diameter [5,9]. Taking into account our findings about the inhibitory effects of various microspheres on the functions of NHOst, it is probable that NHOst can phagocyte PS microspheres as well as macrophages, and in particular, may phagocyte microspheres 5 μm in diameter. Moreover, the effect of the added PS microspheres suggests that NHOst better recognize the microspheres from their lower than upper side. This may explain the reduced functions of NHOst co-cultured with the pre-coated 5 μm PS microspheres.

To estimate the effect of the material composing the microspheres, NHOst were cultured for 1 week on pre-coated PS, PE, alumina and HAp microspheres, all of which have a diameter of around 5 μm. Table 1 shows their number ratio and ALP activities, and the calcium amounts. Pre-coated PS, PE and alumina microspheres showed the potential to suppress functions of NHOst although some of these data did not show statistical differences against NHOst without microspheres. However, when NHOst were cultured with pre-coated HAp, the amount of calcium deposited was almost twice that detected in the cells without microspheres. It was observed that HAp microspheres have no potential to deposit calcium after a 1-week incubation without NHOst (data not shown). Therefore, the increase in calcium deposition by pre-coated HAp may be due to the enhancement in the differentiation of NHOst in contact with HAp. As expected, added various microspheres affected NHOst in a similar manner but less than the pre-coated microspheres (data not shown). We have hypothesized that GJIC of cells in contact with various biomaterials can be used as an index for estimating the biocompatibility of many kinds of biomaterials [5,6,9,11]. In addition, osteoblasts have been reported to communicate with one another *via* GJIC function, and the function is believed to be critical to the coordinated cell behavior necessary in bone tissue development [8,12]. Therefore, effects of these microspheres on the communication of co-cultured NHOst were estimated to consider the relation between this function and the differentiation of NHOst. The FRAP assay revealed that HAp microspheres enhanced the GJIC level of NHOst to 1.8 times as much as that of NHOst alone but others slightly inhibited it, indicating HAp has a potential to enhance homeostasis maintenance function of the NHOst as well as their differentiation. Details of the microspheres effects on GJIC of NHOst will be reported elsewhere [13]. These results indicated that the materials of microspheres affected the differentiation of co-cultured NHOst as well as the diameter of microspheres and their contact with the cells. In addition, microspheres made from HAp, which is a major component of bone tissue and has been shown to have good biocompatibility as bone substitute implants [14], may have the potential to enhance the differentiation of osteoblasts. These results suggest that the estimation of the effects of biomaterials in microsphere form on in vitro cell function may be useful for their in vivo biocompatibility evaluation.

We estimated the effect of sintering, normally used to harden HAp, on the function of NHOst. The estimation revealed that both HAp microspheres enhanced the amount of calcium deposited although the ALP activity of the cells decreased. In addition, when the un-sintered HAp microspheres were incubated with NHOst, the calcium deposition was observed more than sintered HAp. As another index of the differentiation of the NHOst, mRNA expression levels of osteocalcin, which is a well-known protein detected in

Table 1. Effects of a 1-week incubation with pre-coated microspheres on various functions of NHOst.
(Amounts of microspheres = 20 μg/well)

	Control	Polystyrene	Polyethylene	Alumina	Hydroxy Apatite (Sintered)
Diameter (μm)		5.0	6.4	5.1	7.2
The cell number ratio (%)	100.0±5.5	88.2±2.2	92.2±1.3	82.4±2.8	83.0±2.3
Percent ALP activity (activity/proliferation)	100.0±4.7	79.2±5.6	72.7±3.6*	58.2±5.7*	73.8±6.0*
Percent deposited calcium (Calcium percent/proliferation)	100.0±3.7	97.3±4.2	82.3±3.7	90.3±7.8	163.3±18.5*(a

Data are shown as the mean value ± SEM (n = 4 to 22)
* $p < 0.01$, against control group
(a) $p < 0.05$, against NHOst co-cultured with polyethylene and alumina microspheres

differentiated osteoblasts [15], were determined using the RT-PCR technique. Figure 2 shows time profiles of osteocalcin mRNA expression in NHOst cultured with pre-coated PS, PE, alumina, and two kinds of HAp microspheres. As shown in the figure, only the cells co-cultured with sintered HAp microspheres expressed osteocalcin mRNA after a 1-day incubation, while those co-cultured with other microspheres did not express the mRNA. This finding suggests that sintered HAp microspheres have the potential to induce osteocalcin production from NHOst. Neither spontaneous calcium deposition was observed by the incubation of sintered nor un-sintered HAp microspheres without NHOst, so that it is possible that the un-sintered HAp degrade in culture medium with NHOst, resulting in an increase of calcium concentration in the culture medium that enhances the calcium deposition by the NHOst. Therefore, it is suggested that sintered HAp can induce the differentiation of NHOst, and may be a suitable material for inducing osteogenesis rather than un-sintered one.

