パタイトのSEM像である[14]。針状または板状ナノアパタイト微結晶がCNTを基点に放射状に成長している。析出や結晶性、形態は溶液と基材の条件に依存して変化する。同様な現象はいわゆるバイオミメティック・コーティングとして、擬似体液に浸漬したTi表面にリン酸カルシウムが析出する例がよく知られている。

図5はヒト歯牙をリン酸でエッチングしたあとの象牙質で、脱灰により残留したコラーゲン線維が象牙細管内を含め一面にみられ、その上にCNTが吸着している様子が観察される。CNTはコラーゲンほかのタンパク質に対する非特異的な吸着性や親和性を有する[1]。

図6は骨芽細胞様細胞Saos2を培養したときの細胞末端のSEM像で、スカフォールドとしてポリカーボネート膜(a)、CNT(b)を用いた場合を比較したものである。培養細胞はCNT上で高い増殖・伸展性を示し、末端から多数の微細な細胞突起を遠方にまで張り出し強く付着する。同素体のグラファイトでは細胞がきわめて付着しにくく、その血球に対する抗血栓性を生かして人工心臓弁材料として使用されているが、CNT上では細胞はきわめて高い接着性と良好な増殖性を示す[20]。

培養後の増殖細胞数を測定するにはスカフォールドから分離する必要があり、通常細胞外マトリックスの接着性タンパク質を溶解するトリプシン処理がなされる。図7はCNTスカフォールド上の培養細胞のトリプシン処理後の細胞末端のSEM像である。細胞末端から張り出した多数の細胞突起がCNTと機械的に結合しているために、細胞がはく離せず離れない様子が観察される[20]。CNTの細胞に対する高い接着性を反映した現象である。

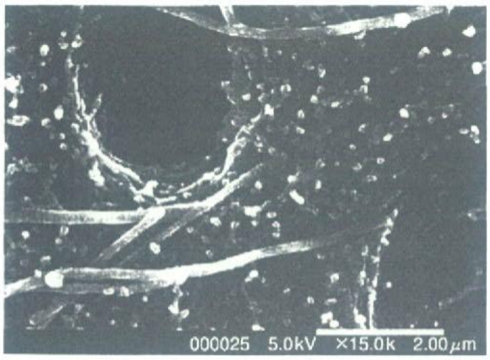

図5 脱灰象牙質表面のコラーゲン線維に吸着するCNT[14]

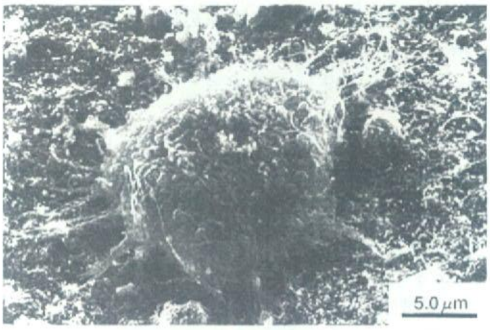

図7 トリプシン処理後のCNTスカフォールド上の培養細胞のSEM像
細胞末端から張り出した多数の突起がCNTと機械的に結合しているために、細胞がはく離できない[20]

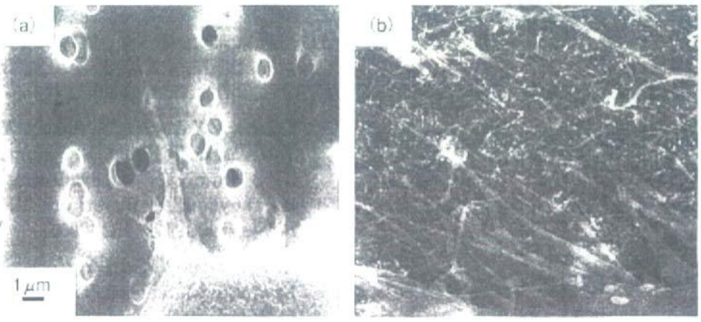

図6 ポリカーボネート膜(a)、CNT(b)の各スカフォールド上で培養した細胞末端のSEM像
CNT上で培養細胞は高い増殖・伸展性を示し、末端から多数の細胞突起を張り出し強く付着する[20]

また細菌に対しても付着性を示す結果が得られている。

9 まとめ

筆者らは2000年以来、ナノ/マイクロ微粒子の生体反応性を調べ、これに基づくバイオ応用を提起してきた。この間、2003年にはナノトキシコロジーという用語が現れ、2005年にはアスベスト問題が再燃した[19]。ナノテクノロジーの人体へのバイオ応用にはあらかじめミクロ/ナノ微粒子の生体反応性に関する適切な理解と指針が必要である。材料のナノサイジングは化学反応を昂進するから、人間の意向とマッチすれば高機能性として働き、一方意図せずして為害性に働くナノトキシコロジーとして現れたとしても現象の本質は同じであり不思議ではない。ナノ微粒子は生体防御機構が想定していない対象である可能性があり、高機能性と刺激性の両面を併せ持ち、その制御が重要である。CNTについては以下のような特性が認められる。

(1) サイズ的には呼吸器系中の気管支を通過し肺胞に到達する可能性がある。

(2) アスベストの微粒子形態は顕著な直線状を示しほとんど湾曲することがなく、また親水性で容易に分散するのに対し、CNTでは屈曲性に富み、疎水性で凝集しやすい点で、やや異なる挙動を示す。

(3) CNTは材質的には純炭素で基本的にはバイオイナートであり、作製時に用いられるNi、Feなどの触媒や分散に用いられる界面活性剤の方が刺激性が大きい場合がある。

(4) 触媒金属や界面活性剤等を取り除いたCNT自体はほかのバイオアクティブ、バイオイナートなミクロ/ナノ微粒子と同様に、材料に非特異的な物理的サイズ効果に由来する微弱な刺激性を示す。

(5) 細胞刺激性、起炎性においてサイズ依存性、結晶構造依存性を示す。

(6) 以下のようなバイオ応用に適した特性を有する。

①(人工)体液中でアパタイトが表面に析出する。

②糖鎖、タンパク質に対する親和性が高く、非特異的な吸着特性を示す。

③細胞に対する高い細胞付着・伸展性を示す。

(7) 長期体内に残留した場合の体内挙動はアスベストとはやや異なるものの、今後の解明が必要である。

〔引用・参考文献〕

1) F. Watari, K. Tamura, A. Yokoyama, K. Shibata, T. Akasaka, B. Fugetsu, K. Asakura, M. Uo, Y. Totsuka, Y. Sato and K. Tohji：Handbook of Biomineralization, Vol.3, Ed. E. Bauerlein, Wiley-VCH, Weinheim, p.127-144 (2007).

2) 亘理文夫：バイオマテリアル―生体材料―, **24** (5), 300-310 (2006).

3) F. Watari, M. Inoue, T. Akasaka, N. Sakaguchi, H. Ichinose and M. Uo：6th Proc. Asian BioCeramics Symp. 2005, p.142-145 (2006).

4) 佐藤義倫, 宇尾基弘, 横山敦郎, 柴田健一郎, 亘理文夫, 田路和幸：表面, **44** (3), 87-103 (2006).

5) 佐藤義倫, 横山敦郎, 柴田健一郎, 亘理文夫, 田路和幸：炭素, **225**, 364-372 (2006).

6) M. Ushiro, K. Uno, T. Fujikawa, Y. Sato, K. Tohji, F. Watari, W. Chun, Y. Koike and K. Asakura：*Physical Review*, B 73, 144103/1-11 (2006).

7) Y. Sato, K. Shibata, H. Kataoka, S. Ogino, B. Fugetsu, A. Yokoyama, K. Tamura, T. Akasaka, M. Uo, K. Motomiya, B. Jeyadevan, R. Hatakeyama, F. Watari and K. Tohji：*Molecular BioSystems*, **1**, 142-145 (2005).

8) 熊澤龍一郎, 亘理文夫, 戸塚靖則：歯科材料・器械, **20** (6), 356-365 (2001).

9) R. Kumazawa, F. Watari, N. Takashi, Y. Tanimura, M. Uo and Y. Totsuka：*Biomaterials*, **23**, 3757-3764 (2002).

10) K. Tamura, N. Takashi, R. Kumazawa, F. Watari and Y. Totsuka：*Materials Transactions*, **43** (12), 3052-3057 (2002).

11) 亘理文夫, 田村一央, 高師則行, 宇尾基弘, 赤坂司, 戸塚靖則, 古月文志, 佐藤義倫, 田路和幸：ナノ学会会報, **2** (1), 33-37 (2003).

12) Y. Sato, A. Yokoyama, K. Shibata, Y. Akimoto, S. Ogino, Y. Nodasaka, T. Kohgo, K. Tamura, T. Akasaka, M. Uo, K. Motomiya, B. Jeyadevan, M. Ishiguro, R. Hatakeyama, F. Watari and K. Tohji：*Molecular BioSystems*, **1**, 176-182 (2005).

13) A. Yokoyama, Y. Sato, Y. Nodasaka, S. Yamamoto, T. Kawasaki, M. Shindoh, T. Kohgo, T. Akasaka, M. Uo, F. Watari and K. Tohji：*Nano Letters*, **5** (1), 157-161 (2005).

14) T. Akasaka, F. Watari, Y. Sato and K. Tohji：*Material*

Science & Engineering C, **26**, 675–678 (2005).

15) K. Kiura, Y. Sato, M. Yasuda, B. Fugetsu, F. Watari, K. Tohji and K. Shibata：*J. Biomed. Nanotechnology*, **1**, 359–364 (2005).

16) I. D. Rosca, F. Watari, M. Uo and T. Akasaka：*Carbon*, **43**, 3124–3131 (2005).

17) W. Wang, M. Omori, F. Watari and A. Yokoyama：*Dent. Mat. J.*, **24** (4), 478–486 (2005).

18) B. Fugetsu, S. Satoh, A. Iles, K. Tanaka, N. Nishi and F. Watari：*The Analyst* (London), **129**, 565–566 (2004).

