bone resorption (Lang et al., 2000). We reported that the expressions of RANKL and OPG mRNAs in gingival epithelial-like cells were not changed when the cells were exposed to Ti ions (1–9 ppm) (Mine et al., 2010). However, an *in vitro* experiment showed that Ti ions significantly enhanced the inflammatory cytokine production of macrophages (Taira et al., 2006). In addition, toll-like receptors (TLR), including −4 and −9, increased in the interface membrane around loosening total hip replacement implants, where Ti particles may exist (Takagi et al., 2007). TLRs can be receptors for microbial organisms and endogenous ligands (Kaisho and Akira, 2000; Beutler, 2002). These data indicate that Ti ions, together with other outer stimuli like microorganisms or their products such as lipopolysaccharide (LPS), alter the physio-

Abbreviation: Ti, titanium; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; TLR, toll-like receptor; LPS, lipopolysaccharide; LDH, lactate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction; *Pg*, *Porphyromonas gingivalis*; ICP, inductively coupled plasma spectroscopy; EGF, epidermal growth factor; PBS, phosphate-buffered saline; ICAM-1, intercellular adhesion molecule 1; CCL2, chemokine (C–C motif) ligand 2.

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logical function of gingival epithelial cells. Therefore, there is a possibility that Ti ions released from dental implants may be involved in, or facilitate the inflammation of gingival tissues with peri-implantitis,

Hence, in the present study, we investigated the effects of Ti ions on the viability of gingival epithelial-like cells using the MTS assay, the lactate dehydrogenase (LDH) release assay, and the caspase 3 activity assay. We also determined the mRNA levels of receptors for microbes and cytokines related to inflammation and bone resorption in the presence or absence of LPS derived from *Porphyromonas gingivalis* using the real-time reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Preparation of Ti ions and P. gingivalis LPS

A Ti standard solution for inductively coupled plasma spectroscopy (ICP) was purchased from Merck (Darmstadt, Germany). For the experiments, the Ti–ICP standard solution was diluted with cell culture medium, under pH monitoring, according to the method described by Taira et al. (2006). Neither a significant change in the pH of the medium, nor visual precipitations accompanying the supplementation of Ti ions were observed.

P. gingivalis LPS (*Pg*-LPS) was purchased from InvivoGen (San Diego, CA, USA); LPS was diluted with sterile endotoxin-free water and stored at −20 °C until use.

2.2. Culture of GE-1 cells

The GE-1 cell line was obtained from the RIKEN Bioresource Center Cell Bank (Tsukuba, Japan). This cell line was established from temperature-sensitive SV40 large T-antigen gene transgenic C57BL/6 mouse gingival epithelium (Hatakeyama et al., 2001). GE-1 cells were cultured and maintained in SFM-101 culture medium (Nissui, Tokyo, Japan) supplemented with 1% fetal bovine serum and 10 ng/ml epidermal growth factor (EGF) (Sigma–Aldrich, Tokyo, Japan). GE-1 cells were seeded onto 96- or 24-well plates at a density of 4.0×10^3 and 2.5×10^4 cells/well, respectively. In addition, GE-1 cells were inoculated on pure titanium discs at a density of 2.5×10^4 cells/well. The Ti discs were set on the bottom of a 24-well plate. Pure wrought Ti (cp-titan) discs (JIS, Japan Industrial Specification H 4600, 99.9 mass% titanium, 15 mm diameter; Kobelco, Kobe, Japan) were purchased; the diameter of these cylindrical-shaped Ti discs fitted within the wells of a standard 24-well tissue culture plate. GE-1 cells were maintained at 33 °C under 5% CO_2/95% air for each experiment.

2.3. MTS assay of GE-1 cells

GE-1 cells were seeded onto 96-well plates, maintained until confluent, and then exposed to Ti ions (1–19 ppm) for 6 h. Suspended cells were removed by gentle rinsing with phosphate-buffered saline (PBS), and the number of adherent cells remaining in each well was quantified using a coupled enzymatic assay which resulted in the conversion of a tetrazolium salt into a red formazan product (MTS assay, CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA). The absorbance at 490 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA) (Makihira et al., 1999).

