です．大阪大をもうリタイアされていて，でも講義をする．講義していただいた後，松田先生にお礼を申し上げましたら，来年はもっと準備しますからとおっしゃられて，びっくりしました．松田先生がこういう気持ちを持っていらっしゃるということは，もうみなさん方がそういう意識にしだいに変わってこられているのでしょう．

これも岡野先生のお力が大きいと思うのですが，大阪大医学部の澤 芳樹先生，松田先生に対して，われわれ工学の研究者が，工学だけではなく，誠意を持ってこういう分野を一生懸命働きかけてきたと思うのです．いまの時代になってきて，医学部の先生方が，やはり自分たちも同じ土俵で学ばねば先の治療，医療をする者がいないという意識に変わってきたのです．それを今度は若い人が敏感に感じて，教育面ではかなりうまくいくようになってきているのではないでしょうか．工学部の方々も，医学部の先生方に対して，いい意味での畏れはあるけれど，怖いという意味の恐れはなくなってきている．そのような雰囲気はありませんか．

米山　そういう環境としてはよい面も出ているかもしれません．逆に言うと，ただ興味を引かれてそういう融合領域のところへ行ったけれども，結局どっちもわからないというような学生も出てくるのではないでしょうか．

明石　どっちもわからなくなってきているというよりは，新しい分野に対する興味を持っている人が増えてきていると思います．われわれ団塊の世代とくらべて，いまは外国語を非常に自然に受け入れています．それと同じように，このようなバイオサイエンスやバイオテクノロジー，バイオエンジニアリングというのを非常に普通のものとして受け入れる時代が来ているのではないかと思います．

岡野　お手本があれば真似はしやすいのです．ある世代，つい最近までは，先生がいて，先輩がいて，先生や先輩の真似をしていれば間違いがなかった．なにかできたわけです．日本はみんな，自分の専門ですといって小さなフィールドで，そこから出ない方がむしろよい人生が描けたし，それが成功者になりました．ところがそういう限られたところというのは，中国・韓国の人たちがどんどん出てきて，追いつかれています．アメリカはどうしているかというと，縦割りではない学際領域に出ていって，新しいフィールドをどんどん立ち上げていっているわけです．むしろ先生や先輩がやらないことをどうやってやろうかということを，アメリカの若い人たちは本気で考えています．日本は先生と先輩のやったことしかやらない，それがよいことだと思い込んでしまっているのです．

そこにいま大変なギャップがあります．バイオマテリアルというのは医学と工学のちょうど境界領域のようなところにあって，そういう場所こそが，つぎの新しい時代をつくるということが最近ようやくいろいろな実績から注目されるようになってきました．ところが教育は変わっていないから，本気で取り組むにはなかなか勇気が要るわけです．

東京女子医大の清水達也先生は循環器のお医者さんで，しばらく臨床はやめて，細胞で心筋をつくりたいといって私の研究所に来ました．現在講師で活躍しています．泌尿器のお医者さんだった白柳慶之先生は，助手を辞めて，細胞で膀胱をつくるから大学院の学生にしてくれといって，新しい再生医療の研究をはじめたりしています．そういう人も出てくるのです．

医師にとっては，これまでのやり方とは変わったやり方というのをやるにはかなり勇気が要るわけです．それでも夢があるからやるという人が出てきたわけです．工学サイドでも，夢があるからこういうバイオマテリアルをやるという人たちが出てきたわけです．時代がやはりそういう人たちを必要としているし，そういう人たちが出てきて成功していく時代になってくればますます人材が集まってくる．やはり，お手本どおりの生き方でよいのかどうかです．本当に賢い人は新しいフィールドへ出てきてチャレンジしたら面白いのではないかと言ってあげたいです．

米山　おそらく，お手本どおりのことをやっていても，自分がお手本となるべき年齢になったときにそこの場所はないという状況が，いまの展開では充分ありえると思います．さまざまな授業や講演などもあるので，そういうところに積極的に出席して，どういうところがあるのかを自分で探せということでしょう．

松下　ありふれたことですが，やはり最後までやり遂げる粘り強さが大切です．先ほど岡野先生がおっしゃった，自分はこれをしたいという，やりたいことに対する情熱をどれだけ燃やせるかという精神構造になったときに，最後まで粘り強くやれるかどうかでしょう．

よく言われるように，途中でやめたらそれは失敗で，粘り強く最後まで行き着いたらそれは成功だと．その成功というところへ行き着くための粘り強さというのは，なかなか普通はできないと思いますが，本人がまず情熱を持ってその努力をする．組織の場合は，それを今度は上司がサポートする．先がみえない場合でも，激励することで限りなく力が出てくると思います．そういう組み合わせが必要です．本人の情熱と努力

は最も重要だと思いますが，それだけではなく，その二つの組み合わせがないと最後まで行き着かないのではないかと思います．

明石　社会としての受け皿を用意するように組んでいきたいです．そういう人たちの活きる道を与えようということです．

松下　道をみせるような感じです．

堤　大学で新しい教授を迎えるときに，実績で評価します．論文が多いとか，引用数が多いとかありますが，陥りやすいのは，その人の先生が偉くて，その先生の仕事を一所懸命やってきたという候補者ばかりが目立ちやすいのです．そういう人よりも，まさに岡野先生がおっしゃったような，変わった人，自分の発想でやってきた人を発掘しようとしているのに，逆にそうした人材がまだまだ少ないというのも困ったものです．

若い人には大いに自分らしさを発揮する研究を粘り強くやっていただきたいと思います．熱意を持っている人はかなり増えてきましたが，他人と違うことを言うと叩かれるのはやむをえません．そこで打たれ強くなるためには，情熱もそうですが，理論を持たなければいけません．こうあるべきだという，従来と違う自分の哲学をつくるような，粘り強くとことん頑固でありながら，間違いとわかれば正しい方向へと豹変できる勇気もある，そういう人を待望しています．

米山　現実として，Ph.Dを取った後に助手のポストが充分にない場合には，どんな気持ちで頑張れと先生方は助言なさいますか．

岡野　私は工学部を卒業しましたので，医学部に行くときに，周り中からどうかしているのではないかと言われました．医学部で万年助手をやるつもりかと言われて出てきたのです．現在，東工大の赤池先生と東大の片岡先生と，3人で助手をやっていた時代があるわけです．そのように言われながらも，いま3人とも教授になっています．

時代とともにそういうものは変わるし，自分が大丈夫だと思っても窓際になってしまうこともあります．それなら，ポストのために研究をするのはやめて，自分が信じられる場所でやったほうがよいと思うのです．ポストがあるからとか，教授になれるからというのは研究が好きなのではありません．本当に好きな研究をやって，そこで頑張りつづけていれば，ポストはどこかでついて回ってくるのではないでしょうか．結局私はアメリカまで行って，働く場所をアメリカに求めて，そこまで追い詰められても好きなことをやりつづけたのです．そうするとなにかが変わります．ですからやはり研究が好きだったら，あるいはやることが必要だと思ったら，先ほど堤先生がおっしゃった理論的なバックグラウンドをきちんと持つことが大事だと思います．こういうことをやりたいという夢に向かって努力する若者は，社会が必ず必要とするから，いつかポストは回ってくる．ポストのために自分を曲げる必要はまったくないと思います．

明石　バイオマテリアル分野の若手研究者は自分で道を拓けと言ってしまってもいいかもしれません．先駆者たちはそうしてきたのです．先ほど申し上げた，ポストを社会で用意できたらというのは願望で，若い人に言うべきこととしては，哲学を持てとか，自分で道を拓けというのが，この分野としてはふさわしいのかもしれません．

米山　若い人が，これがよいと思うのが一番正しい方向かもしれません．

明石　そういう人でないと生き残れないし，拓いていけない分野であることは間違いないと思います．いまの教育システムはそれなりに意味があると思います．ただ，国民の利益を考えるとしたら，もう少し受け皿を用意して，教育システムを充実させて，そういうところに人材がうまく流れるように持っていったほうが国益に適うと思いませんか．

米山　産業のほうまで影響するような知的なバックグラウンドを整えて，国際競争力のある新産業創出にという方向につなげるためには，重要なご指摘だと思います．

岡野　電機というフィールドは非常に日本は頑張っている．ところが少し自分で工夫が必要だったり，創造や新しい挑戦が必要だったりするフィールドが，日本では懸案事項になっていて，ゲリラ的な戦いになっている．バイオマテリアル研究では平均値でアメリカにやられていますが，トップレベルの研究も負けているかというと必ずしもそうではなくて，世界で通用するような，という世界をリードするような研究がたくさんあるのです．このフィールドの底上げという意味では，社会整備をきちんとしていれば優秀な若者が入ってくるはずです．ほかのフィールドにとられてしまうのは，先駆者たちがわるいのではないでしょうか．企業でもそうです．優秀な人材をたくさん採れば自分の事業部も一気に大きくなって，会社も発展するわけです．それを電機会社や自動車会社にとられてしまうのは工夫がないからです．大学も同じではありませんか．

米山　生体材料を標榜する大学研究室，学科はますます増えてきておりますので，会社のニーズを満たしたような卒業生がつぎつぎと巣立ちできて，よりどりみどり…となれればいいですね．

岡野　行政サイドでも，そういう人が必要なのです．おそらく土屋先生のような，バイオマテリアルを本当に専門でやってこられた人が行政サイドに立ってやったらもっとよくなるはずです．しかしそういう人を出す仕組みがないのです．

土屋　はい．審査官も 300 人定員のところ 190 人しかいないのです．

明石　埋まっていないのですか．

米山　無理に埋めても仕方ないからです．

土屋　そうです．募集で受けられるのですが，落とされるようです．医療機器の生物系もそうです．現在，2 人ぐらいの審査官ですから，そのあたりが今後も課題だと思います．

若い人というのは上の人の一言で変わるので，エンカレッジして，この人は駄目だと決めつけないで，その人の能力を最大限に引き上げてあげることを常に考えてあげるとかなり違います．

米山　若手に対するというよりはわれわれに対する助言ですね．

土屋　私のところには大学院生が 1 人いますが，学生さんはいません．さまざまなところを経由したPh.Dがいますが，前の教授が非常に明るく育てた人と，いじめられたと思っているような人とではかなり違うのです．そこで自信を持たせて，あなたならできると言ってあげるのです．一見元気がなさそうでも，本日ここにいらっしゃる先生方は成功例だからご自分の経験としてはおわかりにならないかもしれませんが，一言，元気づけてあげれば人は変わります．若手は特に変わります．そうすると日本のパワーになります．そこがまず非常に重要です．人材育成をもっともっと重要視していただきたい．優秀になれる可能性がある人がたくさんいます．全員にその資格があり，よいところはあるわけですから，よい面をみて育ててください．