19) 亘理文夫編：厚生労働科学研究補助金　萌芽的先端医療技術推進研究事業　ナノメディシン分野，ナノチューブ，ナノ微粒子，マイクロ微粒子の組織応用性とバイオ応用，平成14～16年度総合研究報告書p.1–377 (2005)．同 総括・分担研究報告書，(平成16年度) p.1–227 (2005)，(平成15年度) p.1–262 (2004)，(平成14年度) p.1–168 (2003)．

20) N. Aoki, A. Yokoyama, Y. Nodasaka, T. Akasaka, M. Uo, Y. Sato, K. Tohji and F. Watari：*Chem. Let.*, **35**, 5, 508–509 (2006).

＜亘理　文夫＞

酸化チタン・有機高分子複合人工骨の開発

Development of Artificial Bone Composed of TiO_2 Nanoparticles and High Density Polyethylene

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Abstract

Composite of TiO_2 nanoparticles with high-density polyethylene (HDPE/TiO_2 composite) has been developed as a bone substitute with analogous mechanical properties to those of the bone. The bending yield strength and Young's modulus increased with increasing pressure applied during shaping for the silane-treated HDPE/TiO_2 composite (max. bending yield strength = 65 MPa, Young's modulus = 10 GPa). The use of the silane-coupling agent and the increase of the hot pressing pressure for shaping the composite facilitated the penetration of polymer into cavities between individual TiO_2 particles, which increased the density of the composite. And also, the bone bonding was observed for the silane-treated HDPE/TiO_2 composite. This kind of bioactive material with similar mechanical property to human cortical bone is expected to be useful as load bearing bone substitutes such as vertebra and cranium.

キーワード：人工骨，酸化チタン，ポリエチレン，複合体，機械的強度，骨結合能

1　はじめに

一般に，人工材料を骨の欠損部に埋入すると，生体はそれを線維性被膜で取り囲み，周囲の骨から隔離しようとする．これは生体の正常な防御反応であるが，この反応が生じる限り人工材料を長期に渡って骨と強固に結合させることはできない．しかし，1971年頃に初めて，Hench らにより線維性被膜に覆われることなく骨と化学的に結合する性質，即ち生体活性を示す Na_2O-CaO-SiO_2-P_2O_5 系ガラスからなる Bioglass® [1] が開発された．その後さらに，焼結水酸アパタイト (HAp：$Ca_{10}(PO_4)_6(OH)_2$) [2]，アパタイトとウォラストナイトの結晶化ガラス Cerabone®A-W [3] 等，少数ながら様々な生体活性材料が開発された．これらの材料は，既に有用な骨修復材料として整形外科等の分野で広く臨床応用されるまでに至っている．

これら生体活性材料の多くは，生体内でその表面に骨類似アパタイト層を形成し，それを介して骨と自然に結合することを特徴としている．そのため，人工材料が骨と結合するためには，その表面にアパタイト層を形成することが極めて重要な条件の一つであることが明らかにされた [4]．さらに，小久保らにより，このアパタイトの形成がヒトの体液にほぼ等しい無機イオン濃度を有する擬似体液 (SBF) 中でも再現できることが示され [5]，この SBF を用いて新規材料の生体活性発現の有無を *in vitro* で簡便に評価できるようになった．

しかし，これまでに開発された人工骨の多くは，主にセラミックスや金属材料で構成されているため，高い強度と柔軟性を併せ持つ骨特有の性質を持たず，**表 1** に示すように，ヒト骨と大きく異なった機械的強度を有している [6)−8)]．特に，これら材料は骨に比べて弾性率が非常に高いため，生体埋入後に長期間人工骨に荷重が集中し，応力刺激が人工骨を支えている周囲の骨に加わらなくなると，骨の再生が抑制され，周囲の骨の吸収や萎縮を生じるストレスシールディングという現象が生じてくる．そのため，骨の長期固定性に問題があることが知られている [9]．

そのため，今日ではヒト骨に類似した力学的特性を有する人工骨の開発が望まれている．そこで，ヒト骨に倣い，無機と有機成分を複合化することにより，

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表1 ヒト骨および代表的な既存の人工骨の機械的強度

	曲げ強度/MPa	圧縮強度/MPa	破壊靭性/MPa·m$^{1/2}$	弾性率/GPa
皮質骨	50~150	100~230	2~6	7~30
Bioglass®	40~60	—	<1.0	30~35
HAp	115~200	500~1000	1	86~110
A-W	215	1080	2	118
アパタイト被覆 Ti-6Al-4V	780~1050 (引っ張り)	—	~80	105
HAPEX®	20~26 (引っ張り)	—	3	4

よりヒト骨に近い力学的特性を有する人工骨の開発が試みられるようになった．その代表的なものに，高密度ポリエチレン (HDPE) 中にミクロンオーダーのHApを分散させたHAPEX®[8),10)]がある．このHAPEX®は，既に耳小骨として実用化されており，優れた臨床成績をおさめている．しかし，HAPEX®は，もともと高靭性を付与することを目的として設計されており，ヒト骨に比べるとその強度は低い．このHAPEX®以外にも，今日では様々な無機–有機複合体の開発が試みられているが，未だヒト骨に類似した機械的強度を有する材料は開発されていないのが現状である．

そこで筆者らは，ナノサイズの無機フィラーを用い，ヒト骨のように無機と有機成分をナノレベルで複合化させることにより，ヒト骨に類似した力学的特性と生体活性を有する人工骨の開発を試みた．まず無機成分としてはアナタース型酸化チタン (TiO_2) に着目した．その選択理由には，① 高い生体活性能を有していること[11)]，② HApに比べ高い弾性率を有していること，③ ナノサイズの粒子が比較的手に入り安いことが挙げられる．特に① の特性は，人工骨にとって必須であり，本特性により，複合体に生体活性を付与することが可能となる．次に② の特性も重要で，本特性により，既存のHAPEX®より高い弾性率を有し，より生体骨に類似した機械的強度を有する複合体が得られると期待される．また③の特性により，このナノ粒子を用いることで，高充填化・高強度化が可能となり，また同じ充填量でもTiO_2の比表面積を大きく増大させ，高い生体活性を有する複合体が得られると期待される．一方，有機成分としてはポリエチレンに着目した．その選択理由には，① 生体に安全な材料であること，② 優れた切削性と高い伸張性を有していることが挙げられる．特に① の特性については，既に臨床応用に至っているHAPEX®にも使用されている．② の特性により，手術現場で患部形状に合わせて容易に加工を行える低侵襲で，有効破壊エネルギーの大きい複合体が得られると期待される．

以上のように，ナノサイズのフィラーであるTiO_2粒子をポリエチレン等の熱可塑性樹脂に分散して作製した複合材料が上記特性を発現するためには，作製段階で発生した複合材料中のTiO_2粒子の凝集，樹脂中のボイド，樹脂割れまたはフィラーと樹脂との界面剥離等の欠陥を除去することが重要である．

そこで本研究では，ナノサイズのTiO_2とHDPEを用いてHDPE/TiO_2複合体の調製を試み，TiO_2のシラン処理および成形時のホットプレス圧力を変化させた場合に，複合体の機械的強度に与える影響を調べた．また，複合体の骨結合能を家兎の脛骨に埋入することにより調べた．

2 HDPE/TiO_2複合体の調製

2.1 使用したTiO_2とHDPEの原料

原料には，アナタース型のTiO_2 (ST-41, 平均粒径 200nm, 石原産業株式会社製) とHDPE (KM698A(MFR=8.0)，ペレット状，日本ポリオレフィン株式会社製) を使用した．TiO_2は，あらかじめシランカップリング処理を行ったものを使用した．シランカップリング処理は，γ–メタクリロキシプロピルトリメトキシシランのエタノール溶液をTiO_2に噴霧した後，乾式混合し，その後，熱処理するこ

とにより行った.

2.2 TiO_2とHDPEの混練および複合体の調製

TiO_2とHDPEの混練には，バッチ式加圧ニーダ(PBV0.3, 入江商会製) を使用した．まず200℃に加熱したバッチ式加圧ニーダ中でHDPEを溶融・軟化させて高粘性状態にし，次にロータを回転させて強いせん断力を加え，そこにTiO_2を徐々に添加してHDPEとTiO_2の混練を行った．混練温度，回転数，時間，添加方法を最適化し，高充填量のTiO_2が均一に分散した混練物を得ることに成功した[12]．本実験では，TiO_2充填量を40体積%とし，種々の条件で混練を行い、得られた混練物を230℃, 圧力2.5から5.8MPaで1時間ホットプレスすることにより目的の複合体プレートの成形を行った[13]．

2.3 HDPE/TiO_2複合体の機械的特性評価

機械的特性評価用の曲げ試験片を，ダイヤモンドカッターで先の2.2で調製した複合体プレートから切り出し，#400の研磨紙で表面仕上げを行い，大きさ55×10×4mmに作製した．3点曲げ試験を，万能材料試験機（5582型, インストロン社製）を用い，すべて室温，大気雰囲気中で行った．試験条件としては，クロスヘッドスピードを1.0mm/min，支点間距離を30mmとし，55×10mmの面に引っ張り応力が加わるように荷重を加えた．試験片数を1条件につき最低5個以上とした．得られた荷重–変位曲線から曲げ強度および曲げ弾性率を算出した．曲げ弾性率に関しては，荷重–変位曲線の初期の線形領域の傾きから算出した．また，曲げ試験後の破断面を走査型電子顕微鏡 (SEM) により観察した．

2.4 HDPE/TiO_2複合体の骨結合能

骨結合能評価用試験片を，ダイヤモンドカッターで先の2.2で調製した複合体プレートから切り出し，#200の研磨紙で表面研磨を行い，大きさ$10×15×2mm^3$に加工した後，蒸留水で洗浄，乾燥させることにより作製した．その後，照度$10mW/cm^2$の紫外線を9時間照射した．

動物実験には，日本白色家兎を用いた．無菌環境下で膝内側部に約3cmの皮切りを加え，筋膜，骨膜を切開，翻転して脛骨近位部を露出した．歯科用バーを用いて内側から外側へ向けて広さ$16×2mm^2$の骨孔を脛骨の長軸に平行に開け，貫通させた (図1(A))．両足の脛骨にHDPE/TiO_2複合体を挿入し