In conclusion, microspheres made from various materials had an effect on the differentiation of NHOst. The level of the effect varied with the size, amount, and composition of the microspheres. Microspheres made from PS, PE and alumina showed a potential to suppress the proliferation and the differentiation of co-cultured NHOst. On the other hand, microspheres made from HAp, especially sintered HAp, enhanced the differentiation of co-cultured NHOst, and showed their potential to maintain their homeostasis. Estimating the effect of various microspheres on the differentiation of osteoblasts will provide valuable information on the effects of wear debris from artificial hip joints as well as estimating their effects on osteoclast function.

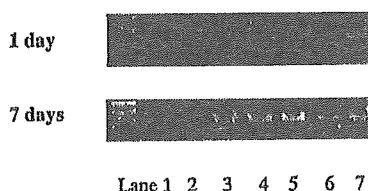

Figure 2. Expression of osteocalcin mRNA extracted from NHOst cultured on various microsphere pre-coated dish. Lane 1: Collagen-coated culture dish, 2: methanol-treated dish, 3: PS, 4: PE, 5: alumina, 6: un-sintered HAp, 7: sintered HAp.

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一般報文

ラット頭蓋冠由来骨芽細胞のALPase活性を促進する硫酸化ヒアルロン酸の効果

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Enhancement Action of Sulfated Hyaluronan on the ALPase Activity of Rat Calvarial Osteoblasts

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Abstract : The purpose of this study was to clarify the effect of hyaluronan (Hya) and sulfated hyaluronan (SHya) on rat calvarial osteoblast (rOB) cells proliferation and differentiation *in vitro*. rOB cells were cultured in the presence of Hya with different molecular weights (0.2, 2, 30, 90, 120 x 10^4) for 10days. Hya did not affect the proliferation of rOB cells. However, SHya suppressed the proliferation of rOB cells. The alkaline phosphatase (ALPase) activity of rOB cells cultured with SHya for 10 days was significantly enhanced in comparison with control (in the absence of polysaccharides) and with Hya. Hya suppressed the ALPase activity of rOB cells. As a result, SHya can control rOB cells proliferation and differentiation. SHya suppressed the rOB cells proliferation in a few culture days and promoted the differentiation. It was suggested that these effects were based on the sulfate groups of SHya. Therefore, it is considered that SHya is useful for the biomedical material, which promotes the differentiation of rOB cells.

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1. 緒　言

硫酸化多糖であるヘパリン（Heparin; Hep）やヘパラン硫酸（Heparan sulfate; HS）はheparin-binding growth factors（HBGFs）と複合体を形成し，組織が損傷を受けた場合，速やかにHBGFsを放出して，周辺の組織を活性化することが明らかとなっている[1-3]．HBGFsは，骨の修復にも重要な役割を果たしていることが知られ，骨芽細胞の増殖や分化の過程でオートクライン，パラクライン的に骨の形成や吸収を制御する[4-6]．病気やケガなどで骨組織が損傷した場合，修復するために人工骨や人工関節などの人工材料が用いられている．しかし，これらの人工材料は様々な問題があり，近年組織工学的手法を用いた骨再生が期待されている．この手法を用いて骨組織の再生に利用される細胞は，骨組織を形成する骨芽細胞である．骨芽細胞は間葉系由来の細胞で，未分化な間葉系の細胞から骨原性細胞を経て次第に成熟した骨芽細胞へと分化する．骨が大きく欠損した場合，細胞が増殖，分化するための足場が失われるため，仮の足場が必要となる．しかし，足場が優れていても細胞の数が少ないと十分な組織の再生は望めない．そこで、生体材料と増殖因子の組み合わせによる組織再生の方法が，近年多く報告されている[7, 8]．しかし，これらの増殖因子はたんぱく質であり，生体内での寿命が短く，不安定であるため，増殖因子を保持する担体が必要である．グリコサミノグリカンの構成成分であるヒアルロン酸（Hya）は，眼球、関節をはじめとする多くの結合組織に存在し，細胞外マトリックスの構成成分として，組織の創傷治癒や形態発生に重要な働きをしていることが報告されている[9-11]．近