明石　土屋先生は激励型ですか．先生はもしかしたら怒るタイプかと思っていました．

土屋　若いころはそうでした．室長のころは必死ですから，そうすると若い人を厳しくみてしまいます．それで反省したのです．

米山　若手をエンカレッジするので，若手自身は自分を信じて粘り強く頑張れと．

土屋　定員は少ないわけですから，最大限活躍してもらうように，いままで一つで済んだことを三つも四つもやれるような人材を育てないとやっていけません．

米山　このエリアはチャンスが多いということですか．

岡野　バイオマテリアル学会に来ると普段会えないような人と会えるという学会にして，そういう人が集まっている学会だから，常に未来のテクノロジーの創出に向けて，エンカレッジメントをしながら学会で若い人を育てていくというのも大切ですね．

米山　学会の懇親会にも出ましょう．

土屋　そうすると，周りの方々に理解していただけてよいかもしれません．

堤　アメリカの学会では本当に学生を大事にする部会がたくさんあります．論文の書き方の講習とか，もちろん就職相談のコーナーもあります．

明石　バイオマテリアル学会も落ち着いてきたようなので，若手の育成というのを次期は入れていただいたらどうですか．

米山　本誌 24 巻 3 号(2006 年 5 月号)に若手の特集が組まれておりますので，是非そちらも参考にしていただいて，バイオマテリアルの未来を背負う人材がますます育っていくように，学会をあげて取り組みましょう．

本日は貴重なお話をいただき，ありがとうございました．

再生医療・繊維工学・人工臓器に使用される医療用材料の安全性・有効性に関する基本的考え方

Standpoints and Principles for the Evaluation of Safety and Efficacy of Biomaterials Applied for Tissue Engineered Products

土屋利江

1. はじめに

国内外共に開発競争が盛んな医療材料の一つとして生分解性材料があげられる。限られた紙面の都合上、ここでは、注目されている生分解性材料において、現在、安全性・有効性において問題になっている点を中心に述べる。生分解性材料は、やがては生体内で消失し、残存しないという長所があるものの、吸収性材料であるが故に、クラスⅣに分類されるハイリスク医療材料である。最近の不具合報告や前臨床試験研究からも、解決すべきいくつかのポイントがあるので、現在考えられる基本的考え方について述べる。

2. 安全性

各種モノマーから合成される高分子系では、使用される触媒の選択が安全性・有効性を考える上で第1のキーポイントとなる。環境および生体ハザードとして有名な触媒を使用している例がある。生分解性材料を医療材料として使用した場合、生体内で吸収される。使用される材料のトータル量は医療材料の使用目的によって数mgから数g程度まで異なっている。量が多くなると安全性上のリスクも高くなる。安全性上問題はないのか最新の知見を十分検討して合成のスキームを描いていただきたい。また、医療材料として使用する場合、使用する部位、使用する量、分解速度によって安全性に関するリスクレベルは異なる。慎重な検討を御願いしたい。

3. 経済性と安全性

医療材料も、エコマテリアルであることが、理想的であるが、通常、工業製品用のエコマテリアルとなると数百から数千トン単位で合成され、地球環境系へ放出される。また、使用量も多く、できるだけ低コストで生産されることになる。工業界では、安価な触媒で効率よく重合できる製法が望まれる。安全性に対する検討事項もあるが、医療材料として使用される場合とは試験選択項目が異なる。分解性という点でエコマテリアルの候補と考えられるポリグリコール酸には、環境ハザードで有名な有機錫で合成された製品が販売されている例がある。有機錫は、ppbあるいはそれ以下のオーダーで神経毒性を示す触媒である。作用域が低濃度であるため、残留物の分析では、一般化学分析では、同定・定量することは容易ではない。製造工程が示してあれば、容易に知ることが可能であるが、カタログ掲載の製品では、使用されている触媒が、明示されていないし、たづねても回答がかえってこないケースが多い。世の中に登場しているエコマテリアルと称される材料が、安全性上、危惧される点が解決できた製造工程で合成されているか確認する必要がある。環境中に放出されれば、触媒は、土壌中に残留することになり、海にも流れ、やがては、魚貝類に蓄積し、食物連鎖によりやがてはヒトの健康への悪影響が懸念される。これらの点は、逆の発想をすれば、有効性の高い材料開発のアイデアともなりうる。すなわち、毒性のある触媒の代わりに、有効性の高い触媒を使用すれば、安全で有効性の高い材料開発の創製となるかもしれない。

海外では、すでに無触媒条件下で分子量100万のレベルまで重合した生分解製材料を高価な価格で販売しているという。この分野の国際情報の流通がわるい。

4. 生体適合性

第2のポイントは、生体内に埋植すると炎症反応を惹起しやすい性質を示す材料がある。炎症反応が起これば、例えば、バイオ軟骨において、動物に細胞組み込み型生分解性スキャホールドを埋植し、炎症がおきれば、スキャホールドに播種した軟骨細胞等の分化発現や機能維持の目的を達成できない。従って、このような材料に起因した炎症反応を回避あるいは消失させるための創意工夫をしていただきたい。炎症反応惹起の有無はin vivoで確認する必要がある。ある生分解性高分子からなる材料に軟骨細胞を播種してin vitro培養すると分化を非常によく促進する。次に、動物の軟骨欠損部分に移植したところ、組織再生がうまくいかない、材料や細胞組み込み型材料を埋植すると組織再生は遅延し、それらをまったく埋植しない軟骨欠損のまま

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の群の自然治癒率がもっとも優れていたということも実際ありうる。Tissue Engineering関連の論文で掲載されたデータで、in vitroでどんなに成績が良くても、生体内で、同じように作用するか否かは、別の問題となる。in vitroで優れた成績を示している必要性はあるが、必ずしもin vivoで成功するとは限らない。生体内は、複数の細胞・組織のネットワークで営まれております。in vitro系は、特定の細胞の反応をみていること、さらに、検出している指標のみを解析しているにすぎないことを、いつも考慮しておく必要がある。

5. 更なる生体適合性

第3のポイントは、合成高分子には、ゲッシ類で比較的高い腫瘍発生率を示す材料があることに留意して、新たな材料開発を行うべきである。たとえば、京都大学の研究成果では、ポリグリコール酸は、フィルムをラット皮下に長期間埋植しても、腫瘍の発生を認めなかったが、ポリ乳酸フィルムを、ラット皮下に埋植した結果、埋植ラットの40%に腫瘍発生を認めている。ポリカプロラクトンとの共重合体では、50%の腫瘍発生頻度であった。一方、材料発癌では、20年以上昔の現象から、フィルム状のものをゲッシ類の皮下に埋植すると、腫瘍を発生する。との古典的な説がある。フィルムがすべて同程度腫瘍化するわけではない。シリコンフィルムでは、手術群に近く、低発生率である。フィルムで腫瘍発生する生分解性材料を、粒子状にして埋植した結果、やはり腫瘍を発生し、発生率は、埋植量に比例した。私は、埋植材料の化学組成、物理学的性質、残留性(分解速度)、血管系の有無などがサイトカイン産生、コネキシン機能変化、炎症反応による活性酸素産生、修復能などに影響を与え、腫瘍発生率に関係すると考えている。

医療材料の安全性評価においては、ガイドラインにある一通りの試験項目について受託機関等により試験し、すべて陰性結果を得ると、安全であると考えやすいが、その試験が適切な抽出やサンプル適用方法でおこなわれていない限り、意味のない試験結果となることを強調しておく。生分解性材料の場合に、そのオリゴマーの安全性についても評価することが、長期予測をおこなう上で重要である。

第10回高分子分析討論会(高分子の分析及びキャラクタリゼーション)－研究発表募集－

主　催：日本分析化学会高分子分析研究懇談会　　**協　賛**：㈳繊維学会　　**日　時**：平成17年10月27日㈭・28日㈮
会　場：工学院大学新宿校舎〔東京都新宿区西新宿1-24-2、交通：JR(山手線・中央線・埼京線)、京王線、小田急線、地下鉄(丸の内線・都営新宿線)「新宿」駅下車西口より徒歩5分。大江戸線「都庁前」駅直結〕
http://www.kogakuin.ac.jp/mnp/shinjuku/map_shinjuku.pdf
発表申込締切：7月1日㈮　　**発表要旨締切**：10月7日㈮
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(独)産業技術総合研究所　計測標準研究部門　有機分析科　高分子標準研究室　松山重倫
TEL：029-861-4617、FAX：029-861-4618、E-mail：polymer@m.aist.go.jp

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第41回X線分析討論会－講演募集－

主　催：日本分析化学会X線分析研究懇談会　　**協　賛**：㈳繊維学会ほか
日　時：平成17年10月21日㈮・22日㈯
会　場：京都大学福井謙一記念研究センター(京都市左京区高野西開町34-4)
講演申込締切日：8月10日㈬　　**講演要旨締切日**：9月16日㈮
詳細については下記にお問い合せください。
〒141-0031　品川区西五反田1-26-2　五反田サンハイツ304号　日本分析化学会X線分析研究懇談会
TEL：03-3490-3351　FAX：03-3490-3572　E-mail：ktanaka@jsac.or.jp

「高分子材料の耐久性評価」に関する講習会

主　催：日本材料学会　　**協　賛**：㈳繊維学会ほか　　**日　時**：平成17年7月22日㈮　9：30～14：40
会　場：工学院大学新宿校舎28階第1会議室　〒163-8677　東京都新宿区西新宿1-24-2(TEL：03-3342-1211)
プログラム、参加申込の詳細については、下記にお問い合せください。
〒606-8301　京都市左京区吉田泉殿町1-101　日本材料学会「高分子材料の耐久性評価」講習会係
TEL：075-761-5321　FAX：075-761-5325　E-mail：jimu@jsms.jp

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Improved Biocompatibility of Titanium–Zirconium (Ti–Zr) Alloy: Tissue Reaction and Sensitization to Ti–Zr Alloy Compared with Pure Ti and Zr in Rat Implantation Study

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Titanium–zirconium (Ti–Zr) binary alloy has better corrosion resistance and mechanical properties than commercially pure Ti. The present study was designed to determine the biocompatibility of Ti–Zr alloy by an implantation test in animal bodies in comparison with pure Ti, Zr, and chromium (Cr) implants as positive controls. Sample specimens were placed in a subcutaneous position in rats for 8 months. No significant decreases in body weight, the weight of any organ, or the weight of any organ relative to body weight were found in the implant groups compared to a no-implant control group. On hematological examination, small differences in several parameters were found in some groups, but these changes were not attributable to the materials implanted. Mitogen-induced blastogenesis was observed in similar degrees among all implant groups. These results suggest that the implantation of test samples did not cause systemic toxicity or a decrease in immune activity. The fibrous capsule membranes around the Ti and Ti–Zr alloy implants were thinner than those around Cr implants. The numbers of macrophages, inflammatory cells, and other cells involved in immune responses in and around the fibrous capsules of the Cr- and Ti-implant groups were higher than those of the Ti–Zr alloy- and Zr-implant groups. The Ti–Zr alloy had the lowest total score of tissue responses among the materials tested. None of the animals from the Ti-, Zr-, and Ti–Zr alloy-implant groups exhibited a skin reaction following exposure to Ti or Zr salt solutions. These results indicate the Ti–Zr alloy has better biocompatibility than Ti for use as an artificial surgical implant.