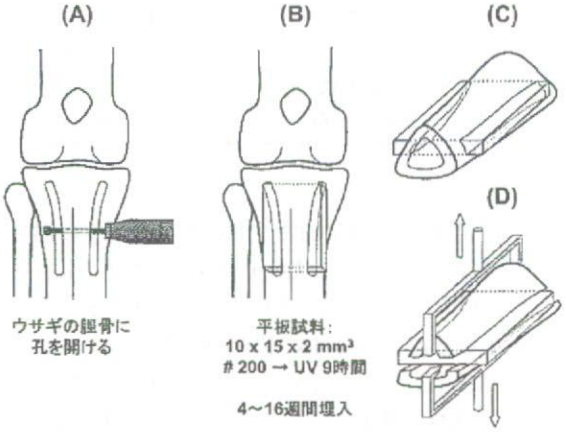

図1 動物実験手術手技および引き剥がし試験 (A) 骨孔作製 (B) サンプル埋入 (C) 屠殺後，サンプル回収 (D) 引き剥がし試験

(図1(B))，洗浄後，上筋膜，皮膚を縫合した．術後4, 8, 16週で7羽ずつ屠殺し，周囲の骨と一体となったHDPE/TiO_2複合体を骨ごと取り出した (図1(C))．

引き剥がし試験のため，取り出したHDPE/TiO_2複合体周辺の骨を除去し，骨にフックをかけて上下に牽引力を加え，接着強度（単位；N）を測定した (図1(D))．

引き剥がし試験後，切片を厚さ50μm前後にまで研磨した後，10%ギムザ液で染色し，光学顕微鏡により観察した．

3 HDPE/TiO_2複合体の機械的強度

まず，複合体破断面のSEM観察を行った結果，図2に示すように，個々のTiO_2粒子表面をポリエチレンが被覆しており，TiO_2粒子とポリエチレンとの相互作用が生じ，フィラーと樹脂との界面剥離が低減されていると考えられる．具体的には，複合体の表面を削って得た粉末をKBr法によるフーリエ変換赤外分光分析を行った結果，TiO_2表面が確か

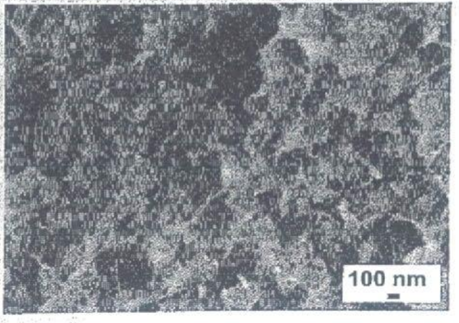

図 2 HDPE/TiO_2 複合体の破断面の SEM 写真

にシランカップリングで処理されていることを確認した．また，TiO_2 粒子と HDPE が化学的相互作用をしていることも確認できた[13]．

次に，シラン処理およびホットプレス圧力の違いによる影響を調べるため，異なるホットプレス圧力で HDPE/TiO_2 を作製した．その複合体の 3 点曲げ試験を行った結果，プレス圧を 2.5MPa から 5.0MPa に上げると，破壊荷重が優位に高くなり，破壊に至るまでの変位量が大きくなり，有効破壊エネルギーが増大した (図 3)．また，曲げ強度および弾性率も

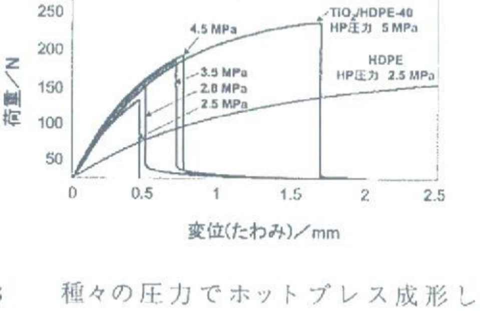

図 3　種々の圧力でホットプレス成形した HDPE/TiO_2 複合体の荷重–変位曲線

増大し，プレス圧が 5.0MPa の時，それぞれ 65MPa および 10GPa を示し，世界で初めてヒト皮質骨の機械的強度範囲に入ることがわかった (図 4)．これは，プレス圧の増大により，複合体中のボイドが除去された結果，複合体の密度が高くなり，理論密度値と同じ値を示したためと考えられる (図 5)．

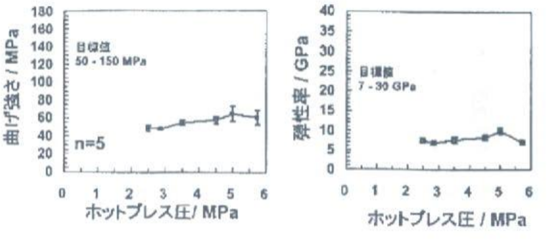

図 4　種々の圧力でホットプレス成形した HDPE/TiO_2 複合体の曲げ強度および弾性率

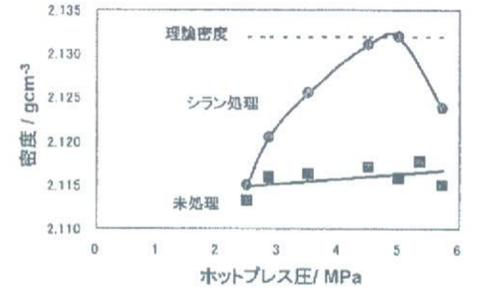

図 5　種々の圧力でホットプレス成形した HDPE/TiO_2 複合体の密度

4　HDPE/TiO_2複合体の骨結合能

家兎の脛骨に 8 週間埋入後の骨と HDPE/TiO_2 複合体の界面の光学顕微鏡写真を図 6 に示す．複合体

図 6 家兎の脛骨に 8 週間埋入した HDPE/TiO_2 複合体と骨の界面の光学顕微鏡写真

は骨と直接結合していることが確認でき，本複合体は生体活性能を有する材料であることがわかった．次に，埋入期間の増加にともなう骨と複合体との接着強度の変化を図 7 に示す．埋入期間が増加しても，接着強度はほとんどかわらず約 8N であることがわかった．引き剥がし試験後の骨と複合体の界面

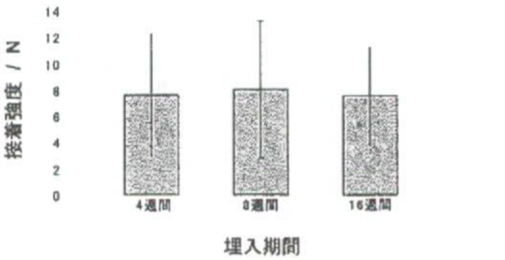

図7 HDPE/TiO_2 複合体と骨の接着強度

をSEMにより観察し，破壊箇所を確認したところ，複合体表面のTiO_2粒子が骨側に付着していたことから，複合体内で破壊が生じていることがわかった．そのため，埋入期間が増加しても接着強度に変化がなかったと考えられる．今回，複合体表面に生体活性を付与するために，表面を研磨後，紫外線処理を行った．本処理はTiO_2粒子を露出させるためには有効であるが，骨との高い接着強度のためには，紫外線処理条件や他の表面処理方法も検討する必要があると考えられる．

5 おわりに

バッチ式加圧ニーダ混練法により，HDPE中に40体積%のナノサイズのTiO_2を均一に分散させることができた．HDPEにTiO_2を40体積%混練し，プレス圧5.0MPaの条件下でHDPE/TiO_2複合体を作製することにより，ヒト皮質骨の機械的強度の範囲に入る複合体を得ることができた．この複合体は，市販のHAPEX®よりもはるかに高い力学的特性を示した．また，得られた複合体は家兎の脛骨に埋入すると骨と直接結合することがわかった．従って，骨結合能とヒト骨に類似した機械的強度を併せ持つHDPE/TiO_2複合体の調製に成功した．しかし，骨との接着強度は人工骨として実用化するにはまだ不十分なため，HDPE/TiO_2複合体の表面処理方法および条件を改良し，人工骨としての可能性を評価する予定である．

謝辞

・本研究は，(独) 新エネルギー・産業技術総合開発機構 (NEDO) の委託による生体親和性材料の一環として実施したものである．

・動物実験は，京都大学医学研究科の中村孝志教授研究室にて行われたものである．

［参考文献］

1) L. L. Hench, R. J. Splinter, W. C. Allen and T. K. Greenlee: *J. Biomed. Mater. Res. Symp.*, **2**, 117 (1972).

2) M. Jarcho, J. L. Kay, R. H. Gumaer and H. P. Drobeck: *J. Bioeng.*, **1**, 79 (1977).

3) T. Kokubo, M. Shigematsu, Y. Nagashima, M. Tashiro, T. Nakamura, T. Yamamuro and S. Higashi: *Bull. Inst. Chem. Res., Kyoto Univ.*, **60**, 260 (1982).

4) T. Kokubo: Bone-Bonding Biomaterials, Reed Healthcare Communications, Netherlands, p.31 (1992).

5) T. Kokubo, H. Kushitani, S. Sakka, T. Kitsugi and T. Yamamuro: *J. Biomed. Mater. Res.*, **24**, 721 (1990).

6) L. L. Hench: *J. Am. Ceram. Soc.*, **81**, 1705 (1998).

7) 小久保正，佐藤温重ほか編：バイオマテリアルと生体，中山書店，pp.39-146 (1998).

8) M. Wang, D. Porter and W. Bonfield: *Brit. Ceram. Trans.*, **93**, 91 (1994).

9) 中村孝志，佐藤温重ほか編：バイオマテリアルと生体，中山書店，pp.164-176 (1998).

10) W. Bonfield, M. D. Grynpas, A. E. Tully, J. Bowman and J. Abram: *Biomater.*, **2**, 185 (1981).

11) S. Fujibayashi, T. Nakamura, S. Nishiguchi, J. Tamura, M. Uchida, H. -M. Kim and T. Kokubo: *J. Biomed. Mater. Res.*, **56**, 562 (2001).

12) M. Hashimoto, H. Takadama, M. Mizuno and T. Kokubo, *J. Mater. Sci: Mater Med.*, **18**, 661 (2007).

13) M. Hashimoto, H. Takadama, M. Mizuno and T. Kokubo, *Mat. Res. Bull.*, **41**, 515 (2006).