年，Hyaのレセプターとして CD44が発見されて以来，Hyaを介した生物学的機能の研究が盛んに行われている[12-14]．Pilloniらは，骨芽細胞の前駆細胞である間葉系細胞を用いて，分子量の異なるHyaの影響を検討しており[15]，Hyaは骨芽細胞の石灰化を促進すると報告している．しかし，細胞の増殖性，分化マーカーについての詳細な検討は行っていない．そこで本研究では，骨再生用材料の開発を目的として，生体適合性の高い多糖類を用いて骨組織の再生を試みた．本研究では、ヒアルロン酸と硫酸化多糖の機能を併せ持つ高分子量の硫酸化多糖を作製し，ラット頭蓋冠由来骨芽細胞[rat calvarial osteoblast (rOB cells)]の初期骨分化マーカーであるAlkaline phosphatase (ALPase) に対する影響について検討を行った．

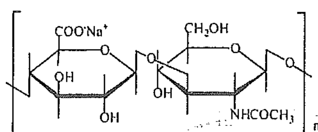
2. 実験方法

2.1 材料

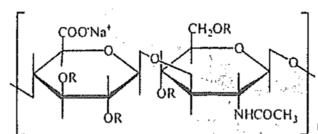
SHyaは以前に報告した方法にて合成した[16]．使用した硫酸化多糖の硫酸化度（D.S.；2糖残基当たり硫酸基の量）をTable 1に示した．HyaX(Xは分子量を示す)の分子量は0.2, 2, 30, 90, 120 x 10^4のもの使用した．Hya, SHya, コンドロイチン硫酸 typeC (Chs-C), Hepは0.5 mg/lの濃度になるように培地に溶解し，0.22 μm の孔径を有するfilterで滅菌をおこなった．HyaおよびSHyaの構造式をFig.1に示した．

Table 1 Characteristics of polysaccharides

Polysaccharides	Number of sulfate groups per two saccharide rings	MW ($x10^4$)
Hya	0	0.2-120
1.2SHya	1.2	55
2.1SHya	2.1	20
3.4SHya	3.4	5
Chs-C	1	0.5
Hep	2.5	1



Hyaluronan (Hya)



Sulfated hyaluronan (SHya)

R = $SO_3^-Na^+$ or H^+

Fig.1 Structure of hyaluronan and sulfated hyaluronan

2.2 細胞培養

生後48時間以内のウィスター系ラット(Charles River)の頭蓋冠から、酵素消化法によりrOB cellsを分離した[17]．その後，10% fetal bovine serum (FBS, GIBCO) を含むDulbecco's modified Eagle's medium(DMEM, Nissui-seiyaku) を用いて，初代培養を行った．3日毎に培地を交換しながら通常の継代培養を行い，継代数4-6のrOB cellsを実験に使用した．

2.3 細胞増殖

多糖類と10% FBSを含むDMEMを用いて調製したrOB cells ($1x10^4$ cells/well, 24 multiwell plate) を播種し，5% CO_2下，37℃で培養した．所定時間培養後の細胞数を，下記のタンパク質量測定によって計測した．上澄みを除去し，wellをphosphate-buffered salines (PBS; pH7.6) で3回洗浄した。0.04% nonidet P-40 (NP-40, Nacalai tesque) を含む1ml PBSを各wellに添加し，37℃で10分間インキュベートした．懸濁液を超音波破砕機を用いてホモジナイズした後，1000rpm，4℃，5分間遠心を行った．この上澄液を細胞溶液として，Bio-Rad protein assay (protein assay, Bio-Rad Lab.) 法により，595nm の吸光度を EIA READERを使って総タンパク質量を測定した．細胞数とタンパク質量の検量線を作成し，検量線により総タンパク質量から細胞数を算出した．検量線の作成法を以下に示す．0, 1, 5, 10, 30x10^5 cells/ml に調製した細胞懸濁液を各試験管に入れ，1000rpm，4℃，5分間遠心を行った．上澄みを除去し，0.04% NP-40を含む1ml PBSを各試験管に入れ，総タンパク質量を求め，細胞数と総タンパク質量の検量線を作成した．