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Keywords: *titanium alloy, titanium, biocompatibility, inflammation, hypersensitivity*

1. Introduction

Stainless steel and cobalt–chromium (Co–Cr) alloys have been widely used as materials in orthopedic and dental implants because of their biocompatibility, physical properties, and manufacturing ease.[1)] In general, these metallic alloys have excellent corrosion resistance and are not believed to cause any local or systemic responses. However, fretting corrosion of metallic implants is sometimes observed in contact with biological systems, causing the release of metallic ions from the implants.[2,3)] Elevated levels of metal ions have been found in blood, urine, and tissues of patients and animals that have received metal implants.[2–6)] Metallic ions, such as nickel (Ni), Co, and Cr, are known to cause adverse tissue reactions and allergy.[7–13)]

Titanium (Ti) and its alloys are currently considered the most attractive metallic materials for orthopedic and dental surgery. The use of Ti alloys is increasing due to their excellent mechanical strength, corrosion resistance, and good biocompatibility.[14–17)] These properties are attributable mainly to the formation of a stable titanium oxide (TiO_2) layer on the surface.[18,19)] However, the mechanical/tensile strength of commercially pure Ti is insufficient for its use as an artificial hip joint, pin, or screw,[20)] and its wear resistance is also inferior to that of stainless steels and Co–Cr alloys.[20)] The appearance of increased wear debris from Ti has been associated with inflammation, bone resorption, and pain.[18,19,21–24)] To improve mechanical strength and wear resistance, various elements have been added to create new Ti alloys. Ti–6Al–4V alloy is a high-strength Ti alloy, but its biocompatibility is considered lower than that of commercially pure Ti. The wear resistance and corrosion resistance of Ti–6Al–4V alloy are inferior to those of Ti, and Ti–6Al–4V alloy releases compounds and wear debris containing vanadium (V) or V ion, both of which are toxic.[18)] At present, it is difficult to avoid the wear and/or fretting of implanted alloys in a living body, resulting in the release of elements contained in the alloy and the formation of wear debris. Therefore, it is preferable not to use highly toxic elements in alloys.

Zirconium (Zr) belongs to the VIa group in the periodic table, as does Ti, and is known to have chemical properties similar to those of Ti.[20)] An insoluble oxide is formed on the surface in the air, and the surface oxide composition (zirconia) influences corrosion behavior. There is general agreement that Zr compounds have no local or systemic toxic effects.[25)] Based on this apparent lack of toxicity, Kobayashi *et al.* selected Zr as an alloying element to improve the properties of commercially pure Ti, and prepared a Ti–Zr binary alloy as a material for use in medical devices, such as artificial joints or bone plates.[20)] The hardness of Ti–50%(atom%) Zr alloy is 2.5 times as large as that of commercially pure Ti, suggesting the alloy's superior mechanical strength.[20)]

Besides mechanical properties, the biocompatibility of an alloy is important if it is to be used in implant devices. We previously observed that animals were sensitized to Cr by long-term implantation of corrosive Cr alloys.[26)] The animal model is a prevalent tool in examining tissue responses to implant material. The present study examined the biocom-

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patibility of the Ti–Zr alloy compared with those of Ti and Zr by implantation tests using rats. Pure chromium (Cr) was used as a positive control material. After 8 months' implantation to a subcutaneous position, the reaction of the tissue around the specimen was studied hematologically and histologically. Since cases of sensitization to pacemakers which are made of Ti have been reported,[27,28] we topically applied a metal salt solution to rats in order to detect hypersensitivity to the metal. Further, the *in vitro* proliferation of spleen lymphocytes was also measured to evaluate immune system activity.

2. Materials and Methods

2.1 Test materials

Specimens of Ti, Zr, Ti–Zr binary alloy (Ti containing 50 atom% Zr), and Cr were used. The preparation of these specimens was reported in detail by Kobayashi *et al.*[20] Briefly, the specimens were prepared from sponge Ti (>99.8 mass% purity), Zr (>99.5 mass% purity), and electrolytic Cr metal (>99.98 mass% purity) by arc melting with a non-consumable tungsten electrode on a water-chilled copper hearth under an ultra-high-purity argon atmosphere. To prevent the macroscopic composition gradient by insufficient mixing, ingots were turned over and re-melted at least five times. To minimize mechanical trauma during implantation, the specimens were prepared in the form of plates with a diameter of 14 mm and a thickness of 1 mm, and were cut out from these button ingots using a silicon carbide wheel cutter. The plate specimens were polished mechanically to a mirror finish using emery paper (gradually finer, up to 600 grit) followed by 0.03 mm alumina paste. Each specimen was washed with 70% ethanol and ultra-pure water, and autoclaved.

2.2 Animals

Female F344/DuCrj rats, 5 weeks old, were obtained from Charles River Japan Inc. (Kanagawa, Japan) and randomized into five groups of six rats each. The animals were housed in air-conditioned facilities (temperature 23 ± 2°C, relative humidity 55 ± 5%, light cycle 12 h/day). Diet (F-2, Funabashi Farm Co., Chiba, Japan) and water were available ad libitum throughout the experimental period. After a 1-week acclimation period, all animals were used in the implantation test. All animals were cared for according to the Japan animal rights act and the NIHS-Japan guidelines for the care and use of laboratory animals.

2.3 Implantation procedure[26,29]

The animals were anesthetized with 50 mg/kg Nembutal (sodium pentobarbital, Dainabot Co., Ltd., Osaka, Japan) *via* intraperitoneal injection. After the induction of anesthesia, the hair on the back was shaved around the implantation site and the skin was sterilized by brushing with a 70% ethanol solution. An incision was made with scissors on the right side of the shaved back skin, and a specimen was inserted subcutaneously. Control animals were treated by a sham operation with no implantation. After the operation, the incision was sutured. During the experimental period, body weight and health conditions were monitored.

2.4 Hematological and histological examination

At the end of the experimental period, the animals were anesthetized with diethyl ether, and total blood (approximately 4–5 ml) was collected from the abdominal aorta. The hematological data, such as red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet count (PLT), and white blood cell count (WBC), in the blood sample were measured by a fully automated blood cell counter (M-2000, TOA Medical Electronics Co., Ltd. [Today's Sysmex Co.], Hyogo, Japan). In addition, differentiation of white blood cells was examined using an automated hematology analyzer (MICROX HEG-120A, Omron Tateisi Electronics Co., Tokyo, Japan; Sysmex Co. is presently acquiring Omron's blood image analysis business). According to the common method, spleen, liver, kidney, and the tissue surrounding the specimen were excised from each sacrificed animal. These tissues, along with the specimen, were fixed in 10% formalin and embedded in paraffin. After the specimen was removed gently, the tissue was sectioned and stained with hematoxylin and eosin for microscopic examination. The histological sections were examined microscopically, and the tissue responses—*i.e.*, the distributions of fibroblasts, neutrophils, eosinophils, macrophages, giant cells, lymphocytes, and plasma cells—in the fibrous capsule that formed around the specimen were recorded, as was the infiltration of inflammatory cells around the capsule.[26,29] The intensity of these histological items was ranked as $-$ = no frequency (0), $+$ = low frequency (1), $++$ = moderate frequency (2) and $+++$ = high frequency (3). The total score for the eight items was derived. The thickness of the fibrous capsule that formed around the specimen was also measured by a micrometer.[29] The thickness was determined in the orthogonal direction of the boundary between the capsule and the hole left by the specimen, and expressed as a mean value of 10 spots.[29]

2.5 Evaluation of sensitization response

At 8 months after implantation, a 50 µl aliquot of 2% titanium chloride ($TiCl_4$) in ethanol, 10% zirconium chloride ($ZrCl_4$) in 70% ethanol solution, or 0.02% potassium dichromate ($K_2Cr_2O_7$) in 25% ethanol solution was applied to the shaved skin of the back using a closed-patch dressing (Trii Pharmaceutical Co., Ltd., Tokyo, Japan) for 24 h. Salt of each metal was used as an application chemical for the determination of skin reaction in the patch testing.[26,30] The challenge concentrations of metal salts were determined by a preliminary irritation test, and they were the maximum concentrations to not elicit responses in normal animals. An application vehicle for metal salts at each concentration was prepared to maximize the content of ethanol in water. The skin reactions of erythema and oedema were evaluated by visual assessment at 24 h after removing the closed-patch dressing.

After 8 months of implantation, the spleen was excised and placed on a sterile 200-mesh stainless steel gauge in a 35 mm plastic dish. After 2 ml of Hanks' balanced salt solution (pH 7.4, Sigma-Aldrich Inc., St. Louis, MO, USA) was added to the dish, the spleen was crushed mechanically using

Table 1 Body and organ weights of rats after implantation of each material for 8 months.

Material	Weight (g) (Mean ± SD, n = 6)					
	Body	Thymus	Spleen	Liver	Kidney	
					Right	Left
Absolute weight						
Control	213.8 ± 16.3	0.085 ± 0.009	0.35 ± 0.03	5.15 ± 0.47	0.61 ± 0.04	0.62 ± 0.04
Ti	223.7 ± 20.7	0.086 ± 0.010	0.39 ± 0.01*	5.63 ± 0.59	0.65 ± 0.05	0.65 ± 0.04
Ti–Zr	221.0 ± 12.4	0.082 ± 0.013	0.37 ± 0.01	5.78 ± 0.32*	0.64 ± 0.03	0.64 ± 0.03
Zr	231.5 ± 8.1*	0.091 ± 0.013	0.38 ± 0.01*	5.30 ± 0.39	0.65 ± 0.05	0.64 ± 0.05
Cr	227.7 ± 7.4	0.087 ± 0.012	0.38 ± 0.02	5.96 ± 0.26**	0.67 ± 0.03*	0.65 ± 0.04
Relative weight (organ/body × 1000)						
Control		0.40 ± 0.03	1.66 ± 0.19	24.2 ± 2.5	2.88 ± 0.16	2.90 ± 0.10
Ti		0.39 ± 0.03	1.75 ± 0.13	25.2 ± 1.8	2.93 ± 0.08	2.92 ± 0.20
Ti–Zr		0.37 ± 0.05	1.67 ± 0.10	26.2 ± 0.7	2.88 ± 0.10	2.92 ± 0.10
Zr		0.39 ± 0.05	1.66 ± 0.10	22.9 ± 1.1	2.82 ± 0.15	2.77 ± 0.20
Cr		0.38 ± 0.05	1.69 ± 0.12	26.2 ± 1.2	2.94 ± 0.09	2.87 ± 0.12

*, **: Significantly different from the control group ($p < 0.05$, $p < 0.01$).

Table 2 Hematological data of rats implanted with alloys.