Behavior of in vitro, in vivo and internal motion of micro/nano particles of titanium, titanium oxides and others

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To clarify the effect of micro/nanosizing of materials onto biological organism, the particle size dependence of reaction of cells and tissue as investigated by both biochemical cell functional test and animal implantation test. Especially for nanoparticles the behavior of invasion and internal diffusion inside body was visualized using an XSAM (X-ray Scanning Analytical Microscope). The increase of specific surface area is usually counted as nanosizing effect which causes the enhancement of chemical reactivity and therefore toxicity of materials such as carcinogenicity found in 500 nm Ni particles for the long term implantation in the soft tissue of rat. Even biocompatible materials such as Ti and TiO_2 shows stimulus with the decrease of particle size. They cause phagocytosis to cells and inflammation to tissue when the size of particles is below 3 μm. For the size below 50 nm, they may invade into the internal body through the respiratory or digestive system and diffuse inside body. After compulsory exposure test of 30 nm TiO_2 particles through the respiratory system, the Ti mapping by XSAM showed the internal diffusion inside the whole body. Nanoparticles injected from caudal vein diffused with time course to lung, liver and spleen. The uptake of 30 nm TiO_2 particles through the digestive system and diffusion into these organs was also confirmed. These phenomena observed in biocompatible or bioinert materials are the nonspecific, physical particle and shape effects which occur independent of materials. Nanoparticles might be the objects whose existence has not been assumed by the living body defense system.

Key-words : Nanoparticle, Phagocytosis, Inflammation, Internal diffusion, Biocompatibility, Nanotoxicology, Cytokine, Size effect

[Received October 15, 2007; Accepted November 18, 2007]

1. Introduction

1.1 Development of nanotechnology

Nanotechnology has been intensively developed and applied in the fields of electronics, chemistry and others. For materials nanosizing brings in quantum effect for less than about 1.5 nm and the formation of activity points such as contained in some catalysts. However the most unambiguous and influential effect is the surface area effect. It is well-known that the specific surface area which is defined as surface area for unit volume is increased with the decrease of particle size and chemical reactivity is pronounced. Therefore high throughput is expected in the functions of material properties and performances of devices. In these developments it is usual that the merit side is emphasized and demerit is neglected.

1.2 Necessity to establish principle for biomedical application

However it is natural that demerit appears as well as merit since merit and demerit are dependent on whether they work as usefulness or not for the purpose of human beings, although both originate from the same mechanism. Especially for the biomedical application of nanotechnology it is necessary to elucidate the phenomena and establish the proper principle in advance to step out to human application. The reaction of biological organism to proteins and saccharides including virus, bacteria, enzyme, and phamacological agents has been investigated in biology and medicine. For materials the reaction to the usual cases, that is, the macroscopic size is well investigated. But the reaction to the micro/nano sizing is not so clear.

1.3 Chemical reactivity enhancement effect

One of the most important factors to affect on the biocompatibility of materials in macroscopic size is ionic dissolution and this is also true for micro and nano size. This is closely related to the specific surface area and becomes apparent in most cases as stimulus or toxicity to nanosizing. Ni is known to cause allergy in macroscopic size. We found that 500 nm Ni particles cause the formation of tumors after one year when implanted in the soft tissue of rats. This is the typical example and nanosizing effect onto biological organism has been usually interpreted from this aspect.

1.4 Stimulus effect in non-soluble materials

On the other hand corrosion-resistent and biocompatible Ti causes inflammation in abraded fine particles[1),2)] which are produced from artificial joint, and asbestos,[3)] a kind of clay minerals, induces mesothelioma after a long-term, large quantity of exposure. These phenomena cannot be explained by the specific surface area effect and understood as the different effect from the material properties of either toxicity or biocompatibility, that is, physical size and shape effect. The abraded fine particles may diffuse inside the body through the cardiovascular system. There is also the possibility that the uptake of nanoparticles occurs through the respiratory and digestive systems.

These strongly suggest the necessity to reveal the micro/nanosizing effect other than the specific surface area effect,[4] such as the biological reactivity of micro/nanoparticles and their internal dynamics.

1.5 Nanotoxicology and DDS originated from internal particle diffusion

Meanwhile Drug Delivery System (DDS) is one of the most typical biomedical applications of nanoparticles. The development of DDS is expected for the administration of anticancer agent and gene transfection. The behavior of nanoparticles in the internal body is necessary to investigate for the assessment of nanotoxicology and this is, in turn, essential to comprehend the diffusion path of DDS to reach the diseased target. Thus internal diffusion is significant from both demerit and merit aspects of nanotechnology.

1.6 Purpose

In the present study both biochemical cell functional test and animal implantation test were done to clarify the particle size dependence of reaction of cells and tissue, and micro/nanosizing effect with the primary attention focussed on non-soluble materials such as Ti and TiO_2.[5),6)] In addition for nanoparticles, the behavior of invasion and internal diffusion inside body was visualized using XSAM (X-ray Scanning Analytical Microscope)[7),8)] for the level of the whole body and organs.

2. Materials and methods

2.1 Specimens

99.9% pure Ti, and TiO_2 particles of the various size were principally used throughout. For in vitro and in vivo implantation tests Fe, Ni, TiO_2 and carbon nanotubes[9),10)] were also used. The particles of nominal size from 500 nm to 150 μm were used for Ti. Usually these contain the size distribution to the considerable amount. To reduce the size distribution as small as possible and equalize the experimental conditions among materials such as metallic Ti, Fe and Ni, the particles of 0.5, 3, 10 μm were extracted by sedimentation method and those less than 300 nm were extracted by ultrafiltration from particle powders of nominal size.

2.2 Dissolution test of Ti particles

After Ti particles were immersed in HBSS (Hanks balanced salt solution) at 37°C for 1 month, the supernatant was filtered through a 0.45 μm membrane to remove Ti particles and then elemental analysis was done by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) using ICPS - 8100, Shimadzu, Tokyo.

2.3 Biochemical analyses of cellular reaction to materials

Human neutrophils, which play a central role in the initial stage of inflammation in a non-specific manner against foreign bodies, were used as probe cell. Particles smaller (0.5, 3 μm) and larger (10, 50, 150 μm) than neutrophils were used to determine the relationship between cell and particle size with respect to cytotoxicity.

Cell survival rate and lactate dehydrogenase (LDH) values, superoxide anion (O^{2-}) production per 10^6 neutrophils were measured. Cytokines of TNF-α and Il-1β were measured using ELISA kits (Endogen, Inc. USA). Morphological change of neutrophils mixed with HBSS containing various particles was observed by optical microscopy (OM: Zeiss, Axioskop, Germany) and scanning electron microscopy (SEM: Hitachi S-4300, Tokyo).

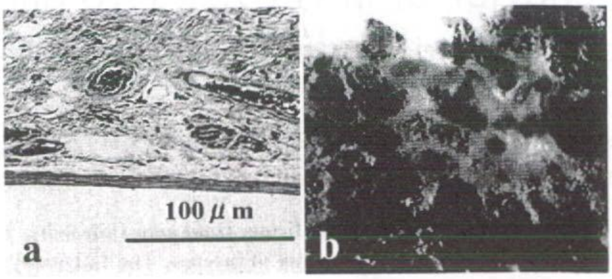

Fig. 1. Comparison of the reaction of rat soft tissue to the macroscopic Ti implant (a) and 3 μm Ti particles (b) by histological observation.

2.4 Animal experiments

Particles were inserted in the subcutaneous connective tissue in the abdominal region of Wistar rats aged between 11 and 12 weeks (weight 350–380 g). Specimens were prepared through the usual process of fixation, embedding, sectioning, staining with hematoxylin-eosin, and then histopathologically observed.

2.5 Visualization of internal distribution of nanoparticles

The compulsory exposure test to the respiratory system was performed to rats using 30 nm TiO_2 particles. The uptake of nanoparticles through the digestive system was also tested for mice by mixing agar gelatin containing 30 nm TiO_2 particles to their foods. To inspect internal diffusion more simply, the experiments were done for mice by injecting nanoparticles directly to the cardiovascular system from caudal vein. The observation of internal distribution of nanoparticles was conducted for the whole body and each organ by elemental mapping in air using X-ray Scanning Analytical Microscope (XSAM: Horiba XGT-2000V, Tokyo) without the pretreatments of fixation, dehydration and staining after sectioning. The distribution inside the organ was inspected by elemental mapping using energy dispersive X-ray spectroscopy (EDS) installed to SEM. The experiments of internal diffusion were also done for the particles Ti, Fe, Ni, Pt, TiC, Fe_2O_3.

3. Results

3.1 Comparison of tissue reaction to macroscopic and nanosize materials

Figure 1 shows the histological observation of the reaction of rat soft tissue to the macroscopic Ti implant (a) and 3 μm Ti particles (b) after 8 weeks, comparatively. For the macroscopic size, Ti implant was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk Ti. For 3 μm Ti numerous inflammatory cells appeared. The macrophages and adjacent collagen show degenerative changes in morphology. Ti particles, observed as small black dots, were phagocytized into the cytoplasm by a macrophage.

3.2 Particle size dependence of cell reaction

Figure 2 shows the SEM images of human neutrophils in HBSS (Hanks balanced salt solution) (a) and exposed to 500 nm Ti particles (b). Figure 2b showed the neutrophil extending its pseudopod to phagocytize Ti particles for the size below 3 μm. For the particles larger than 10 μm, phagocytosis was not observed.

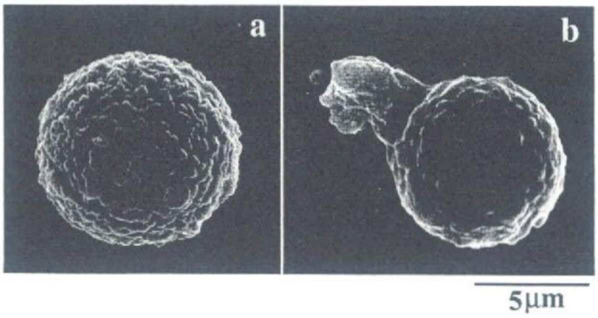

Fig. 2. SEM images of human neutrophils. a: control, b: exposed to particles of 500 nm Ti.