2.4. LDH release assay of GE-1 cells

GE-1 cells inoculated onto 96-well plates at a density of 4.0×10^3 were maintained until confluent. At confluence, the medium was changed with fresh culture medium and then exposed to Ti ions (1–19 ppm) for 6 h. LDH released into the culture supernatant during the 6 h incubation was isolated from the cultured GE-1 cells and measured using an LDH release assay kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega). Absorbance of the developed colorimetric substance in a 96-well plate was measured at 490 nm using the microplate reader.

2.5. Measurement of caspase 3 activity in GE-1 cells

Caspase 3 activity was measured using a kit (CaspACE Assay System; Promega) that detects caspase 3-mediated specific cleavage at the C-terminal side of the aspartate (D) residue in the sequence DEVD (DEVDase activity). GE-1 cells were cultured in 96-well plates in the presence or absence of Ti ions (1–19 ppm) for 6 h. Cells were solubilized with a cell lysis buffer on ice for 15 min, and three repeats of a freeze/thaw cycle. The amount of caspase 3-specific DEVDase activity in the extracted sample was determined by monitoring the release of free chromophore from substrate (OD 405 nm) using the microplate reader.

2.6. Real-time quantitative RT-PCR

Total RNA from each treatment group was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from total RNA (100 ng) using ReverTra Ace (Toyobo, Osaka, Japan). The cDNA was then amplified by BIOTAQ DNA polymerase (Bioline, Randolph, MA, USA). Real-time quantitative RT-PCR analyses for TLR-2, TLR-4, intercellular adhesion molecule-1 (ICAM-1), chemokine (C–C motif) ligand 2 (CCL2), RANKL, OPG and β-actin were performed using Rotor-Gene™ 6000 (Qiagen, Tokyo, Japan). β-actin was chosen as an internal control to standardize the variability in amplification owing to slight differences in starting total RNA concentrations. Sequences of primers and probes used in the present study are listed in Table 1. The sequences of primers and probes for β-actin have been described previously (Mine et al., 2010).

2.7. Data analysis

The MTS and LDH release assays were repeated as triplicate independent experiments. Caspase 3 activity assays were repeated as duplicates independent experiments. Data represent the means ± standard deviations (SD) of samples assayed in triplicate. Real-time quantitative RT-PCR analyses for the expression levels of CCL2, RANKL, and OPG mRNAs were repeated as duplicate or triplicate experiments. Data were normalized to the expression

Table 1
Primers and probes used for real-time RT-PCR.

Target name	Primer and probe	Sequence (5′–3′)
CCL2	Primer F	ATGCAGGTCCCTGTCATGC
	Primer R	CATCTTGCTGGTGAATGAGTAGC
	Probe	6FAM-TCTGGGCCTGCTGTTCACAGTTGC-TAMRA
TLR-2	Primer F	GGCTTCACTTCTCTGCTTTTCG
	Primer R	AGATCCAGAAGAGCCAAAGAGC
	Probe	6FAM-TCTGGAGCATCCGAATTGCATCACCG-TAMRA
TLR-4	Primer F	CTTCTCCTGCCTGACACCAG
	Primer R	GGACTTTGCTGAGTTTCTGATCC
	Probe	6FAM-AGCTTGAATCCCTGCATAGAGGTAGTTCCT-TAMRA
ICAM-1	Primer F	AGATCCTGGAGACGCAGAGG
	Primer R	ACTGTGGGCTTCACACTTCAC
	Probe 6	FAM-ACTTGGCTCCCTTCCGAGACCTCCAG-TAMRA

Note. Forward primers (Primer F), reverse primers (Primer R) and probes are listed.

levels of β-actin mRNA, which was the internal control. Data represent the means ± SD of samples assayed in triplicate. Differences between average values for groups were subjected to a one-way analysis-of-variance (ANOVA) and Tukey's multiple range tests. Significance was achieved at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Effects of Ti ions on cell viability