Item	Group				
	Control	Ti	Ti–Zr	Zr	Cr
RBC ($\times 10^4$/ml)	812 ± 27	729 ± 13*	765 ± 24*	779 ± 37	769 ± 16*
HGB (g/dl)	14.8 ± 0.5	13.6 ± 0.2*	14.4 ± 0.4	14.6 ± 0.6	14.4 ± 0.2
HCT (%)	41.8 ± 1.1	38.2 ± 0.8*	40.0 ± 1.7	40.3 ± 1.8	39.9 ± 0.8*
MCV (fl)	51.5 ± 0.6	52.4 ± 0.4*	52.3 ± 0.8	51.7 ± 0.3	51.9 ± 0.2
MCH (pg)	18.2 ± 0.2	18.7 ± 0.3*	18.8 ± 0.2*	18.8 ± 0.2*	18.8 ± 0.3*
MCHC (g/dl)	35.4 ± 0.5	35.6 ± 0.7	35.9 ± 0.5	36.4 ± 0.2*	36.1 ± 0.5*
PLT ($\times 10^4$/μl)	61.1 ± 4.9	65.8 ± 5.7	66.3 ± 6.8	70.8 ± 3.1*	60.0 ± 4.2
WBC ($\times 10^2$/μl)	20.0 ± 7.0	14.0 ± 2.0	18.0 ± 4.0	19.0 ± 6.0	15.0 ± 3.0
Differential cell counts (%)					
Neutrophil-band	0.5 ± 0.7	0.1 ± 0.3	0.3 ± 0.5	0.1 ± 0.2	0.1 ± 0
Neutrophil-segmented	31.5 ± 5.8	34.4 ± 7.7	28.8 ± 6.6	28.9 ± 3.8	30.6 ± 4
Basophil	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Eosinophil	2.5 ± 1.3	2.2 ± 1.3	2.5 ± 1.2	1.3 ± 0.8	1.5 ± 1
Lymphocyte	63.1 ± 6.6	61.3 ± 6.7	65.7 ± 5.9	67.8 ± 3.6	65.7 ± 4
Monocyte	2.4 ± 1.0	2.1 ± 1.3	2.8 ± 1.5	1.9 ± 1.3	2.2 ± 2

Data represent mean values ± SD (n = 6).
*Significantly different from the control at $p < 0.05$.

a syringe, and spleen cells were released. The cell suspension was transferred into a 15 ml tube, and the tube was centrifuged at 1200 rpm for 5 min at 4°C. After the supernatant was removed, the cells were treated with 2 ml of 0.83% ammonium chloride-tris solution (pH 7.65) for 5 min at 37°C for hemolysis. The cells were washed twice by centrifuge at 1200 rpm for 5 min with Hanks' balanced salt solution and resuspended in RPMI-1640 culture medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. The cell suspensions (5×10^5 cells/200 μl) were seeded into 96-well culture plates (three wells per group) and cultured with 10 μl of 10^{-8} mol/L $K_2Cr_2O_7$, 10^{-5} mol/L $TiCl_4$, 10^{-5} mol/L $ZrCl_4$, or 5 μg/mL Con A and 9.25 KBq [^{3}H]methyl thymidine (^{3}HTdR) for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation, the cells were collected from each well on a glass filter (Type Filter Mat 11731, SKATRON Instruments AS, Lier, Norway) using an automatic cell harvester (Type 11025, SKATRON Instruments AS). The incorporation of ^{3}HTdR into cells (counts per minute, cpm) was determined by liquid scintillation counting (LSC-5101, Aloka Co. Ltd., Tokyo, Japan).

3. Results

3.1 Body and organ weights

After 8 months of implantation, no significant visual changes such as rust or cracks were found on the surface of any specimen. In all implant groups, no decreases in body and organ weights were found compared with the control group (Table 1). The body weight in the Zr-implant group was higher than that in the control group. The thymus, spleen, liver, and kidney of some animals in the implant groups weighed more than those of control animals. However, the organ weights relative to body weight were not significantly different from group to group.

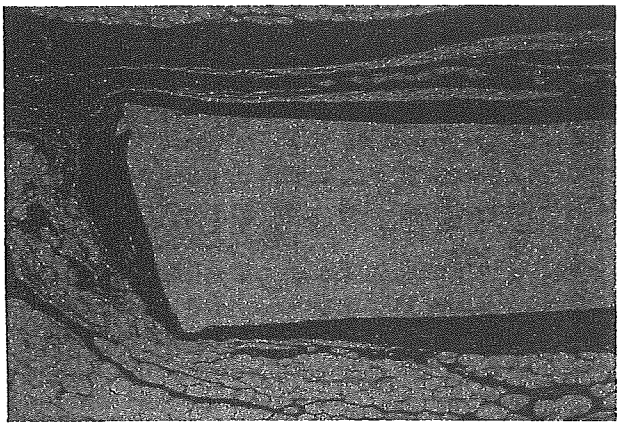

Fig. 1 Histological section of subcutaneous tissue around Ti (hematoxylin and eosin, original magnification × 16). The central lumen was the site of the implant, and the tissue surrounding the lumen formed a fibrous capsule. The thickness of the fibrous capsule was expressed as the mean ± standard deviation (SD) of 10 spots.

3.2 Hematological examination

Table 2 shows the results of analyses of blood constituents, such as RBC, PLT, and WBC counts, and HGB concentration. In the Ti-implant group, slight decreases in WBC count as well as HCT and HGB concentrations were found compared with the control group. Although some implant groups showed small changes in several parameters, none of the specimens showed a clear increasing or decreasing trend in any of these parameters. With regard to hematograms of WBC, no significant difference was found between the control and implant groups.

3.3 Histological examination

Figure 1 is a photograph of a histological section of tissue surrounding a Ti specimen. The central lumen was the specimen's extraction mark. Mature fibroblasts were found to form a distinct capsule between the soft tissue and the Ti specimen. Figure 2 is a typical image of tissues surrounding some specimens at high magnification (×100). The lower part is the specimen's extraction mark. Two of the six animals in the Ti-implant group showed a moderate inflammatory response, and the image of animal no. 3 is shown in (a). Various types of cells infiltrated the fibrous membrane. The photograph in Fig. 3(b) is a tissue image of animal no. 7 of the Ti–Zr alloy-implant group. All animals in this group had levels of tissue reaction similar to those shown in Table 3(b). A fibrous capsule was formed, but the frequency of cell infiltration into the membrane around the Ti–Zr alloy implant was lower than that with the other materials. In the Zr-implant group, the frequency of cell infiltration into the surrounding tissue was higher than that in the Ti–Zr alloy-implant group but lower than that in the Ti-implant group (photograph not shown). The strongest tissue response was observed in the Cr-implant group, and many inflammatory cells were present in the fibrous layer (photograph not shown).

Table 3 summarizes the histological findings of the tissue responses to each material. The distribution frequencies of inflammatory cells or fibroblasts in or around the fibrous capsule were ranked and the total score was derived for each

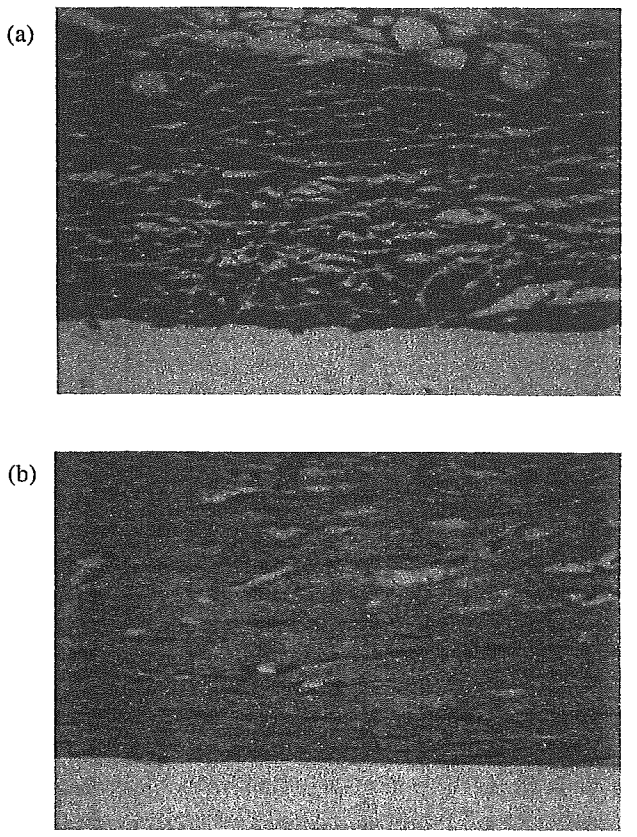

Fig. 2 Histological image of tissue around Ti (a) and Ti–Zr alloy (b) at high magnification (×100).

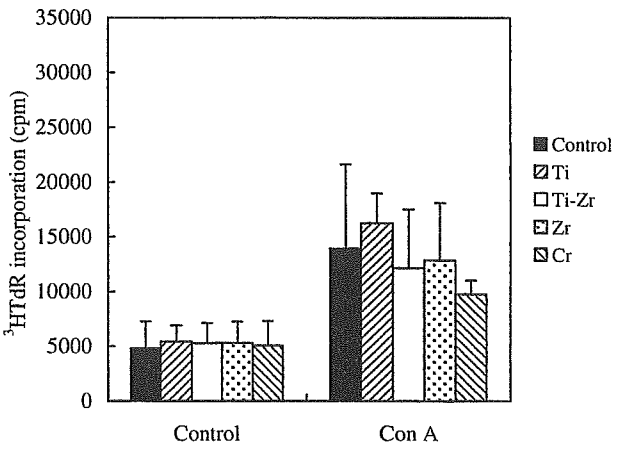

Fig. 3 Con A-induced blastogenesis of spleen lymphocytes in rats implanted with the material for 8 months. Single cell suspension of spleen cells (5×10^5 cells) was cultured with 5 μg/ml Con A and [^{3}H]methyl thymidine (^{3}HTdR) for 48 h. The culture was terminated by automatic cell harvesting, and the ^{3}HTdR incorporation (cpm) was determined. The data are the mean ± SD of 6 animals.

material. The cell distributions in tissues surrounding the materials observed in this study were not as strong as those in our previous implantation study, which used a 4-month experimental period.[26] Large amounts of fibroblasts were observed in all animals in the Cr-implant group and in some animals in the Ti-implant group. Greater numbers of macrophages, the main inflammatory cell types, infiltrated the fibrous capsules around the Cr or Ti specimens compared

Table 3 Histological findings of the tissues around materials implanted for 8 months.