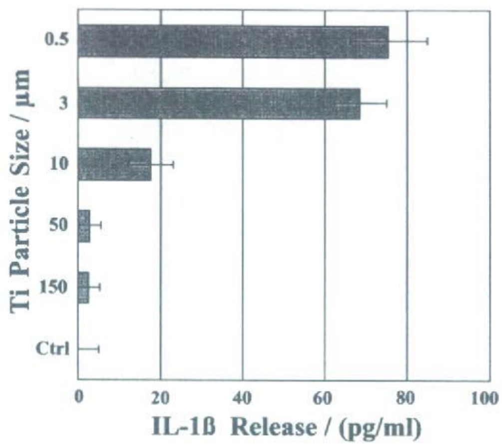

Fig. 3. Dependence of Il-1β release from neutrophils on Ti particle size.

Figure 3 shows the amount of Il-1β released from neutrophils in HBSS containing Ti particles. Il-1β is one of the most representative cytokines of inflammation. Il-1β showed the increase against the decrease of particle size. The increase was pronounced for 0.5 and 3 μm. The release of LDH, superoxide and cytokine TNF-α showed the similar behavior as Il-1β, while cell survival rate showed the inverse decreasing tendency. ICP elemental analysis showed that the dissolution from Ti particles was negligible below detection limit. The pronounced phenomena of biochemical cell reactivity observed for the particle size below 3 μm in Fig. 3 are closely related to the phagocytosis shown in Fig. 2.

3.3 Particle size dependence of tissue reaction

The histological image of tissue reaction of rat to the different size of Ti particles for the long term implantation test showed the similar size dependence to those in vitro shown in Figs. 2 and 3. **Figure 4** is the tissue reaction to 10 μm (a) and 150 μm Ti (b) particles after 30 week implantation. For 150 μm Ti, each particle was surrounded by fibrous connective tissue layer, which is similar to the case of macroscopic Ti implant shown in Fig. 1a. Tissue reaction to 10 μm Ti was inflammatory where there was inflammatory cell infiltration as well as fibrous connective tissue formation.

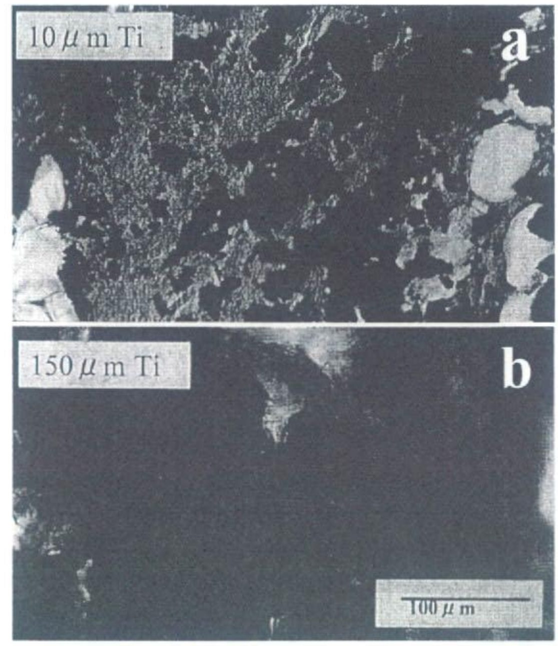

Fig. 4. Histopathological image of tissue reaction to 10 μm (a) and 150 μm (b) Ti particles after 30 week implantation.

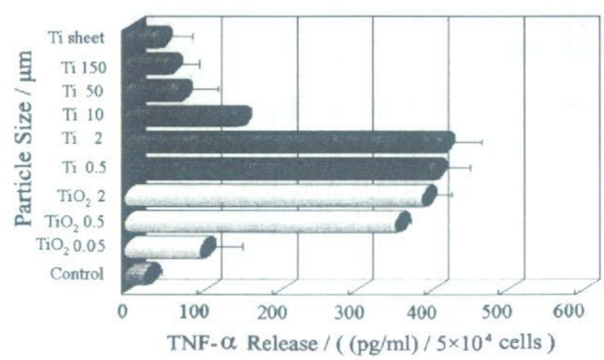

Fig. 5. Dependence of TNF-α release from neutrophils on particle size down to nm size.

3.4 Stimulus in nm size

Figure 5 shows the dependence of TNF-α release from neutrophils on particle size down to nm size. Stimulus, represented as amount of TNF-α release, which is pronounced below 3 μm, exhibited the maximum from around μm down to 500 nm, similar to the case of Il-1β shown in Fig. 3, and then for further smaller size decreased below 200 nm. This means that the biophylactic system does not work well any more against the invasion of nanoparticles into the inside of body.

3.5 Internal diffusion of nanoparticles

Figure 6 is the Ti mapping of the internal whole body of rats by XSAM after compulsory exposure test to respiratory system, and reveals the distribution of 30 nm TiO_2 particles. The condensation occurred from the respiratory system to urinary bladder by diffusion in the body through the cardiovascular system after the direct uptake into blood

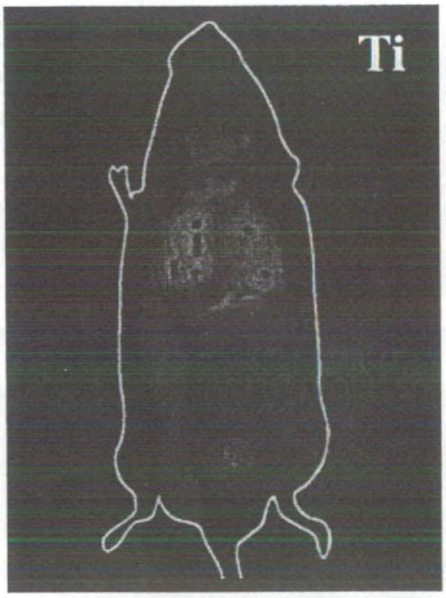

Fig. 6. XSAM Ti mapping of internal distribution of 30 nm TiO_2 particles after compulsory exposure test to respiratory system.

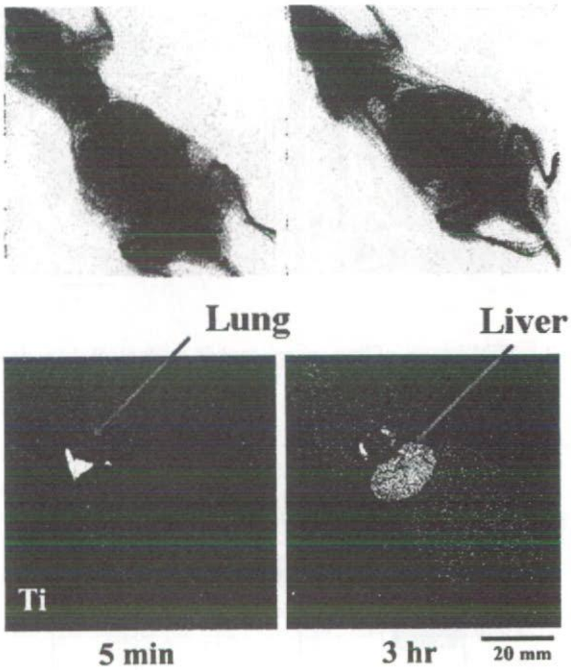

Fig. 8. Time course of internal diffusion of 30 nm TiO_2 particles after injection to caudal vein.

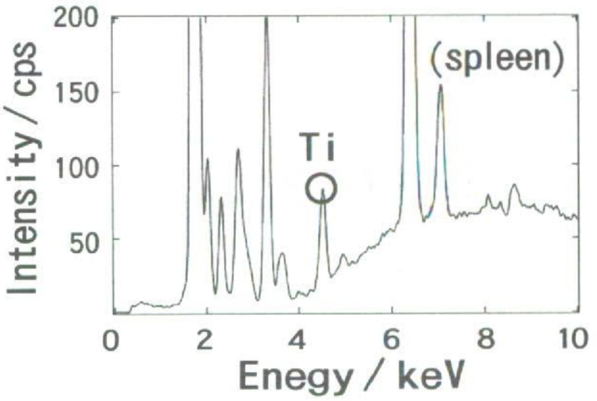

Fig. 7. Elemental analysis of spleen of mouse by XSAM after 10 days of oral administration of 30 nm TiO_2 particles.

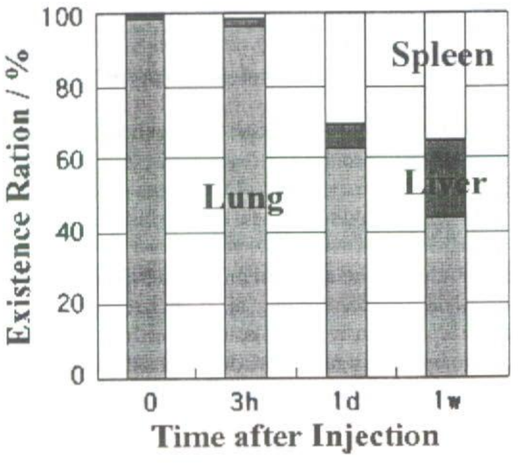

Fig. 9. Change of existence ratio of TiO_2 particles in each organ with time.

vessels from lung cells.

Figure 7 is the XSAM elemental analysis from spleen for the case after 10 d of oral administration of 30 nm TiO_2 particles. Although peak height is small in this case, Ti-Kα peak undoubtedly exists other than Fe-Kα peaks around 6.5 keV and peaks of incident X-ray from Rh target below 4 keV. This confirms the phenomenon that nanoparticles were taken into the internal body through digestion system.

Figure 8 shows the X-ray transmission image and the corresponding Ti elemental mapping by XSAM for 5 min and 3 hr after injection of 30 nm TiO_2 particles to caudal vein. TiO_2 nanoparticles diffused to lung just after injected from caudal vein, then liver and spleen with time course.

Figure 9 shows the change of existence ratio of TiO_2 particles in each organ with time. Particles reach lung shortly after injection, then the content in lung decreases and the content in liver and spleen increases with time.

To observe the more detailed distribution of nanoparticles in each organ, EDS elemental mapping was applied. **Figure 10** is the SEM image (a) and corresponding Ti elemental mapping by EDS (b) for spleen of mouse at 3 hr after injection of 30 nm TiO_2 particles to caudal vein. The distribution is not uniform and in dotted manner.