2.4 Alkaline phosphatase (ALPase) 活性

ALPase活性の測定は以下のようにして行った．細胞増殖の測定時に得られた細胞溶解液0.1mlと基質水溶液0.4ml (16mM p-nitrophenylphosphate disodium salt hexahydrate) を混合して，30分間，37℃でインキュベートした．その後，反応を停止するため，混合液に0.2N NaOH水溶液を0.5ml添加し，410nmの吸光度をEIA READERを用いて測定した．総タンパク量はBio-Rad protein assayによって測定し，Albumin(Bovine Albumin Fraction V) の検量線から算出した．

全ての実験において，実験数n=6として測定を行い，その平均値を求めた。

3. 結果

分子量の異なるHyaを添加したrOB cellsの増殖曲線を，Fig.2に示した．培養7日目までは，Hyaの分子量に関係なくrOB cellsは増殖し，コンフルエントに達した．しかし，培養10日目になると，高分子量のHyaを添加したrOB cellsにおいて，わずかに細胞数の増加が示され

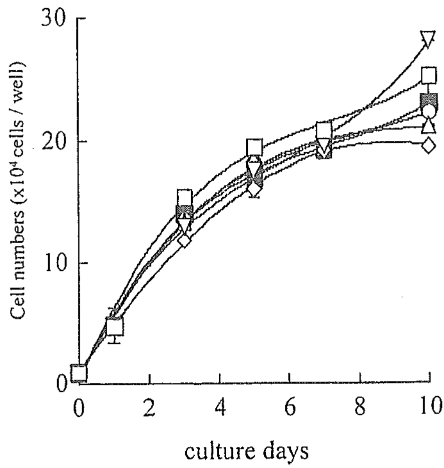


Fig.2 Effect of 0.5mg/ml hyaluronan on the proliferation of rOB cells

■ none ○ Hya0.2 △ Hya2 ◇ Hya30 ▽ Hya90 □ Hya120

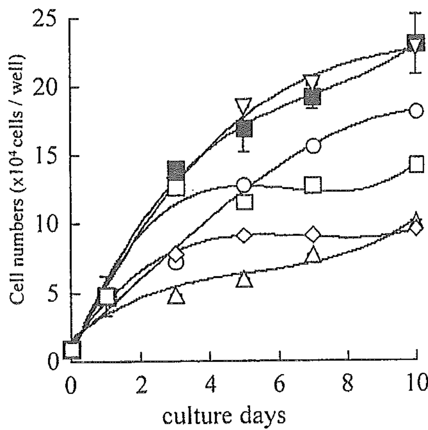


Fig.3 Effect of 0.5mg/ml sulfated polysaccharides on the proliferation of rOB cells

■ none ○ 1.2SHya △ 2.1SHya ◇ 3.4SHya ▽ Chs-C □ Hep

た.

Fig.3に，硫酸化度の異なるSHyaを添加したrOB cellsの増殖曲線を示した．Hyaを添加した場合と異なり，SHyaを添加したrOB cellsは，培養3日目から非添加系に比べて増殖が抑制された．さらに，SHyaの硫酸基の導入率が高くなるほど，rOB cellsの増殖は抑制された．これに対し，同じ硫酸基を有する多糖類であってもChs-Cではほとんど影響は見られず，Hepでも抑制効果は小さかった．

Fig.4に，Hyaを添加したrOB cellsのアルカリフォスファターゼ(ALPase)活性の経時変化を示した．Hyaは分子量に関係無く，骨芽細胞の初期分化マーカーであるALPaseの活性は非添加系に比べて低い値を示した．Fig.5に、硫酸化度の異なるSHyaを添加したrOB cellsのALPase活性を示した．Hyaとは異なり，SHyaを添加したrOB cellsのALPase活性は非添加系に比べて上昇が認められた．特に、高硫酸化度になるほど，ALPase活性の