Sample	No.	In fibrous capsule							Around fibrous capsule	Total score	(mean ± SD)	Thickness of fibrous capsule (μm)	(mean ± SD)
		Fibroblasts	Neutrophils	Eosinophils	Macrophages	Giant cells	Lymphocytes	Plasma cells	Inflammatory cells				
Ti	1	+++	+	+	++	+	++	+++	++	15	9 ± 6	83	119 ± 48
	2	+	+	+	+	−	+	−	+	6		71	
	3	+++	++	+	+++	++	+++	++	+++	19		80	
	4	+	+	−	+	−	+	−	+	5		170	
	5	++	+	−	+	−	+	−	+	6		179	
	6	+	−	−	+	−	+	−	+	4		129	
Ti–Zr	7	+	+	−	+	−	+	−	+	5	5 ± 0	119	128 ± 16
	8	+	+	−	+	−	+	−	+	5		121	
	9	+	+	−	+	−	+	−	+	5		140	
	10	+	+	−	+	−	+	−	+	5		108	
	11	+	+	−	+	−	+	−	+	5		153	
	12	+	+	−	+	−	+	−	+	5		126	
Zr	13	+	+	−	+	−	+	−	−	4	5 ± 1	141	153 ± 11
	14	++	+	+	+	−	++	−	+	8		165	
	15	+	+	−	+	−	+	−	+	5		143	
	16	+	+	−	+	−	+	−	+	5		165	
	17	+	+	−	+	−	+	−	+	5		160	
	18	+	+	−	+	−	+	−	−	4		146	
Cr	19	+++	++	+++	++	−	+++	−	++	15	12 ± 6	222	170 ± 36
	20	+++	+	++	++	−	++	−	+	11		161	
	21	++	+	+	+	−	++	−	++	9		167	
	22	+++	+++	++	+++	+	+++	+++	+++	21		198	
	23	++	+	−	+	−	+	−	−	5		118	
	24	+	+	++	+	−	++	−	+	8		155	

Each parameter was scored as − = no frequency (0), + = low frequency (1), ++ = moderate frequency (2), and +++ = high frequency (3).

with the Ti–Zr or Zr specimens. A few giant cells were found in the Ti- and Cr-implant groups, but not in the Zr-implant and Ti–Zr alloy-implant groups. Increased numbers of neutrophils, eosinophils, and lymphocytes were found especially in the Cr-implant group compared with the Ti–Zr alloy- and Zr-implant groups. The total scores in the Ti–Zr alloy- and Zr-implant groups were significantly lower than that in the Cr-implant group.

The capsule membrane formed around the Ti specimen was thinner than those around the Zr and Cr specimens. Individuals varied considerably in membrane thickness in the Ti-implant group, so no statistically significant differences were obtained between the Ti- the Ti–Zr alloy-implant group. The membrane around the Cr specimen was the thickest ($170 \pm 36\,\mu m$) among the materials tested and differed significantly from that around the Ti–Zr alloy specimen ($126 \pm 16\,\mu m$). A statistical difference in membrane thickness was also found between the Ti–Zr alloy- and the Zr-implant groups.

3.4 Sensitization

A patch test was performed to assess the sensitization to each metal. The animals were topically challenged with 0.02% $K_2Cr_2O_7$, 2% $TiCl_4$, or 10% $ZrCl_4$ solution on the skin. None of the animals exhibited positive skin reactions to any of these metal salt solutions, so sensitization to Cr, Ti, or Zr did not develop as a result of implantation of the metal specimens.

Spleen lymphocyte proliferation is an *in vitro* indicator in the assessment of immune function and provides information useful for diagnosing contact allergy. The proliferation activity without mitogens or metal salts was similar among the test groups. The addition of $K_2Cr_2O_7$ solution did not stimulate lymphocyte proliferation in the Cr-implant group. In the same way, the stimulation index obtained by the addition of $TiCl_4$ or $ZrCl_4$ were almost all 1.0 in the Ti-, Zr-, and Ti–Zr alloy-implant groups (Table 4). Con A-induced blastogenesis was somewhat lower in the Cr-implant group than in the other groups, but no significant difference was observed (Fig. 3).

Table 4 Lymphocyte proliferative responses by stimulation with each metal salt.

Sample	Stimulation index (mean ± SD)		
	$TiCl_4$ 10^{-6} mol/L	$ZrCl_4$ 10^{-6} mol/L	$K_2Cr_2O_7$ 10^{-9} mol/L
Control	1.04 ± 0.08	1.03 ± 0.06	1.01 ± 0.07
Ti	1.00 ± 0.09	ND	ND
Ti–Zr	0.98 ± 0.03	0.99 ± 0.05	ND
Zr	ND	0.99 ± 0.02	ND
Cr	ND	ND	0.97 ± 0.05

Spleen was collected from each animal, and a single cell suspension of spleen cells was prepared. The cells (5×10^5 cells) were cultured with each concentration of metal salt and 25 μCi [^{3}H]methyl thymidine (^{3}HTdR) for 48 h, and the ^{3}HTdR incorporation (cpm) into cells was determined. A stimulation index, the ratio of ^{3}HTdR incorporation relative to control wells, was derived for each metal salt. The data are mean ± SD for 6 animals.
ND = not determined.

4. Discussion

In a corrosive environment, component elements of metallic materials are released from the surface as ions and chemical compounds, and some of them may cause local adverse tissue reactions and the development of metal allery.[1,14] Commercially pure Ti has generally good biocompatibility,[14–17] but its mechanical strength is insufficient for use in artificial hip joints.[16,20] Furthermore, the appearance of increased wear debris from Ti has been associated with tissue inflammation.[1,2,31] Elements for Ti alloys are classified into three microstructural categories: α-stabilizers [aluminum (Al), oxygen (O), nitrogen (N), carbon (C)], β-stabilizers [molybdenum (Mo), vanadium (V), iron (Fe), Cr, Ni, Co] and neutral [zirconium (Zr)]. The properties of Ti alloys vary according to the composition of the elements. Ti alloys with α and near-α microstructures exhibit superior corrosion resistance but low ambient temperature strength. The α + β and β alloys have high strength and good formability but relatively low corrosion resistance.[18] For example, Ti–6Al–4V alloy was developed as a high-strength material, but its low corrosion resistance in the living body was problematic.[18] Kobayashi *et al.* experimented with Zr, whose chemical properties are similar to those of Ti, and formed an insoluble oxide. They developed a Ti–Zr alloy that has an α + β structure and better mechanical properties than commercially pure Ti. Because of its good mechanical properties, such as tensile strength and hardness, Ti–Zr alloy was presented as a new biomedical material for use in artificial joints or bone plates.[20]

Since orthopedic devices are generally implanted into a corrosive environment for anywhere from several months to the lifetime of the patient, long-term evaluation of biocompatibility is necessary. In a previous study, we investigated sensitization of the rat to Cr by implantation of Cr–Fe alloys into a subcutaneous position for 4 months.[26] Lewin *et al.* also evaluated the local response to bone screws in guinea pigs after 4 months' implantation.[32] Oron and Alter examined the corrosion of metal specimens by implantation into rats for 14 months, and found progressive increase in corrosion concomitant with the length of implantation period.[2] In this study, an implantation period of 8 months was used because Ti–Zr alloy and other metal specimens do not easily corrode and release metallic ions. Furthermore, the early inflammatory responses caused by surgical injury could be disregarded.

The results of body or relative organ weights, hematological examination, and mitogen-induced blastogenesis suggest that the implantation of test materials did not cause systemic toxicity or decrease immune activity (Tables 1 and 2, Fig. 3). The membrane thickness around the Cr specimen was significantly higher than that around the Ti–Zr alloy specimen (Table 3). The membranes that formed around the Ti–Zr and Ti specimens were similar in thickness to that around the 316L-type stainless steel (SUS316L) specimen, which is used clinically.[8,26] Fibroblasts migrated to the injury site around the implants in the early phase and increased in density with an increasing amount of extracellular collagen. The tightly formed fibroblast membrane inhibits circulation of biological substances to inside the membrane, possibly

with negative consequences. However, the membrane thickness is related only to fibroblasts but not to other cells, so it has debatable utility as an index of tissue response in implantation studies that cover long periods.

Local tissue response to a material is the most important aspect of the material's biocompatibility, and is mainly related to inflammatory reaction.[15] It can be evaluated by analyzing the cell population or the morphological characteristics of the tissue around the implant.[8,21,22] Ryhänen *et al.* found no qualitative differences in histology between stainless steel, Ti–6Al–4V, and Ti–Ni alloy 26 weeks after implantation in rats.[8] Macrophages are the main inflammatory cell types in a short-term implantation. They play an important role in acute inflammation and probably in determining the final biocompatibility of an implanted material.[33,34] They release various mediators that influence the activities of fibroblasts, lymphocytes, and other cells.[33,34] In addition, macrophages form multinucleated foreign-body giant cells, which constitute evidence of a specific inflammatory response evoked by a foreign substance.[35] Two of the six animals in the Ti-implant group showed a moderate inflammatory response. In all animals in the Ti–Zr alloy-implant group, the frequency of cell infiltration into the membrane was smaller than in other implanted groups (Fig. 2). The numbers of macrophages and inflammatory cells in the fibrous tissue around the Cr and Ti specimens were higher than those around the Ti–Zr and Zr specimens (Table 3). In the Ti- and Cr-implant groups, giant cells were found. These observations indicate that the Cr and Ti specimens induced inflammatory reactions more strongly than did the Ti–Zr and Zr specimens. Neutrophils, eosinophils, and lymphocytes are involved in immune responses.[8,36] The infiltration frequency of these cells into the fibrous membrane was especially high in the Cr-implant group. A small increase in these cells was observed in the Ti-implant group relative to the control group. However, the number and type of infiltrating cells did not differ among the tested materials. The histological examination did not allow us to clearly say whether or not hypersensitive response occurred in an animal. The total scores obtained in the Ti–Zr alloy- and Zr-implant groups were significantly lower than that in the Cr-implant group. The total score in the Ti–Zr alloy-implant group was the same as or less than those of the Ti-implant and SUS316L-implant groups, the latter of which scored 7.3 ± 1.5 in the previous study.[26] These results suggest that the Ti–Zr alloy has good biocompatibility.

The patch test using metal salts is the most widely used method for evaluating metal allergy.[30,37] The *in vitro* lymphocyte proliferation test is also considered useful for assessing allergic conditions or immune activity.[38,39] If a positive reaction appears following the implantation of a metallic device, the contribution of the device to the development of metal sensitivity should be considered. We previously observed that animals became sensitized by the high amount of Cr ion released from the easily corrosive Cr–Fe alloy implanted in them.[5,26] The stable oxide complex layer that formed on the surfaces of Ti, Zr, and their alloys allowed little release of their ions.[16,18,25] Although highly unusual, cases of contact sensitivity to pacemakers which are made of Ti have been reported.[27,28] A positive skin reaction to titanium chloride was obtained by a sensitization test using guinea pigs.[38] Topical exposure to Ti and Zr salt solutions in rats implanted with Ti, Zr, or Ti–Zr alloy specimens resulted in no skin responses. Ti and Zr salts did not stimulate the proliferation of lymphocytes in these implanted animals (Table 4). None of these materials caused blastogenesis of spleen lymphocytes in the groups implanted with them (Fig. 3). These results indicated that the implantation of the Ti, Zr, and Ti–Zr alloy specimens did not induce sensitization to Ti or Zr ions. This may be explained by the weak sensitization potentials of Zr and Ti ions. Or it may be that the amount of ions released from each specimen was insufficient to cause a sensitization response.

As a result, the Ti–Zr alloy and Zr had better biocompatibility than Ti and Cr. Considering the fragility of Zr, we concluded Ti–Zr alloy as the best material tested in this study. For orthopedic implants, the hardness of metallic materials is important. However, for clinical application, other factors are more important: wear resistance, fretting corrosion resistance, and mechanical/tensile strength such as Young's modulus determine a material's suitability for the targeted position or tissue. In the future, we intend to design new alloys using a Ti–Zr base to serve this purpose.