4. Discussion

4.1 Particle size dependence of reaction of cells and tissue

Comparison of the reaction of tissue to the macroscopic Ti and 3 μm Ti particles in Fig. 1 showed clearly the micro/nanosizing effect on biological organism. Both biochemical cell functional test and animal implantation test showed the toxicity due to fine particles and its size dependence. Both

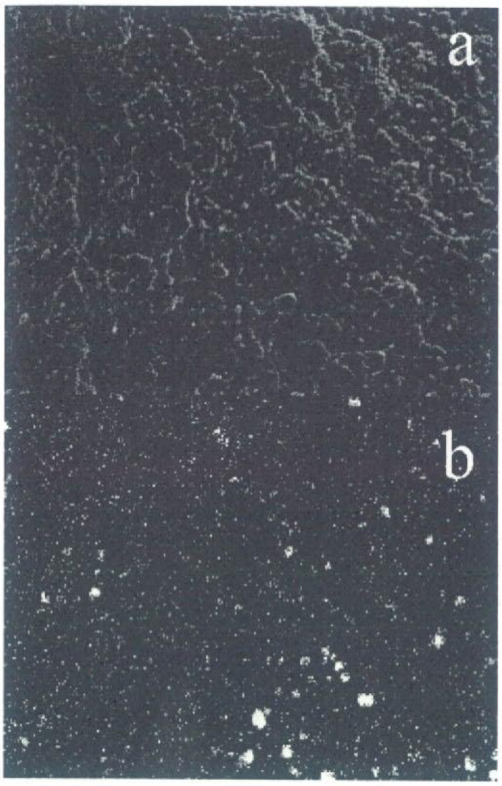

Fig. 10. SEM image (a) and corresponding Ti elemental mapping by EDS (b) for spleen of mouse at 3 hr after injection of 30 nm TiO_2 particles to caudal vein.

results of in vitro and in vivo are in accordance each other in their size dependence. Ti particles larger than approximately 100 μm was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk size of Ti implant.[11),12)] As the particle size was smaller, stimulus was induced due to the physical size effect in the range less than about 100 μm as shown in Fig. 4. The inflammation was especially pronounced when the particle size was typically below 3 μm which is smaller than 10 μm, about the cell size, where phagocytosis was induced.

These phenomena occur commonly in any bioactive and bioinert materials other than Ti, such as Fe and TiO_2 where particles induce nonspecifically phagocytosis to cells and inflammation to tissue for the size below 3 μm. It is different from the usually observed toxicity due to the ionic dissolution effect in the macroscopic size.[4)]

4.2 Stimulus in nm size

Nanosizing effect is usually interpreted by the increase of specific surface area, which pronounces chemical reactivity with the decrease of particle size. Effects related to the ionic dissolution correspond to this category, such as the acceleration of toxicity observed in Ni where tumor was generated in the long-term implantation for 500 nm particles, compared with necrosis occurred in short term for macroscopic size.

Specific surface area effect is based solely on the material properties, and indifferent from biological body, while physical particle size effect has the origin in the relative size relationship between particles and cell/tissue. Stimulus arises by biological process which induces the occurrence of functionality of body defense system.

4.3 Internal diffusion of nanoparticles

Figure 5 shows that particles become less stimulative when the particle size becomes in the level of 50 nm or less and the recognition by body defense system becomes lower. The invasion of nanoparticles into the body occurs for this range of particle size. The present results showed both cases of uptake of nanoparticles through the respiratory (Fig. 6) or digestive system (Fig. 7). Figures 8 and 9 show that nanoparticles diffuse with time course to lung, liver and spleen after injection from caudal vein. SEM image and EDS elemental mapping in Fig. 10 show the distribution of TiO_2 nanoparticles inside the organ of spleen.

5. Conclusions

Particles cause nonspecifically phagocytosis to cells and inflammation to tissue for the size below 3 μm. For the size below 50 nm particles may invade directly into the internal body through the respiratory or digestive system and diffuse inside body. Nanoparticles might be the objects whose existence has not been assumed by living body defense system. Thus the visualization of the internal dynamics of nanoparticles is essential for the proper treatments based on risk assessment and biomedical applications such as DDS. The present study could successfully visualize the internal diffusion of nanoparticles inside the whole body using XSAM.

Acknowledgements Present research was supported by Health and Labour Sciences Research Grants in Research on Chemical Substance Assessment of Ministry of Health, Labour and Welfare of Japan (H18-Chem-General-006).

References

1) Y. Tamura, A. Yokoyama, F. Watari and T. Kawasaki, *Dental Materials J.*, 21, 355-372 (2002).
2) Y. Tamura, A. Yokoyama, F. Watari, M. Uo and T. Kawasaki, *Mat. Trans.*, 43, 3043-3051 (2002).
3) F. Watari, M. Inoue, T. Akasaka, N. Sakaguchi, H. Ichinose and M. Uo: Proc. 6th Asian BioCeramics Symp. 2006, Nov. 7-10, Bangkok, Thailand (2006) pp. 142-145.
4) F. Watari, K. Tamura, A. Yokoyama, K. Shibata, T. Akasaka, B. Fugetsu, K. Asakura, M. Uo, Y. Totsuka, Y. Sato and Tohji, "Handbook of Biomineralization, Vol. 3," Ed. E. Bauerlein, Wiley-VCH, Weinheim (2007) pp. 127-144.
5) R. Kumazawa, F. Watari, N. Takashi, Y. Tanimura, M. Uo and Y. Totsuka, *Biomaterials*, 23, 3757-3764 (2002).
6) K. Tamura, N. Takashi, R. Kumazawa, F. Watari and Y. Totsuka, *Mat. Trans.*, 43, 3052-3057 (2002).
7) M. Uo, F. Watari, A. Yokoyama, H. Matsuno and T. Kawasaki, *Biomaterials*, 20, 747-755 (1999).
8) M. Uo, M. Tanaka and F. Watari: J. Biomed. Mater. Res. Part B, *Appl. Biomater.*, 70B, 146-151 (2004).
9) A. Yokoyama, Y. Sato, Y. Nodasaka, S. Yamamoto, T. Kawasaki, M. Shindoh, T. Kohgo, T. Akasaka, M. Uo, F. Watari and K. Tohji, *Nano Letters*, 5, 157-161 (2005).
10) M. Ushiro, K. Uno, T. Fujikawa, Y. Sato, K. Tohji, F. Watari, W. Chun, Y. Koike and K. Asakura, *Phys. Rev. B*, 73, 144103/1-11 (2006).
11) H. Matsuno, A. Yokoyama, F. Watari, M. Uo and T. Kawasaki, *Biomaterials*, 22, 1253-1262 (2001).
12) F. Watari, A. Yokoyama, M. Omori, T. Hirai, H. Kondo, M. Uo and T. Kawasaki, *Composites Science and Technology*, 64, 893-908 (2004).

Key Engineering Materials Vols. 361-363 (2008) pp 95-98
online at http://www.scientific.net

Internal Diffusion of Micro/Nanoparticles Inside Body

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Keywords: biocompatibility, nanotoxicology, internal diffusion, nanoparticle, titanium, phagocytosis, superoxide, cytokine, neutrophil, macrophage

Abstract. Both biochemical cell functional test and animal implantation test were done to investigate the reaction to fine particles. Particles cause nonspecifically phagocytosis to cells and inflammation to tissue for the size below 10μm. With the size below 50nm particles may invade into the internal body through the respiratory or digestive system and diffuse inside body. Ti mapping by XSAM after the compulsory exposure test to the respiratory system showed the internal diffusion of 30nm TiO_2 particles. They diffused with time course to lung, liver and spleen after injection from caudal vein. Nanoparticles might be the objects whose existence has not been assumed by the biophylactic system.

Introduction

Nanosizing effect of materials onto living organism is usually interpreted in the aspects of the increase of specific surface area, since chemical reactivity is pronounced with the decrease of particle size. This is the chemically enhancing effects of material properties. There are, however, other kind of effects[1,2]. Biocompatible Ti as the form of abraded fine particles[3,4], which are produced in the sliding parts of artificial joints, often cause inflammation in the surrounding tissue. Asbestos, a kind of clay minerals in a view point of composition[5], induces mesothelioma in lung after a long-term, large quantity of exposure. These phenomena can be understood as the physical size and shape effect, apart from the material properties of either toxicity or biocompatibility.

This physical particle effect cause phagocytosis to cells and inflammation to tissue even for biocompatible materials[6,7] such as Ti and TiO_2 when particle size is below 10μm. For the size in the nm range particles may invade into the internal body and diffuse inside body through the cardiovascular system[8]. It is necessary to reveal the internal dynamics for the proper treatments and biomedical applications of particles. In the present study the observation of the internal dynamics of micro/nanoparticles was done using the recently developed X-ray scanning analytical microscope (XSAM)[9,10] for the level of the whole body and organs, and by optical and transmission electron microscopy (OM, TEM) for the tissue and intracellular level.

Materials and Methods

Both biochemical cell functional test and animal implantation test were done to investigate the reaction to fine particles such as Ti, Fe, Ni, Pt, TiO_2, TiC, Fe_2O_3 and carbon nanotubes[11,12]. The compulsory exposure test to the respiratory system was performed using 30nm TiO_2 particles. The experiments of internal diffusion was also done for the particles Ti, Fe, Ni, Pt, TiO_2, TiC, Fe_2O_3 by injection to caudal vein. XSAM observation for the whole body and each organ was conducted in air without the pretreatments of fixation, dehydration and staining after sectioning. Histological and ultrastructural investigations were carried out by OM and TEM for the specimens prepared by ordinary process.

Results

Particle size dependence of cell reaction. Fig.1 shows the amount of TNF-α released from neutrophils in HBSS(Hanks balanced salt solution) containing Ti particles. HBSS was the control. TNF-α increased with the decrease of particle size. The increase was pronounced for 0.5 and 3μm. The release of LDH, superoxide and cytokine Il-1ß showed the similar behavior as TNF-α, while cell survival rate showed the inverse decreasing tendency. ICP elemental analysis showed that the dissolution from Ti particles was negligible below detection limit.

Fig.2 shows the SEM image of human neutrophils exposed to particles of 0.5μmTi. It showed the neutrophil extending its pseudopod to phagocytize Ti particles for the size less than 10μm. For the particles larger than 10μm, phagocytosis was not observed. The pronounced phenomena of biochemical cell reaction observed for the particle size below 10μm in Fig.1 are closely related to the phagocytosis shown in Fig.2.