The results of the MTS assays showed that Ti ions at 1–11 ppm had no significant effects on the viability of the GE-1 cells (Fig. 1A), while Ti ions at 13–20 ppm significantly decreased the viability of the GE-1 cells as compared with unloaded controls (ANOVA; $p < 0.05$, 0.01 *vs.* control; Fig. 1A). The LDH release assay (Fig. 1B) was used to determine whether Ti ions caused GE-1 necrotic cell death. The results of the LDH release assay revealed that Ti ions at concentrations higher than 11 ppm significantly increased LDH release from the GE-1 cells into the extracellular medium (ANOVA; $p < 0.05$, 0.01 *vs.* control; Fig. 1B). To examine whether Ti ions caused GE-1 apoptotic cell death, the activity of caspase 3 was measured in cells exposed to Ti ion stimulation. Ti ions at 1–19 ppm had no significant effect on the caspase 3 activity of the GE-1 cells (ANOVA; $p > 0.05$; Fig. 1C). The results from the three cell toxicity assays indicated that Ti ions were non-toxic at concentrations of less than 9 ppm. Therefore, a concentration of Ti ions that did not reduce cell viability (less than 9 ppm) was used for the following experiments.

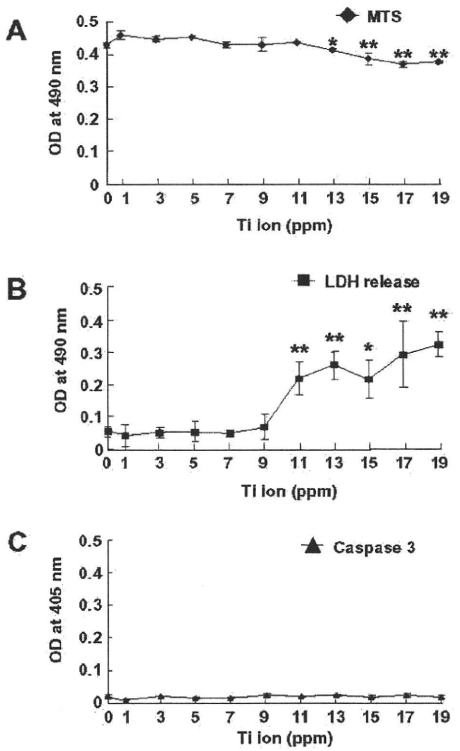

Fig. 1. The effects of Ti ions on the cell viabilities of GE-1 cells were assessed by the MTS assay, by measuring LDH release from the cells into the supernatant, and by measuring caspase 3 activity in the cells. When the cells reached confluence they were exposed to Ti ion (1–19 ppm) stimulation for 6 h. Each supernatant containing LDH released from the cells was collected. After gentle rinsing of the cells with PBS, MTS assays were carried out. The content of LDH released from the cells into the extracellular medium was measured using a LDH release assay kit. In an independent experiment measuring caspase 3 activity, confluent cells were prepared and exposed to Ti ion stimulation for 6 h. The activity of caspase 3 in the cells was measured using a caspase 3 assay kit. Independent experiments for the MTS and LDH release assays were repeated three times. Independent experiments for the caspase 3 activity assay were performed twice. Data represent the means ± SD of triplicate samples. * $p < 0.05$, ** $p < 0.01$.

3.2. Morphological observations of GE-1 cells exposed to Ti ions

When GE-1 cells were cultured with Ti ions at concentrations of 1, 5, and 9 ppm for 6 h, there were no morphological changes compared with the control (data not shown). However, a slight change was observed in GE-1 cells stimulated with 5 and 9 ppm Ti ions (Fig. 2); the borders of cells loaded with 5 and 9 ppm Ti ions became unclear compared with cells treated with 1 ppm Ti or the controls.

3.3. Effects of Ti ions on the mRNA levels of CCL2, RANKL, and OPG in GE-1 cells in the presence of Pg-LPS

The mRNA expression profiles of CCL2, RANKL, and OPG in GE-1 cells were investigated when the cells were exposed to Ti ions and/or *Pg*-LPS.