5. Conclusion

By implanting Ti, Zr, Cr, or Ti–Zr alloy into rats for 8 months, we evaluated the tissue response around the implant and the development of metal sensitization. There was no toxicological change in body or organ weights or in hematological parameters. The tissue inflammatory responses to the Ti–Zr alloy were lower than those to Ti. No sensitization response to the Ti–Zr alloy appeared. The Ti–Zr alloy has better biocompatibility than Ti for use as an artificial surgical implant.

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ORIGINAL ARTICLE

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Biodegradable polymers in chondrogenesis of human articular chondrocytes

Abstract The aim of this study was to evaluate the potential role of polyglycolic acid (PGA), poly(glycolic acid-ε-caprolactone) (PGCL), poly(L-lactic acid-glycolic acid) (PLGA), poly(L-lactic acid-ε-caprolactone, 75:25 (w/w)) [P(LA-CL)25], poly-ε-caprolactone (tetrabutoxy titanium) [PCL(Ti)], and fullerene C-60 dimalonic acid (DMA) in cartilage transplants. After 4 weeks of culture of human articular cartilage, the levels of cell proliferation and differentiation and the expression of cartilage-specific matrix genes were estimated. The relationship between cell differentiation and gap junction protein connexin 43 (Cx43) was also evaluated. All materials except PCL(Ti) retained cell proliferation activities similar to the controls. Cell differentiation levels from the highest to the lowest were in the following order: PGA >> PLGA > PGCL > Control = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C-60 DMA. Expression of the collagen type II gene was selectively upregulated for PGA, PGCL, and PLGA and slightly increased for P(LA-CL)25 polymers but was downregulated for fullerene C-60 DMA. Aggrecan gene expression was strongest with PGA and was consistently expressed with other matrices, especially with PGCL and PLGA. However, the expression patterns of the connexin 43 gene were different from the former two genes. Multiple regression analysis revealed a high correlation between cartilage proteoglycans production and expression levels of these three genes.

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Introduction

A shortage of donor tissue restricts the successful application of tissue reconstruction for various cartilage injuries. Tissue engineering is a relatively new and promising field directed at the evolution of new tissues that will offer hope to orthopedic patients with a variety of injuries. To permit repair of cartilage defects, many researchers are turning toward a tissue engineering approach involving cultured cells and biomaterials. Although these biomaterials, especially polyglycolic acid (PGA) and poly(L-lactic acid) (PLLA), play an increasingly important role in orthopedics, adverse reactions to these biomaterials have been reported in animal experiments. PLLA produces toxic substances due to acidic degradation,[1] and long-term implants of PLLA produced tumorigenicity in rats.[2] Despite these setbacks, numerous studies have documented the biocompatibility of these bioabsorbable polymers.[3–7] PLLA, PGA, and their copolymers also have been used in clinical practice.[5,8] More recent studies have indicated that copolymers of glycolic acid promoted peripheral nerve regeneration in a rat model.[9,10] These polymers are degraded by hydrolysis and enzymatic activity and have a range of mechanical and physical properties that can be engineered appropriately to suit a particular application.

Knowledge of the biological interactions between chondrocytes and biodegradable polymers is needed to design novel biomaterials and to develop new strategies for cartilage repair. Therefore, further experimental elucidation of these polymers, their combination with other biomaterials, and new materials to find good substrates is essential to attain satisfactory conditions for their clinical application. In this study, along with PGA and poly(L-lactic acid-glycolic acid) (PLGA), we investigated the copolymer poly(glycolic acid-ε-caprolactone) (PGCL), the copolymer poly(L-lactic acid-ε-caprolactone) 75:25 (w/w) P(LA-

CL)25, and poly-ε-caprolactone (tetrabutoxy titanium) [PCL(Ti)] to determine their effects on human articular chondrocyte (HAC) proliferation, differentiation, and phenotypic expression with the aim of clarifying their suitability as carriers for future clinical cartilage transplants. Fullerene C-60 dimalonic acid (DMA) has been reported to stimulate[11] and inhibit[12] proliferation and differentiation of rat embryonic limb bud cells and mouse embryo midbrain cells, respectively, and in the present study we also investigated the effect of fullerene C-60 DMA on HACs.

Gap junctions are intercellular channels supporting direct cell-to-cell communication and tissue integration.[13] Connexins, the family of proteins that form vertebrate gap junctions, play key roles during development and in the adult. Among the 19 connexins that have been identified in mammals, the gap junction protein connexin 43 (Cx43) is the most abundant member of the channel-forming proteins in chondrocytes.[14,15] The distribution of Cx43 in hyaline cartilage and in the perichondrium of mouse and rat knee joints suggested a possible involvement of connexins in cartilage development.[16] It has been indicated that the early stage of in vitro chondrocyte differentiation is the formation of cell condensations and the ability to establish cell-to-cell communication. Cx43, together with other molecular mechanisms, mediates the condensation phase of chondrogenesis.[17] In the present study, we investigated the role of gap junctional protein Cx43 in the process of chondrocyte differentiation.

Materials and methods

Materials

HACs from knee joints and chondrocyte growth medium were commercially obtained from BioWhittaker (Walkersville, MD, USA). Chondrocyte growth medium contains bovine insulin, basic fibroblast growth factor, insulin-like growth factor-1, transferrin, gentamicin sulfate, and fetal bovine serum (5% v/v). PGA (mw 3000) and PLGA (mw 5000) were purchased from Nakalai Tesque (Kyoto, Japan) and PGCL (mw 3000) was from Taki Chemical (Hyogo, Japan). P(LA-CL)25 (mw 10000) and PCL(Ti) (mw 130000) were synthesized in our laboratory and fullerene C-60 DMA was obtained from Dr. T. Mashino.[18]

Synthesis of P(LA-CL)25

L-Lactide (Tokyo Kasei Kogyo, Tokyo, Japan) 7.5 g and caprolactone (Wako Pure Chemical Industries, Osaka, Japan) 2.5 g were put into a reactor as monomers. As a catalyst, tetrabutoxy titanium (Wako) 0.03 g was added. Furthermore, *n*-octyl alcohol (Wako) 0.001 g was added. These were completely dissolved in methylene chloride (Wako) 50 mL at room temperature. Methylene chloride was removed by decompression and a uniform mixture was left. The reactor was filled with nitrogen and was sealed. The contents were mixed and heated to 140°C. Polymerization was carried out for 4 h. After the reaction, the reactant was cooled to room temperature, and was dissolved in tetrahydrofuran 100 mL. The solution was dropped into cold methanol and a colorless precipitate was obtained. This was dried under reduced pressure and precipitation was done once again. This was again dried under reduced pressure and the polymer was obtained. The yield was 58.2% (5.82 g).

Synthesis of PCL(Ti)

Synthesis was done using the same method as described for the synthesis of P(LA-CL)25 except that the monomer was only caprolactone (Wako). The yield was 87.1% (8.71 g).

Preparation of materials

PGA, PGCL, PLGA, and P(LA-CL)25 were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 50 μg/0.8 μl of DMSO (Sigma-Aldrich, Irvine, CA, USA) and then dissolved in chondrocyte growth medium to give a final concentration of 50 μg/ml. PCL(Ti) was dissolved in tetrahydrofran (THF) at a concentration of 5 mg of PCL/ml of THF. Glass wells were coated with this solution to give a final concentration of 2 mg PCL(Ti)/well. A homogenous solution of fullerene C-60 DMA was made with the chondrocyte growth medium.

Cell culture

In vitro high-density micromass cultures of HACs were initiated by spotting 4×10^5 cells in 20 μl of medium onto each well of 12-well microplates for tissue culture (Costar Type 3513, Corning, Corning, NY, USA) and PCL(Ti)-coated glass wells (diameter 22 mm). After 2 h in a 5% CO_2 incubator at 37°C, the wells were flooded with chondrocyte growth medium (2 ml/well). The medium was supplemented with DMSO (0.8 μl/ml), PGA (50 μg/ml), PGCL (50 μg/ml), PLGA (50 μg/ml), P(LA-CL)25 (50 μg/ml), or fullerene C60 DMA (50 μg/ml). HACs cultured on tissue culture polystyrene but not exposed to any biomaterials served as a control. The media were changed in every 3 days and culture was continued for 4 weeks.

Cell morphology assay

Cell morphology was determined by inverted light microscopy. Twice weekly observations were done and photographs were taken with Fuji film.

Proliferation assay

Cell proliferation was quantitatively measured by alamar blue (Biosource International, Camarillo, CA, USA) assay after 4 weeks of culture, as previously described.[18] The assay

demonstrates the metabolic activity of the cells by detection of mitochondrial activity. The indicator dye alamar blue is incorporated into the cells and reduced and excreted as a fluorescent product. At the end of the 4-week culture period, the medium from all wells was discarded and the culture wells and three blank wells were filled with 1 ml/well of 5% alamar blue solution in fresh medium. The culture plates were incubated at 37°C for 4 h. After the incubation period, two aliquots of 100 µl of solution from each well were transferred to new wells of a Costar 96-well tissue culture microplate (Costar Type 3595, Corning). The extent of cell proliferation was quantitated by a Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Framingham, MA, USA) at 535 nm for excitation and 590 nm for emission. The intensity of the blue color obtained was directly proportional to the metabolic activity of the cell populations. Blank values were subtracted from experimental values to eliminate background readings.

Proteoglycan production assay

Proteoglycans are typical components of the cartilage matrix. The extent of chondrogenesis was determined by staining the cartilage-specific proteoglycans with alcian blue (Wako) as described previously.[11,19] Briefly, the cultures and three blank wells were stained overnight at 4°C (0.5 ml/well) with 1% (v/v) alcian blue, pH 1.0. The alcian blue solution was then removed and the micromass cultures and blank wells were rinsed with 3% (v/v) acetic acid and distilled water to completely remove the free dye. The cartilage proteoglycans were extracted using 4-M guanidine hydrochloride, and the absorbance was measured at a wavelength of 600 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek Instruments, Winooski, VT, USA). Blank values were subtracted from experimental values to eliminate background readings.

RNA harvest

After the 4-week culture period, RNA was extracted from all matrices except the PCL(Ti) matrix. For the PCL(Ti) matrix, we did not have enough samples to harvest RNA because cells from 50% of the cultured wells became detached overnight following cell spotting. Total cellular RNA was extracted from cultured cells of four wells (for each material) in 0.5 ml Trizol reagent (Life Technologies, Frederick, MD, USA) according to the manufacturer's instructions. The concentration of total RNA was determined using a UV spectrophotometer (Gene Quanta, Pharmacy Biotech, Piscataway NJ, USA) at 260 nm.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The matrix molecules probed as part of this study were collagen type II and aggrecan. The gap junction protein gene Cx43 was also studied. Single-strand cDNA was prepared from 1 µg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After optimization of PCR conditions, subsequent PCR was performed with 4 µg of cDNA in a 20-µl reaction mixture (10 × PCR buffer 2 µl, dNTP 1.6 µl, forward and reverse primer 0.4 µl, Taq DNA polymerase 0.1 µl, and distilled water to make up 20 µl). The codon sequence used for the primer sets was as follows:

Collagen type II:
forward 5′-GGCAATAGCAGGTTCACGTACA-3′
reverse 5′-CGATAACAGTCTTGCCCCACTT-3′

Aggrecan:
forward 5′-TCGAGGACAGCGAGGCC-3′
reverse 5′-TCGAGGGTGTAGCGTGTAGAGA-3′.