Particle size dependence of tissue reaction. In vivo test showed that Ti particles larger than 100μm was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk size of Ti implant. As the particle size was smaller, the inflammation occurred and for less than 10μm, about the cell size, the phagocytosis by macrophages or neutrophils was induced. Numerous inflammatory cells were observed in the surroundings of Ti particles and showed the degenerative changes in morphology.

Fig.3 shows the histological image of tissue reaction of rat to 3μm (a) and 10μm (b) Ti particles after 5 day implantation. For 3μm Ti particles, phagocytosis by macrophages occurred, Ti particles were observed inside cells and the cytoplasm of an inflammatory cell was full of small black particles, whereas the 10μm Ti particles exist outside of cells, phagocytosis was rarely observed and tissue was much less inflammatory. The phenomena of size dependence in vivo observed in Fig.3 is similar to the results in vitro in Fig.2, concerning to the occurrence of phagocytosis.

Fig.4 is the histopathological image of tissue reaction to 3μm Ti particles after 2 and 30 weeks. For this range of particle size the particles scattered in tissue in a short term of implantation (Fig.4a) became gradually agglomerated with the time for the long term by biological process of repeated cycle of phagocytosis and cell death (Fig.4b).

Internal diffusion of nanoparticles. Stimulus, represented as amount of TNF-α release, which is pronounced below 10μm, exhibited the maximum from around μm down to 0.5μm as shown in Fig.1 and for further smaller size decreased below 0.2μm. This looks preferable to the view points of biological application of nanoparticles, since stimulus is decreased. However this, in turn, means that the biophylactic system does not work well any more against the invasion of nanoparticles into the inside of body.

Fig.5 is the Ti mapping of the internal whole body of rats by XSAM after compulsory exposure test, and shows the distribution of 30nm TiO_2 particles. The condensation occurred from the respiratory system to urinary bladder by diffusion in the whole body through the cardiovascular system after the direct uptake into blood vessels from lung cells.

Fig.6 shows the internal diffusion of 30nm TiO_2 particles after injection to caudal vein. Nanoparticles from caudal vein diffused with time course to lung, liver and spleen. The uptake of 30nm TiO_2 particles through the digestive system and diffusion into these organs was also confirmed.

Discussion

The present study clearly showed the cytotoxicity due to fine particles and its size dependence using human neutrophils in vitro and tissue response in vivo. Both results of in vitro and in vivo are in accordance each other in their size dependence. The cytotoxicity was induced due to the physical size effect in the range less than 100μm, which is different from the usually observed toxicity due to the ionic dissolution effect in the macroscopic size[8]. The inflammation was pronounced when the particle size was smaller than 10μm, about the cell size, where phagocytosis was induced.

Nanosizing effect is usually interpreted by the increase of specific surface area, which pronounces chemical reactivity with the decrease of particle size. Effects related to the ionic dissolution correspond to this category, such as the acceleration of toxicity observed in Ni where tumor was generated in the long-term implantation for 0.5μm particles, compared with necrosis occurred in short term for macroscopic size.

Specific surface area effect is based solely on the material properties, and indifferent from biological body, while physical particle size effect has the origin in the relative size relationship between particles and cell/tissue. Stimulus arises by biological process which induces the occurrence of functionality of body defense system. When the particle size becomes in the level of 50nm or less, they become less stimulative and the recognition by body defense system becomes lower. The invasion of nanoparticles into the body occurs for this range of particle size. The present results showed both cases of uptake of nanoparticles through the respiratory (Fig.5) or digestive system. Nanoparticles diffuse with time course to lung, liver and spleen after injection from caudal vein (Fig.6).

Conclusions

Particles cause nonspecifically phagocytosis to cells and inflammation to tissue for the size below 10μm. With the size below 50nm particles may invade directly into the internal body through the respiratory or digestive system and diffuse inside body. Nanoparticles might be the objects whose existence has not been assumed by living body defense system. Thus the visualization of the internal dynamics of nanoparticles is essential for the proper treatments based on risk assessment and biomedical applications such as DDS (Drug delivery System).

Acknowledgements: Research supported by Health and Laybour Sciences Research Grants in Research on Chemical Substance Assessment of Ministry of Health, Laybour and Welfare of Japan (H18-Chem-General-006).

References

[1] R.Kumazawa, F.Watari, N.Takashi, Y.Tanimura, M.Uo, Y.Totsuka: Biomaterials 23(2002), 3757-3764
[2] K.Tamura, N.Takashi, R.Kumazawa, F.Watari, Y.Totsuka: Mat.Trans. 43(2002), 3052-3057
[3] Y.Tamura, A.Yokoyama, F.Watari, T.Kawasaki: Dental Materials J. 21(2002), 355-372
[4] Y.Tamura, A.Yokoyama, F.Watari, M.Uo, T.Kawasaki: Mat.Trans. 43(2002), 3043-3051
[5] F.Watari, M.Inoue, T.Akasaka, N.Sakaguchi, H.Ichinose, M.Uo: Proc.6th Asian BioCeramics Symp.2006, p.142-145, 2006
[6] H.Matsuno, A.Yokoyama, F.Watari, M.Uo, T.Kawasaki: Biomaterials 22(2001), 1253-1262
[7] F.Watari, A.Yokoyama, M.Omori, T.Hirai, H.Kondo, M.Uo, T.Kawasaki: Composites Science and Technology 64(2004), 893-908
[8] F.Watari, K.Tamura, A.Yokoyama, K.Shibata, T.Akasaka, B.Fugetsu, K.Asakura, M.Uo, Y.Totsuka, Y.Sato, K.Tohji: Handbook of Biomineralization, Vol.3, Ed.E.Bauerlein, Wiley-VCH, Weinheim, p.127-144 (2007)
[9] M.Uo, F.Watari, A.Yokoyama, H.Matsuno, T.Kawasaki: Biomaterials 20(1999), 747-755
[10] M.Uo, M.Tanaka, F.Watari: J.Biomed.Mater.Res.Part B:Appl.Biomater. 70B(2004), 146-151
[11] A.Yokoyama, Y.Sato, Y.Nodasaka, S.Yamamoto, T.Kawasaki, M.Shindoh, T.Kohgo, T.Akasaka, M.Uo, F.Watari, K.Tohji: Nano Letters 5 (2005) , 157-161
[12] M.Ushiro, K.Uno, T.Fujikawa, Y.Sato, K.Tohji, F.Watari, W.Chun, Y.Koike, K.Asakura: Phys. Rev. B 73, 144103/1-11(2006)

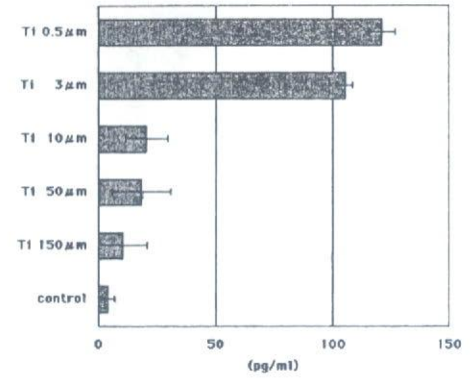

Fig.1 Dependence of TNF-α release from neutrophils human on Ti particle size.

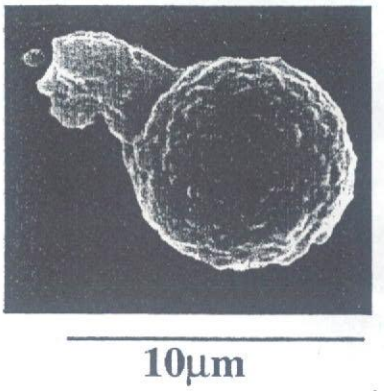

Fig.2 SEM images of human neutrophils exposed to particles of 0.5μm Ti

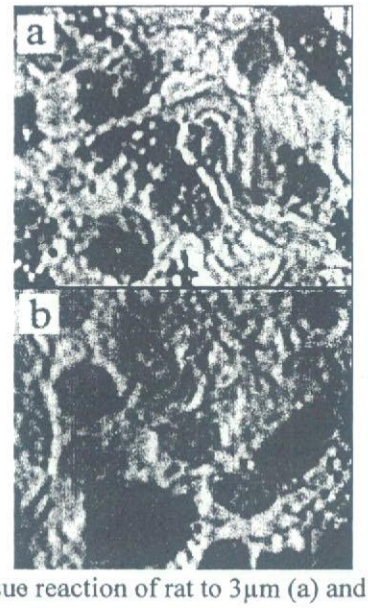

Fig.3 Tissue reaction of rat to 3μm (a) and 10μm (b) Ti particles after 5 day implantation

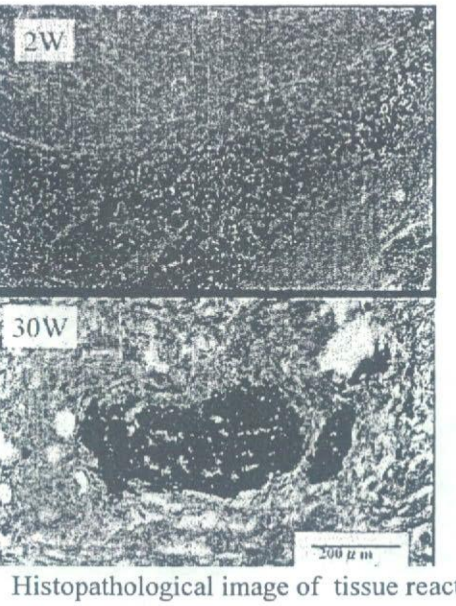

Fig.4 Histopathological image of tissue reaction to 3μm Ti particles after 2 and 30 weeks

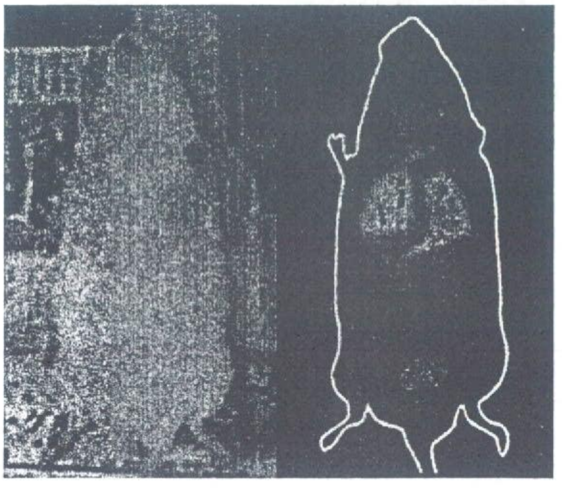

Fig.5 XSAM Ti mapping of internal distribution of 30 nm TiO_2 particles after compulsory exposure test