Pg-LPS (5–25 μg/ml) significantly increased the level of CCL2 mRNA compared with the control (ANOVA; $p < 0.01$). The enhanced level of CCL2 mRNA induced by *Pg*-LPS peaked at 10 μg/ml (Fig. 3A). The level of expression of RANKL mRNA was significantly increased by 10 μg/ml *Pg*-LPS in comparison to the levels at 0, 5, 15, 20, and 25 μg/ml *Pg*-LPS (ANOVA; $p < 0.01$; Fig. 3A). *Pg*-LPS had no significant effect on the abundance of OPG mRNA (ANOVA; $p > 0.05$; Fig. 3A). Similarly, Ti ion (2–9 ppm) stimulation had no significant effect on the mRNA levels of CCL2, RANKL, and OPG (ANOVA; $p > 0.05$; Fig. 3A).

Pg-LPS alone (10 μg/ml) significantly increased the levels of expression of CCL2 and RANKL mRNAs (ANOVA; $p < 0.01$, *vs.* control and Ti alone; Fig. 3B). The addition of Ti ions synergistically elevated the enhanced level of CCL2 mRNA in GE-1 cells stimulated by *Pg*-LPS (Fig. 3B). There were no significant changes in the abundances of RANKL mRNA in GE-1 cells loaded with *Pg*-LPS alone and *Pg*-LPS plus Ti ions, or in the levels of OPG mRNA in GE-1 cells treated with *Pg*-LPS and/or Ti ions (Fig. 3B).

3.4. Comparison of the levels of CCL2 mRNA in GE-1 cells cultured on a plastic dish compared with a Ti disc

CCL2 mRNA in GE-1 cells was investigated to examine the differences in the cellular response to *Pg*-LPS in GE-1 cells culture on a standard plastic culture dish or on a pure Ti disc. *Pg*-LPS increased the abundance of CCL2 mRNA in GE-1 cells cultured on a plastic dish and a Ti disc, compared with their respective controls; however, these increases in mRNA levels were not significant (ANOVA; $p > 0.05$; Fig. 4).

3.5. Effects of Ti ions on the mRNA levels of TLR-2, TLR-4, and ICAM-1 in GE-1 cells

To examine the cellular responses in the membrane proteins of GE-1 cells when recognizing microorganisms in the presence of Ti ions ranging from 1 to 9 ppm, the mRNA levels of TLR-2, TLR-4, and ICAM-1 were analyzed by real-time RT-PCR. In the present study, Ti ions at a concentration of 9 ppm significantly enhanced the levels of expression of TLR-4 (Fig. 5, closed square) and ICAM-1 mRNAs (Fig. 5, closed triangle) (ANOVA; $p < 0.05$, *vs.* control), while concentrations of 1 and 5 ppm Ti ions did not increase these levels

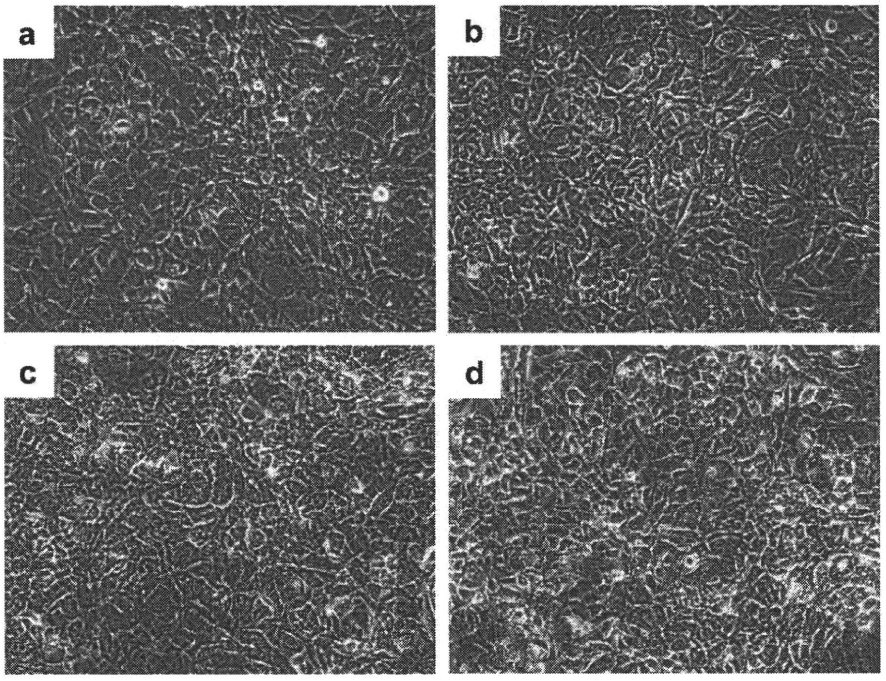

Fig. 2. Phase contrast microscopic observations of GE-1 cells cultured in the presence of Ti ions: (a) control, (b) 1 ppm of Ti ions, (c) 5 ppm, (d) 9 ppm) for 24 h (magnification: 100×.