Connexin 43 (*Homo sapiens*):
forward 5′-ATGGGTGACTGGAGCGCCTTAGGCAA ACTC-3′
reverse 5′-GACCTCGGCCTGATGACCTGGAGATC TAG-3′

For collagen type II and Cx43, an initial denaturation step at 94°C was carried out for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. For aggrecan, an initial denaturation at 95°C was carried out for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min), with a final extension at 72°C for 5 min. The polymerization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was accomplished by 25 cycles with a corresponding PCR program. Electrophoresis of PCR products was done on 3% agarose gel for visualization of collagen type II and aggrecan and on 1% agarose gel for Cx43 after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). The relative intensity of signals from each lane was analyzed with a computerized scanner. For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene, GAPDH:

forward 5′-CCCATCACCATCTTCCAGGAGCGAGA-3′
reverse 5′-TGGCCAAGGTCATCCATGACAACTTTG G-3′.

Statistical analysis

Comparing the control with samples exposed to various materials assessed the statistical significance of the cell proliferation and cartilage proteoglycans production. Student's *t* test was used to assess the statistical significance. Statistical significance was taken as $P < 0.05$. Data were indicated as the mean ± SD (standard deviation). Four or five cultures were run for each biomaterial. All experiments were repeated at least twice, and similar results were obtained.

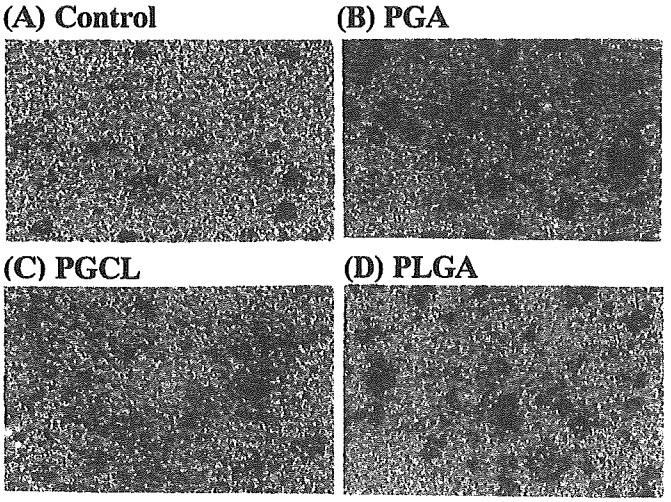

Fig. 1A–D. Light microscopic appearance of cultured human articular chondrocytes spotted as a high-density micromass culture with different biodegradable polymers for 4 weeks. **A** Control, **B** polyglycolic acid (PGA), **C** poly(glycolic acid-ε-caprolactone) (PGCL), **D** poly(L-lactic acid-glycolic acid) (PLGA). Original magnification ×200

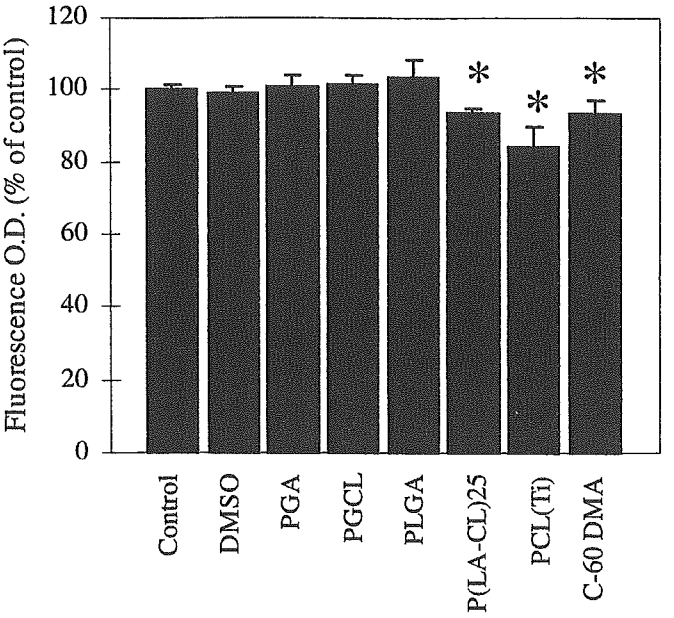

Fig. 2. Cell proliferation of human articular chondrocytes as determined by alamar blue assay after culturing with synthetic biodegradable polymers for 4 weeks. The proliferation in all samples exposed to dimethyl sulfoxide (*DMSO*) and biomaterials were calculated as a percentage of control values. *P(LA-CL)25*, poly(L-lactic acid-ε-caprolactone) 75:25 (w/w); *PCL(Ti)*, poly-ε-caprolactone (tetrabutoxy titanium); *C-60 DMA*, fullerene C-60 dimalonic acid. $*P < 0.05$ and *error bars* represent standard deviations of the mean

Results

Cell morphology

Cells were aggregated as high-density micromass cultures 2 h after cell spotting. After 4 weeks of culture, the chondrocytes mainly formed a uniform sheet of chondrogenic cells with nodules. The cartilage nodules were first observed in the first week of the culture. These nodules were better visualized by staining the proteoglycans with alcian blue after 4 weeks of culture. The control cells showed less nodule formation and they were poorly defined (Fig. 1A). The cultures exposed to the PGA and PLGA had more distinct nodules and greater numbers of nodule formations than the controls (Figs. 1B and 1D). The nodules formed in the culture exposed to PGCL were less distinct and fewer in number than the nodules in the cultures exposed to PGA and PLGA, but were more distinct and numerous than the nodules of the control cultures (Fig. 1C). After alcian blue staining, light microscopic examination also revealed that PGA-, PGCL-, and PLGA-treated cultures contained denser extracellular matrix (ECM) than the controls. Cells extended from the edge of all micromass cultures, and the extending cells were spindle-shaped.

Cell proliferation assay

The proliferation rates of all the matrices are shown in Fig. 2, with error bars representing the standard deviation of the mean. All values for the samples exposed to the biomaterials were expressed as a percentage of the control average value, which was taken as 100%. The effect of DMSO on cell proliferation was not significant (99.3% ± 1.6%). The cell proliferations for PGA, PGCL, and PLGA were fairly parallel to that of control cell proliferation. The cell proliferation for P(LA-CL)25, PCL(Ti), and fullerene C-60 DMA were significantly inhibited compared to the control. The inhibitions for P(LA-CL)25 and fullerene C-60 DMA were mainly due to the small variation of the standard deviation. Despite being significantly different from the control, both proliferation values were fairly close to the control proliferation value.

Therefore, from the standpoint of cell proliferation, all materials except for PCL(Ti) remained viable candidates for tissue engineering. The values of cell proliferation for the samples exposed to PGA, PGCL, PLGA, P(LA-CL)25, PCL(Ti), and fullerene C-60 DMA were 101% ± 2.7%, 101.6% ± 2.2%, 103.5% ± 4.8%, 93.2% ± 1.4%, 84.3% ± 5.1%, and 93.6% ± 3.7%, respectively.

Proteoglycan synthesis

The proteoglycans bound with alcian blue were extracted with 4-M guanidine hydrochloride. Their levels were expressed as a percentage of the average control value, which was taken as 100% (Fig. 3). The intensity of alcian blue staining was found to be higher in PGA-, PGCL-, and PLGA-containing cultures than in the control culture. Among the biomaterials, PGA caused a significant 3.1-fold increase in cartilage proteoglycans compared to the control ($P < 0.05$). The samples exposed to PGCL (116.2% ± 10.1%) and PLGA (128.4% ± 11.1%) also produced

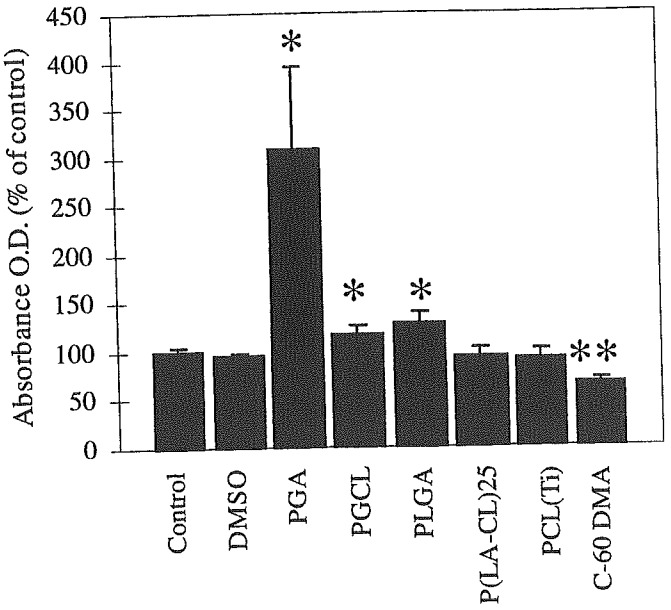

Fig. 3. Cartilage proteoglycan content of human articular chondrocytes as determined by the alcian blue staining method after culturing with synthetic biodegradable polymers for 4 weeks. The values are expressed as a percentage of control values. $*P < 0.05$ and $**P < 0.01$

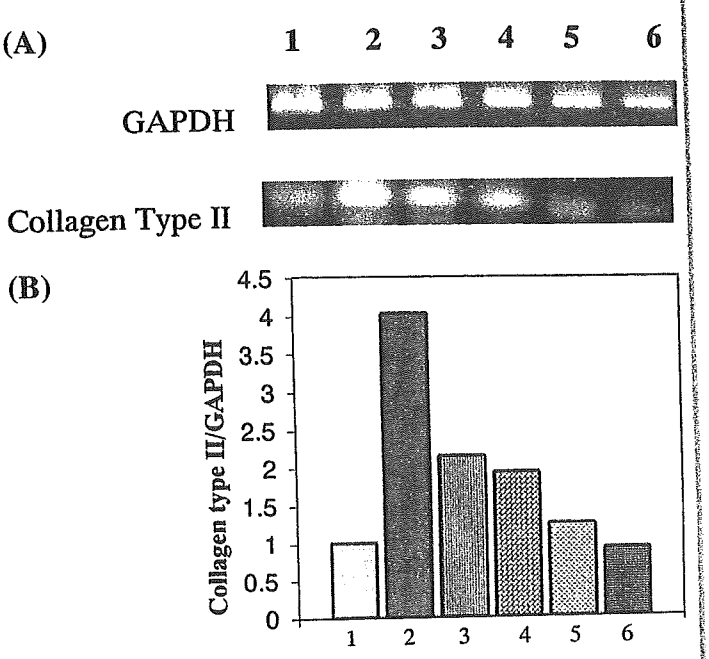

Fig. 4. Reverse transcription polymerase chain reaction (RT-PCR) analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of collagen type-II gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable polymers. The mRNA expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for comparing the level of expression. **A** Lane 1, Control; lane 2, PGA; lane 3, PGCL; lane 4, PLGA; lane 5, P(LA-CL)25; lane 6, Fullerene C-60 DMA. **B** Bar 1, Control; bar 2, PGA; bar 3, PGCL; bar 4, PLGA; bar 5, P(LA-CL)25; bar 6, Fullerene C-60 DMA

significantly higher cartilage proteoglycans than the control. Copolymers P(LA-CL)25 (92.7% ± 10.5%) and PCL(Ti) (90.8% ± 9.1%) did not induce significant changes in cartilage proteoglycans compared to the control. Fullerene C60 DMA acted as a potent inhibitor (66.1% ± 4.7%) and caused a significant inhibition of cartilage proteoglycans ($P < 0.01$) compared to the control. The effect of DMSO (96% ± 1.1%) on cell differentiation was negligible.