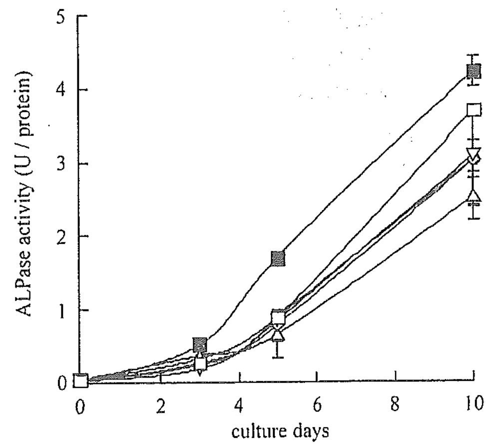


Fig.4 Effect of 0.5mg/ml hyaluronan on the ALPase activity of rOB cells

■ none ○ Hya0.2 △ Hya2 ◇ Hya30 ▽ Hya90 □ Hya120

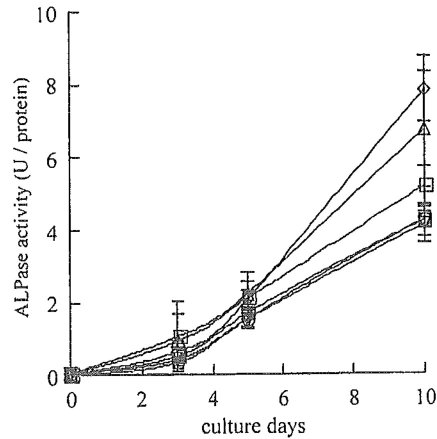


Fig.5 Effect of 0.5mg/ml sulfated polysaccharides on the ALPase activity of rOB cells

■ none ○ 1.2SHya △ 2.1SHya ◇ 3.4SHya ▽ Chs-C □ Hep

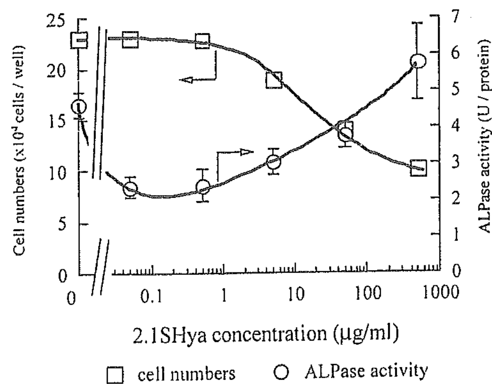


Fig.6 Dose-dependence of 2.1SHya on the proliferation and ALPase activity of rOB cells after 10days

上昇率は高かった．そこで，rOB cells の増殖と ALPase 活性に対する 2.1SHya の添加濃度の影響を検討した（Fig.6）．高濃度の SHya は rOB cells の増殖を抑制し，ALPase 活性を促進させたのに対し，低濃度の SHya は増殖を促進し，ALPase 活性を抑制させることが認められた．

4. 考 察

本実験で我々は，分子量の異なるHyaを骨芽細胞に添加し，骨芽細胞の増殖とALPase活性について検討を行った．分子量2000から120万のHyaを rOB cells に添加したところ 非添加系とほとんど変わりなく増殖し，Hya添加やその分子量の違いによる影響は見られなかった（Fig.2）．しかし，ALPaseの活性は分子量に関係なく非添加系に比べてすべて低いため（Fig.4），Hya は rOB cells の分化を抑制することが示唆された．Hep, HSは細胞外あるいは細胞表面に広く存在し，多くの種類のタンパク質と特異的な相互作用を示すことが知られている[18]．特に，ヘパラン硫酸プロテオグリカン（HSPG）は，細胞と ECM の相互作用や細胞同士の相互作用を介して，接着，凝集，シグナル伝達などに関与している．このように，多岐にわたる HSPG の機能の中で，増殖因子との相互作用については多くの報告があり，注目されている[1-3]．FGF, transforming growth factor-β(TGF-β), bone morphogenetic protein(BMP)などの細胞増殖因子は，Hep や HS などの硫酸化多糖と相互作用し，細胞の増殖を制御することが報告されている[1,4]．Hep, HS, Chs の分子量は，Hyaに比べて非常に小さい．そこで本研究では，高分子量である SHya の骨再生用材料への応用を目的として，SHya 単独での rOB cells に対する影響を検討した．SHya の硫酸化度が高くなるにつれ，細胞の増殖は抑制され，Hepもある程度の抑制効果を示した(Fig.3)．ALPase活性に対しては，硫酸化度が高くなるにつれて活性が上昇した(Fig.5)．これより，硫酸化多糖は細胞の増殖を抑制し，分化を促進させることが示された．次に，影響が最も大きく現れた2.1SHyaを用いて，濃度依存性について検討を行った．Fig.6より，2.1SHyaは低濃度では細胞の増殖を促進し，高濃度になるにつれ増殖を抑制した．これに対して ALPase 活性は低濃度では活性が低く，高濃度になるにつれ上昇した．これより，2.1SHyaは濃度を変化させることで，rOB cells の機能を制御することが可能であることが示された．Hep, HSと増殖因子との協同的な作用の細胞の増殖に対する影響も，濃度によって大きく異なることが報告されている．Blanquaert らは，Hep 及び硫酸化多糖の RGTA(Heparin-like polymers derived from dextran)と増殖因子との協同作用による，マウス頭蓋冠由来骨芽細胞 MC3T3-E1 への影響を報告している[1]．RGTA は増殖因子と共に用いることで，増殖に対しては抑制的に働き，ALPaseの活性が上昇することを明らかにした．この作用は RGTA のみでも影響が現れるが，増殖因子が存在することにより，さらに顕著に影響が現れた．今回，我々は SHya 単独の影響を検討したが，彼らの結果と一致する結果が得られた．以上の結果から，Hyaに硫酸基を導入することにより，SHyaは骨芽細胞の増殖や分化機能を制御することが可能であると示された．