(Fig. 5). Exposure of cells to Ti ions (1, 5, and 9 ppm) did not alter the mRNA level of TLR-2 (Fig. 5, closed circle) in GE-1 cells.

4. Discussion

Titanium ions and/or particles from Ti implants directly and indirectly induce cell death and bone resorption, even though Ti is regarded as an excellent biocompatible metal (Bi et al., 2001; Mine et al., 2010; Sommer et al., 2005; Vamanu et al., 2008). Exposure of primary osteoblast cells derived from rat calvaria to Ti ions at 10 ppm and higher for 24 h was reported to be toxic (Liao et al., 1999). Similarly, two-day exposure to 1 ppm Ti reduced the viability of RAW264.7 cells to approximately 60% as compared with control cells (Taira et al., 2006). Therefore, Ti ions or particles may be involved in the prognosis of implants through bone remodeling. It is generally believed that in the oral cavity, gingival epithelia are constitutively exposed to outer stimuli including oral bacteria. In the present study, to investigate the effects of Ti ions and oral bacteria on gingival epithelial cells, the GE-1 cell line and LPS derived from *P. gingivalis* were used. The GE-1 cell line was established from temperature-sensitive SV40 large T-antigen gene transgenic C57BL/6 mouse gingival epithelium, and possesses the ability to undergo keratinization (Hatakeyama et al., 2001). The present study revealed that 6 h exposure to Ti ions at concentrations over 13 ppm significantly decreased the viability of GE-1 cells. This was thought to be due to the induction of necrosis, since an enhancement of LDH release from GE-1 cells to the supernatant was observed by the LDH release assay, and no change in caspase 3 activity was detected in the cells (Fig. 1B and C). These results suggest that ionized Ti may be incorporated by GE-1 cells and behave as an inducer of necrosis in the cells at concentrations of greater than 11 ppm; a Ti disc which was not ionized had no effect on the growth of GE-1 (data not shown). However, the mechanism by which the Ti ions induce necrosis in GE-1 cells remains unclear.

In a previous study, we revealed that a 24 h exposure of GE-1 cells to Ti ions had no effect on cell viability (as measured by the MTS assay), or on the mRNA expression levels of RANKL and OPG (Mine et al., 2010). In the present study, 9 ppm of Ti ions were not cytotoxic to GE-1 cells after a 6-h incubation, as indicated by the MTS, LDH and caspase 3 assays, but the borders of cells exposed to 9 ppm Ti ions for 24 h became unclear (Fig. 2). This phenomenon suggests that cellular responses were induced by Ti ions at less than 9 ppm within 24 h, although the effects on cellular viability appeared to be negligible. Thus, we chose three concentrations of Ti ions, 1, 5, and 9 ppm, to examine the cellular response of GE-1 cells to Ti ions within 24 h (Mine et al., 2010). We hypothesized that Ti ions at concentrations which do not affect cell viability may modulate the sensitivity of gingival epithelial cells to oral bacteria, since it is reported that TLRs recognizing bacteria were increased in the interface membrane around loosening total hip replacement implants (Takagi et al., 2007).

The emergence of peri-implantitis has been observed as a problem since the oral implant system, initially proposed by a Swedish group, was established (Mombelli and Lang, 1998). Peri-implantitis, periodontal diseases, and the periodontopathic bacteria *P. gingivalis*, have frequently been detected around and on dental implants (Quirynen et al., 2002). It is noteworthy that *Pg*-LPS is involved in bone resorption and inflammation in gingival tissues in periodontitis (Baker et al., 2000). Similarly, *Pg*-LPS may be involved in the pathological condition of peri-implantitis. The ratio of RANKL to OPG is related to bone resorption (Grimaud et al., 2003). Previously, we reported that GE-1 cells express RANKL and OPG, and that Ti concentrations of 1, 5 and 9 ppm did not induce expression of these proteins (Mine et al., 2010). However, in the present study, *Pg*-LPS increased RANKL mRNA levels in GE-1 cells, while 5 ppm Ti ions alone did not. Synergistically enhanced