Extracellular matrix gene expression

RT-PCR and corresponding National Institutes of Health (NIH) image analysis showed that all matrices consistently supported the expression of the collagen type II gene and that the PGA matrix had the strongest induction (Fig. 4). Slight increases in expression of the collagen type II gene were noted with PGCL, PLGA, and P(LA-CL)25 matrices. Expression of the collagen type II gene for fullerene C60 DMA was similar to the control. The PGA matrix also showed the strongest induction of the aggrecan gene (Fig. 5). Aggrecan gene expression was slightly increased in PGCL and PLGA matrices. The P(LA-CL)25 matrix caused an expression of this gene similar to that of the control, but the fullerene C60 DMA matrix caused decreased expression of this gene.

Expression of gap junction protein connexin 43 gene

To determine the expression of gap junctions during in vitro chondrocyte differentiation, RT-PCR and corresponding

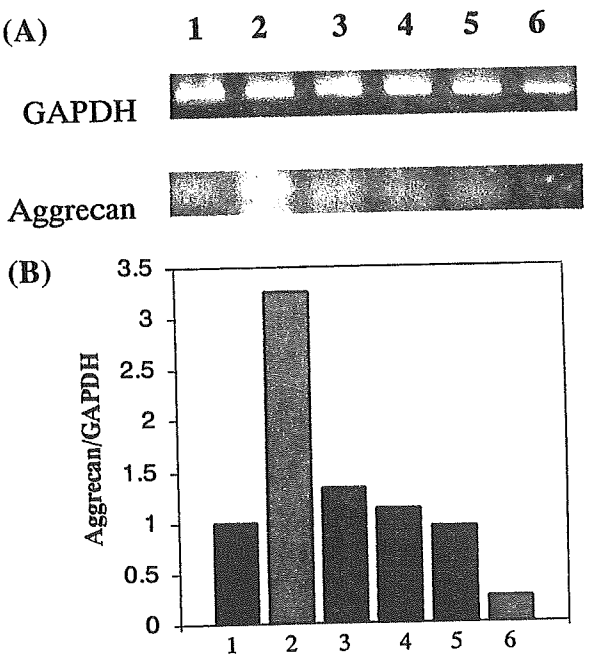

Fig. 5. RT-PCR analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of aggrecan gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable polymers. The mRNA expression of housekeeping gene GAPDH was used for comparing the levels of expression. *Lanes* and *bars* as defined in Fig. 4

Determination of Benzo[*a*]pyrene, Benz[*a*]anthracene and Dibenz[*a*,*h*]anthracene in Creosotes and Creosote-Treated Woods

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The amount of benzo[*a*]pyrene (BaP), benz[*a*]-anthracene (BaA), and dibenz[*a*,*h*]anthracene (DBA) has been restricted to a concentration of 10 μg/g each in creosotes, and 3 μg/g each in creosote-treated woods, respectively, because of the possibility of the risk of skin cancer in consumers, and creosotes can otherwise contain high concentrations of each chemical. We already reported the content of 16 polycyclic aromatic hydrocarbons (PAHs) and phenols in creosotes and creosote-treated wood as determined by gas chromatography-mass spectrometry (GC-MS) and absorptiometry [*Chemosphere*, 60, 1279–1287 (2005)]. However, the limit of determination of each PAH per sample was > 40 μg/g according to that method, the sensitivity of which was insufficient for determining the allowable levels of these 3 compounds. Moreover, a substantial amount of time was needed for GC-MS analysis. In the present study, we improved upon our previous analytical method in order to increase the sensitivity of the test and to reduce the duration of GC-MS analysis. Creosote was extracted from treated wood samples by dichloromethane-soak incubation, and was placed on a Sep-Pak silica cartridge and eluted with dichloromethane. The eluates were evaporated and dissolved in dichloromethane. The sample solution spiked with the internal standard solution was injected into the GC-MS system. The limit of determination of each chemical in the test solution was approximately 0.2 μg/ml, which corresponded to 1–2 μg/g in each sample. The duration of GC-MS analysis was approximately 17 min. A collaborative study was also carried out in order to evaluate the reproducibility of the method for determining low levels of BaP and related compounds in creosotes. The present method was applied for the analysis of certain commercially available creosotes and creosote-treated wood samples in Japan. It was confirmed that some creosotes and railway sleepers contained these compounds in high concentrations, thus exceeding the allowed control value.

Key words —— polycyclic aromatic hydrocarbon, creosote, benzo[*a*]pyrene, GC-MS, wood preservative

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INTRODUCTION

Creosote is a mid-heavy distillate of coal tar.[1] The majority of the creosote produced to date has been used as raw material for carbon black, while much of the remainder has been used as a wood preservative [The Japan Aromatic Industry Association, Inc., http://www.jaia-aroma.com/, Japan Wood Preserving Association (JWPA), http://wwwsoc.nii.ac.jp/jwpa/]. Wood treated with creosote was formerly used for railway sleepers and poles for the transport of electricity, but creosote-treated wood is now commonly used for the foundations of buildings, fences, and stakes for agricultural use, and also for the manufacture of garden furniture and outdoor recreational facilities in parks. However, direct contact with creosote can lead to skin irritation and disease,[2,3] and is likely to be carcinogenic in humans; creosote is therefore classified as belonging to Group 2A among potential human carcinogens, as established by the International Agency for Research on Cancer (IARC).[4] Creosote contains high quantities (up to 85%) of polycyclic aromatic hydrocarbons (PAHs),[1] and the U.S. Environment Protection Agency (U.S. EPA) has defined 16 PAHs as priority pollutants.[5] Benzo[*a*]pyrene (BaP) is one of the most thoroughly investigated PAHs, and is classified as belonging to Group 2A among potential human carcinogens.[6] Therefore, BaP has been chosen as a marker for creosote treatment and is taken as an indicator for the toxicity of creosote. The Scientific Committee on Toxicity, Ecotoxicity and the Environment reported that the cancer risk from exposure to creosote is greater than previously thought.[7] Such concerns have led to a new Directive (2001/90/EC) that was adopted by the European Council,[8] according to which creosote that contains BaP at a concentration of higher than 0.005% (50 μg/g) by mass, as well as water-extractable phenols at a concentration

of higher than 3% by mass, may not be used in the treatment of wood; moreover, wood treated in such a manner may no longer be placed on the market. Each country in the European Union (EU) has thus been restricting the use of creosote since 2003.

In Japan, the recycling of disused railway sleepers as exterior wood for use in gardens has recently become popular. Disused railway sleepers imported from other countries and/or new exterior wood products have been sold at retail stores that deal in carpenter's tools and gardening supplies. Consequently, the opportunity to come into contact with creosote is increasing among the general public in Japan. In our monitoring study, more than 50 μg/g of BaP was found in creosote and creosote-treated wood products available on the market in Japan.[9] To reduce the health risk posed by creosote, the Ministry of Health, Labour and Welfare decided to restrict the use of creosotes containing elevated amounts of carcinogenic PAHs. The health risks were estimated on the basis of the opportunity and time that a child could come into contact with creosote containing BaP in Japan. Based on these considerations, the amount of BaP was restricted to the following concentrations: 10 μg/g in creosotes and 3 μg/g in creosote-treated woods. Both benz[*a*]anthracene (BaA) and dibenz[*a*,*h*]anthracene (DBA) are also classified as belonging to Group 2A, due to their carcinogenic potential. The amount of DBA is lower than that of BaP in creosote, but DBA has a similar toxic equivalency factor relative to that of BaP.[10–12] The toxicity of BaA is not as great as that of BaP, but the amount of BaA in creosotes has been reported to be several times higher than that of BaP.[9] Therefore, the amount of these 2 PAHs are also restricted to the same level as that of BaP in creosotes. Creosotes that are commercially available in Japan undergo alkaline treatment after distillation (personal communication with a manufacturer), such that the content of water-extractable phenols is slight, relative to the EU control value.[9] It is therefore not considered important to measure and restrict the content of the phenols in creosotes manufactured in Japan.

The purpose of this study was to improve our previous analytical method[9] for the simultaneous determination of low levels of various PAHs (primarily BaP, BaA, and DBA) in creosotes and creosote-treated woods. Bestari *et al.* investigated the PAH content in wood products and the leaching behavior of creosote-treated wood by high-performance liquid chromatography (HPLC).[13] Anklam *et al.* used a HPLC system equipped with a fluorescence detector for the determination of the BaP content in creosotes.[14] HPLC determination is sensitive to PAHs, but the identification of individual PAHs by comparison of their retention time is less accurate than the use of gas chromatography (GC); furthermore, it remains difficult to detect 3 PAHs simultaneously when using fluorescence HPLC. A GC system equipped with a capillary column and mass selective-ion-monitoring (SIM) is useful for detecting each chemical selectively and provides sufficient separation for the quantification of the PAHs in a complex.[15–22] Therefore, we used GC-mass spectrometry (GC-MS) for the determination of PAHs in creosotes. With our previous method, the sensitivity of detection was low with respect to the determination of 3–10 μg/g of PAHs in creosote and creosote-treated wood products,[9] and a long period of time was required for GC-MS analysis, *i.e.*, more than 30 min per sample. Therefore, we adopted an evaporation-concentration step and changed the column temperature conditions in order to increase the sensitivity of testing and to reduce the amount of time needed to perform GC-MS.

MATERIALS AND METHODS

Samples —— Four commercially available creosotes (Nos. 1–4) and non-creosote type (creosote alternative) oil-based wood preservative paints were purchased from stores in Tokyo Metropolis, and in Gumma and Kagawa prefectures. Four creosote (Nos. 5–8) were provided by the Japan Aromatic Industry Association, Inc. (JAIA). The three trial products used for the collaborative study (codes A–C) were supplied by a manufacturer. Three new creosote-treated wood products were supplied by the Japan Wood Preservers Industry Association (JWPIA). The JWPIA reported that two of the products (samples B and C) were Kempas, those were used for railway sleepers. The other sample was Japanese hemlock, which was used for building foundations (sample A). Two previously used railway sleepers (samples D and E) that had been treated by creosote penetration and were imported for use in gardening were also purchased. One of these samples was pine wood. Sample F was a brand-new wooden stake made with Japanese cedar, the surface of which was painted with creosote.

Chemicals —— An EPA PAH mixture containing acenaphthene, acenaphtylene, anthracene, Ba