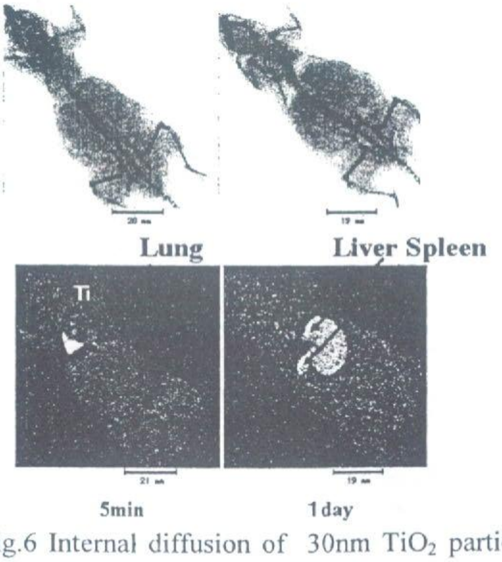

Fig.6 Internal diffusion of 30nm TiO_2 particles after injection to caudal vein

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Journal of Society for Titanium Alloys in Dentistry

Proceedings

The 6th International Symposium on Titanium in Dentistry and The 2nd International Meeting on Titanium in Dental Technology

In conjunction with

The 20th Annual Meeting of the Society for Titanium Alloy in Dentistry

June 5-6, 2007
Kyoto International Conference Center, Kyoto, Japan

Vol.5 No.1

歯科チタン学会

DO-21

Biological Reaction to Micro/Nano Particles of Titanium and Titanium Oxides

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Introduction

Ti is highly corrosion-resistant due to the thin and stable protective oxide layer formed on surface and one of the most biocompatible metals. In this sense Ti is the nearly ideal material for implant and used most commonly in orthopedics and dentistry. Low abrasion resistance is, however, one of the few short points of Ti. The abraded fine particles produced in the sliding parts of artificial joints often cause inflammation in the surrounding tissue. This rather contadictory phenomenon may be originated from particle effects such as physical size and morphology, apart from the material properties of either toxicity or biocompatibility.

The abraded fine particles may diffuse inside the body through the cardiovascular system. There is also the possibility that the uptake of nanoparticles occurs through the respiratory and digestive systems. Meanwhile the development of DDS (Drug Delivery System) is expected for the administration of anticancer agent and gene transfection. The behavior of nanoparticles in the internal body is essential to comprehend for the realization of DDS.

Thus it is necessary to reveal the biological reactivity of micro/nanoparticles and their internal dynamics, and establish the proper principle in advance for their biomedical applications. In the present study the effects of fine particles of Ti and TiO_2 and their size dependence on cytotoxicity in vitro and biocompatibility in vivo were investigated by biochemical functional analyses using human neutrophils as probe cells and the histological observation in animal implantation test.
Human neutrophils, which play a central role in the initial stage of inflammation in a non-specific manner against foreign bodies, were used as probes. Particles smaller (0.5, 3 μm) and larger (10, 50, 150 μm) than the neutrophils were used to determine the relationship between cell and particle size with respect to cytotoxicity.

The observation of the internal dynamics of micro/nanoparticles was then performed using the X-ray scanning analytical microscope (XSAM) for the level of the whole body and organs, and by optical and transmission electron microscopy (OM, TEM) for the tissue and intracellular level.

Materials and Methods

1. Specimens

99.9% pure Ti, and TiO_2 particles of the various size from 50 nm to 150 μm were used for experiments. To equalize the experimental conditions between materials the particles of 0.5, 3, 10 μm were extracted by sedimentation method and those less than 300 nm were extracted by ultrafiltration from the particle group with the size distribution.

2. Dissolution test of Ti particles

After Ti particles were immersed in HBSS (Hanks balanced salt solution) at 37°C for 1 month, the supernatant was filtered through a 0.45 μm membrane to remove Ti particles and then analyzed by ICP-AES (Inductively Coupled Plasma - Atomic Emission Spectrometry) elemental analysis (ICPS-8100, Shimazu, Tokyo, Japan).

3. Biochemical analyses of cellular reaction to materials

Cell survival rate and lactate dehydrogenase (LDH) values, superoxide anion (O^{2-}) production per 106 neutrophils were measured. Cytokines of TNF-α and Il-1β were measured using ELISA kits (Endogen, Inc. USA). Morphological change of neutrophils mixed with HBSS containing various particles was observed by optical microscopy (OM: Zeiss, Axioskop, Germany) and scanning electron microscopy (SEM: Hitachi S-4300, Tokyo, Japan).

4. Animal experiments

Particles were inserted in the subcutaneous connective tissue in the abdominal region of Wistar rats aged between 11 and 12 weeks (weight 350-380 g). Specimens were prepared through the usual process of fixation, embedding, sectioning, staining with hematoxylin-eosin, and histopathologically observed. The compulsory exposure test was also performed using 30 nm TiO_2 particles. The experiments of internal diffusion was also done by injection to caudal vein The observation of internal diffusion of nanoparticles was conducted by elemental mapping in air using X-ray Scanning Analytical Microscope (XSAM: Horiba XGT-2000V, Tokyo, Japan) without the pretreatments of fixation, dehydration and staining after sectioning.

Results and Discussion

1. Particle size dependence in vitro

Fig.1 shows the amount of Il-1ß released from neutrophils in HBSS containing Ti particles. HBSS solution was the control. Il-1ß increased with the decrease of particle size. The increase was pronounced for 0.5 and 3 μm. The release of LDH, superoxide and cytokine TNF-α showed the similar behavior as Il-1ß, while cell survival rate showed the inverse tendency to decrease with the particle size.

The biochemical functional analyses of cell survival rate, LDH, superoxide anion, cytokines and microscopic observation of cellular morphology revealed that the stimulatory effects on neutrophils and inflammation in soft tissue became prominent as the particle size became smaller in the range less than 100 μm, and especially pronounced for smaller than 10 μm, about the cell size, where phagocytosis was induced. ICP elemental analysis showed that the dissolution from Ti particles was negligible. ICP elemental analysis showed that the dissolution from Ti particles was negligible below detection limit.

The OM and SEM observation showed that neutrophils extend their pseudopod to phagocytize TiO_2 or Ti particles for the size less than 10μm. For the particles larger than 10 μm, phagocytosis was not observed. The pronounced phenomena of biochemical cell reaction observed for the particle size below 10 μm in Fig.1 are closely related to the phagocytosis.

2. Particle size dependence in vivo

In vivo test showed that Ti particles larger than 100 μm was surrounded by fibrous connective tissue layer which is the usual reaction for the biocompatible materials such as the bulk size of Ti implant. As the particle size was smaller, the inflammation occurred and for less than 10 μm, about the cell size, the phagocytosis by macrophages was induced. Numerous inflammatory cells were observed in the surroundings of Ti particles. The macrophages or neutrophils showed the degenerative changes in morphology.

3. Internal diffusion of nanoparticles

The Ti mapping by XSAM (Fig.2) showed the internal distribution of 30nm TiO_2 particles, resulting in the condensation from the respiratory system to urinary bladder. Nanoparticles diffused with time course to lung, liver and spleen after injection from caudal vein.

Conclusion

Biocompatible materials such as Ti and TiO_2 cause phagocytosis to cells and inflammation to tissue when the size of particles is below 10 μm. They may invade into the internal body through the respiratory system and diffuse inside body when the size is below 50nm.

References

1) R.Kumazawa, F.Watari, et al.: Effects of Ti ions and particles on cellular function and morphology of neutrophils, *Biomaterials* 23, 3757-3764, 2002

2) K.Tamura, N.Takashi, R.Kumazawa, F.Watari, Y.Totsuka: Effects of particle size on cell function and morphology in titanium and nickel, Mat. Trans.43(12),3052-3057, 2002

3) F.Watari, A.Yokoyama, et al.: Biocompatibility of materials and development tofunctionally graded implant for bio-medical application, Composites Sci.& Tech. 64(6), 893-908, 2004

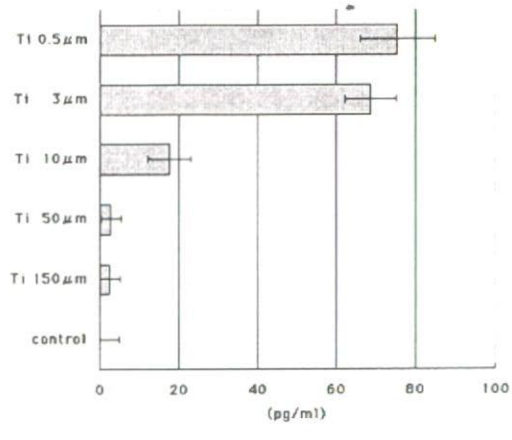

Fig. 1 Dependence of Il-1ß release from neutrophils on Ti particle size [6]

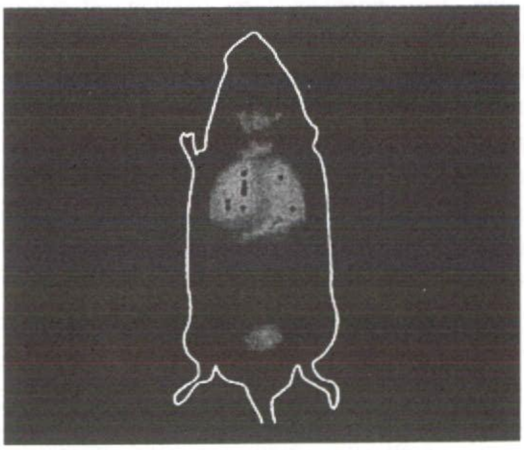

Fig. 2 XSAM Ti mapping of internal distribution of 30 nm TiO2 particles after compulsory exposure test.