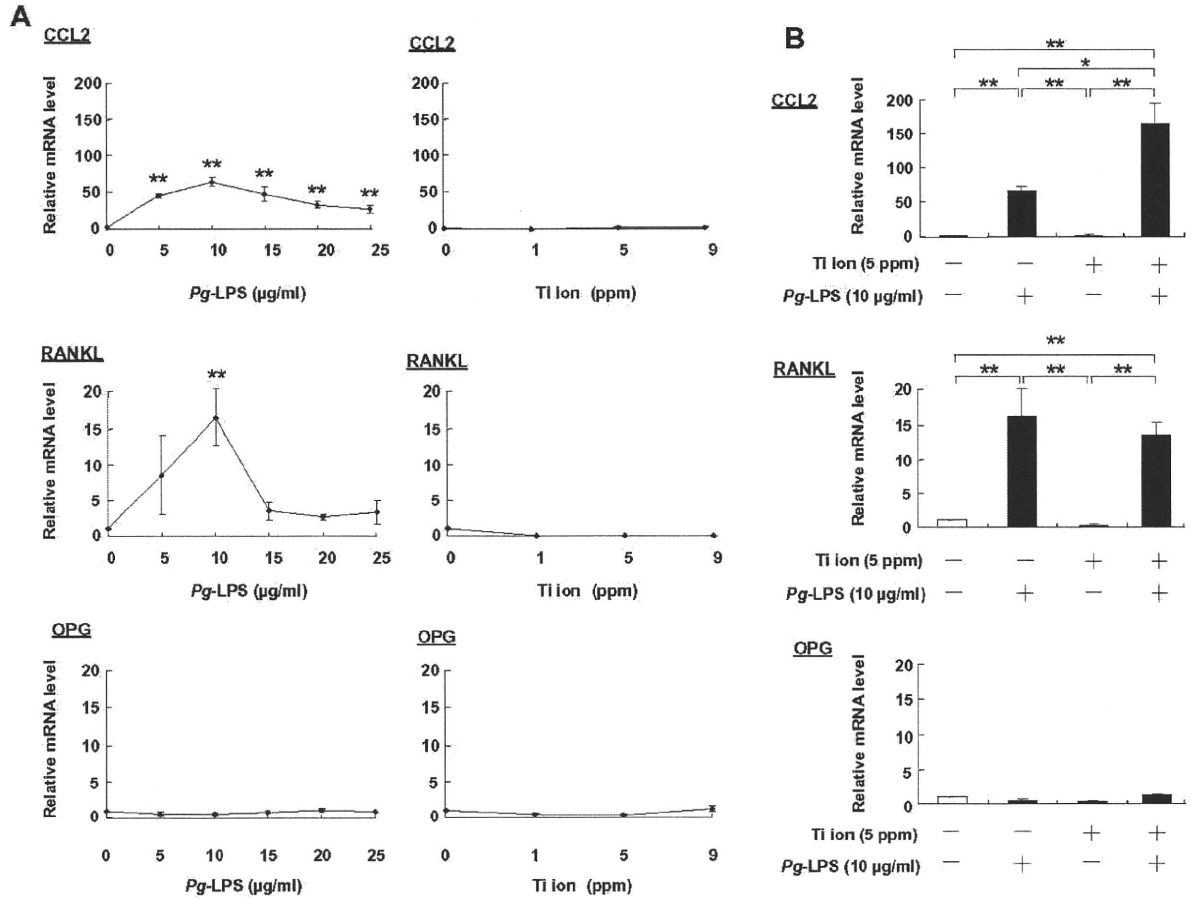

Fig. 3. The effects of Ti ions (5 ppm) and/or *Pg*-LPS (10 μg/ml) on the mRNA levels of the genes encoding CCL2, RANKL, and OPG in GE-1 cells were examined by real-time quantitative RT-PCR (A and B). Once confluent, the cells were exposed to *Pg*-LPS (5–25 μg/ml), Ti ions (1–9 ppm), or *Pg*-LPS (10 μg/ml) with/without Ti ion (5 ppm) stimulation for 6 h, and total RNA was isolated for analysis. The data from real-time quantitative RT-PCR analyses of CCL2, RANKL, and OPG mRNA levels were normalized to the expression levels of β-actin mRNA. Independent experiments were repeated three times. Data represent the means ± SD of triplicate samples. * $p < 0.05$, ** $p < 0.01$.

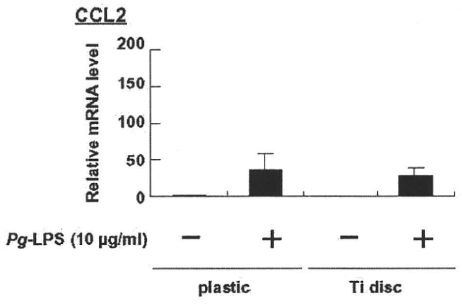

Fig. 4. The effects of *Pg*-LPS on the mRNA expression levels of CCL2 in GE-1 cells cultured on a plastic dish and a Ti disc were examined by real-time quantitative RT-PCR. At confluence, the cells were incubated with or without *Pg*-LPS (10 μg/ml) for 6 h, and total RNA was isolated for analysis. The data from real-time quantitative RT-PCR analyses of the levels of CCL2 mRNA were normalized to the expression levels of β-actin mRNA. Independent experiments were repeated two times. Data represent the means ± SD of triplicate samples.

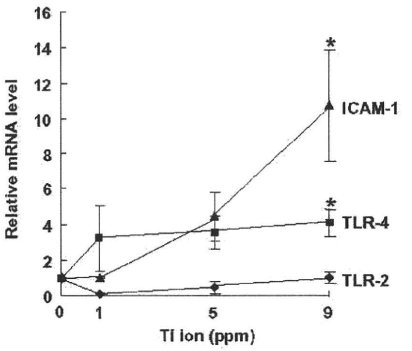

Fig. 5. The effects of Ti ions on the levels of expression of TLR-2, TLR-4, and ICAM-1 mRNAs in GE-1 cells were analyzed by real-time quantitative RT-PCR. Confluent cells were exposed to Ti ion stimulation at the indicated concentrations for 6 h prior to isolation of total RNA. The data from real-time quantitative RT-PCR analyses of TLR-2, TLR-4, and ICAM-1 mRNA levels were normalized to the expression levels of β-actin mRNA. Independent experiments were repeated three times. Data represent the means ± SD of triplicate samples. * $p < 0.05$ *vs.* the control cells.

expression of RANKL mRNA was not observed in GE-1 cells exposed to a combination of 5 ppm Ti ions and 10 μg/ml *Pg*-LPS (Fig. 3). However, Ti ions surprisingly enhanced the expression of CCL2 mRNA in the presence of *Pg*-LPS, in a synergistic manner (ANOVA; $p < 0.01$); CCL2 is a chemotactic cytokine for monocytes (Kim

et al., 2006), which are differentiated multinucleated gigantic cells such as macrophages and osteoclasts (Horowitz et al., 2005). In contrast, the effects of 10 μg/ml *Pg*-LPS on CCL2 mRNA levels in GE-1 cells cultured on a plastic dish and a Ti disc, in the absence of Ti ions, were similar (Fig. 4). Therefore, induction of CCL2 by Ti ions under LPS stimulation may contribute not only to inflammation but also to bone resorption.

Toll-like receptor (TLR)-2 and TLR-4 are competent receptors for LPS (Kaisho and Akira, 2000). We revealed that *Candida albicans* was recognized by ICAM-1 on human primary gingival epithelial cells (Egusa et al., 2005); TLRs and ICAM are important for the recognition of microorganisms in the oral cavity. Therefore, we investigated the expression patterns of TLR2, TLR-4, and ICAM-1 in GE-1 cells exposed to Ti ions. The mRNA expression levels of ICAM-1 and TLR-4 in GE-1 cells stimulated with Ti ions were increased in a dose dependent manner (Fig. 5). Although the mechanism of the synergistic induction of CCL2 by Ti ions and *Pg*-LPS is still unclear, Ti ions may affect the cellular competency of bacterial recognition by altering the sensitivity of epithelial cells to oral bacteria. However, there is inconsistency between the phenomena at the transcriptional and translational levels in cultured cells subjected to external stimuli. Therefore, the influence of Ti ions on the production of cytokines and receptors must be investigated to elucidate the cellular responses to Ti ions within non-cytotoxic concentrations.

5. Conclusion

In summary, we found that the cytotoxic effect of Ti ions on GE-1 cells was partly due to the induction of necrosis. Titanium ions in the presence of *Pg*-LPS enhanced the expression of CCL2 mRNA in a synergistic manner. Moreover, Ti ions alone elevated ICAM-1 and TLR-4 mRNAs in GE-1 cells. These results, taken together, suggest that Ti ions might decrease cell viability and affect inflammation by altering the sensitivity of the epithelia surrounding implants to microorganisms.

Conflict of interest statement

The authors declare that they have no competing financial interests.

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Original article

Impact of titanium ions on osteoblast-, osteoclast- and gingival epithelial-like cells

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Abstract

Purpose: To investigate the effects of titanium (Ti) ions on the cell viability, the cell differentiation and the gene expressions related to bone resorption including Receptor Activator of NF-κB Ligand (RANKL) and Osteoprotegerin (OPG) in the tissues around dental implants, the osteoblast-, osteoclast-, and gingival epithelial-like cells were exposed to Ti ions.
Methods: An MTS assay was carried out to evaluate the viabilities of osteoblast-like MC3T3-E1, osteoclast-like RAW264.7 and epithelial cell-like GE-1 cells. The gene expressions in these cells were analyzed by the use of RT-PCR and real-time quantitative RT-PCR.
Results: Ti ions in the concentration range 1–9 ppm had little effect on the viabilities of MC3T3-E1, RAW264.7 and GE-1, whereas 20 ppm Ti ions significantly decreased the viabilities of all cells. Analyses of RT-PCR and real-time quantitative RT-PCR data revealed that Ti ions at 9 ppm remarkably inhibited the expressions of Runx2, Osterix and type I collagen in MC3T3-E1. In RAW264.7, Ti ions showed no effects on the levels of mRNAs for TRAP and cathepsin K enhanced by RANKL. Ti ions at the range of 1–9 ppm showed no effects on the levels of mRNAs for RANKL and OPG in GE-1, while Ti ions at 9 ppm enhanced the expression of these genes in MC3T3-E1.
Conclusions: These results, taken together, suggested that Ti ions show the biological effects, both on the viabilities of osteoblast and osteoclast and on the differentiation of either the osteoblastic or osteoclastic cells, which may influence the prognosis of dental implants.

Keywords: Dental implant; Titanium ion; RANKL; OPG

1. Introduction

Titanium (Ti) and its alloys are widely used for numerous medical applications, including orthopedic and dental implants, because of their excellent corrosion resistance, mechanical properties and biocompatibility [1,2]. However, Ti ions have been detected at concentrations of up to 21 ppm in fibrous membranes encapsulating implants and 0.5 ppm in synovial fluid [3]. An *in vitro* experiment showed that Ti ions significantly enhanced the inflammatory cytokines production of macrophages, which may directly or indirectly cause bone resorption [4]. The facilitation of osteoclast differentiation by Ti particles is also reported [5]. Moreover, clinical studies have shown that titanium particles, released from orthopedic implants accumulate in the tissues, and that the phenomenon may the aseptic loss of orthopedic implants [6,7]. The results of these reports imply that released Ti ions could play a pivotal role in bone resorption at the interface of tissues and implants [3–7].

In the system of bone metabolism, the balance between the synthesis of bone matrix by bone-forming cells/osteoblasts and bone resorption by bone-resorbing cells/osteoclasts maintains the normal bone remodeling process that occurs throughout life. Skeletal abnormalities such as osteopetrosis and osteoporosis can be caused by increased or decreased bone mass, respectively [8]. In inflammatory diseases such as rheumatoid arthritis and periodontitis, the emergence of lymphocytes causes an imbalance of osteoclast and osteoblast

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