5. 結 論

rOB cells に Hya を添加すると，rOB cells の増殖は促進され，分化は抑制された．しかし，SHyaを添加すると，rOB cells の増殖は抑制され，分化の促進が示された．SHya の効果は SHya の硫酸化度，濃度に大きく依存した．従って，SHyaは骨芽細胞の機能を制御することが明かとなった．骨形成促進作用を持っている BMP, FGF2, TGF-βなどの増殖因子を臨床応用に用いる場合，これらの増殖因子に適した担体の開発が必要である．SHya は分子量が高く，粘性があるため，増殖因子を保持する能力は Hep、HS などの他の硫酸化多糖に比べて高いことが考えられる．今後，SHyaと増殖因子との相互作用について検討を行うことにより，SHyaの分化促進作用の機序を明らかにできると同時に，SHya の骨再生用材料への応用が期待される．

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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NHOst). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NHOst on it although the NHOst on it showed an enhancement in their differentiation level. On the other hand, NHOst on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NHOst was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NHOst on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NHOst to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NHOst on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.
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Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using in vitro techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

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A polyelectrolyte complex (PEC) is a compound made from an electrically neutralized molecular complex of polyanions and polycations [13]. PEC can be prepared in various forms such as a film (2D) and a hydrogel, a microcapsule or a sponge (3D), which can be used as a scaffold in tissue regeneration studies. The effects of PEC films composed of polysaccharides on cell behavior have been studied, and we have already reported that PEC can stimulate differentiation of osteoblasts and periodontal ligament fibroblasts [14–16]. These studies suggest that PEC can be used as a biomaterial for repairing or regenerating tissues. In addition, because the PEC are composed of polysaccharides, PEC is expected not to elicit immune responses against it and to have better biocompatibility with the human body, although this is yet to be proved. Therefore, it is necessary to study the interactions between PEC and cells, especially human-derived, to clarify the usefulness of PEC as a biomaterial.

In this study, normal human osteoblasts (NHOst) were cultured on various PEC prepared on a tissue culture plate from chitosan as the polycation and modified chitins or hyaluronan as the polyanion. It should be generally agreed that estimating not only functional advantages but also safety and biocompatibility of biomaterials is important to develop them for clinical use, but the latter is not always studied. Therefore, we measured changes in gap junctional intercellular communication (GJIC) as well as the cell number and differentiation. GJIC is very important function for almost all cells to maintain their homeostasis [17]. During this decade, we have studied the effects of model biomaterials on the GJIC of cells cultured on them and suggested a possibility that changes in the GJIC can be used as an index of biocompatibility of biomaterials [18–21]. Therefore, we measured changes in GJIC of NHOst on PEC in order to estimate the biocompatibility of PEC from their effects on these cell functions.

2. Materials and methods

2.1. Chemicals

Fig. 1 shows the chemical structures of the polyanions and the polycation. Chitosan as the cationic polysaccharide and carboxymethylated chitin [CM-Chitin: degree of substitution (DS) = 1.0 (1.0 anionic site/saccharide ring)] were purchased from Katokichi Co., Ltd. (Kagawa, Japan). Sulfated chitin (S-Chitin: DS = 1.5), phosphated chitin (P-Chitin: DS = 1.6), hyaluronan (HA), and sulfated hyaluronan (SHA: DS = 1.05) were prepared as previously reported [14–16,22].

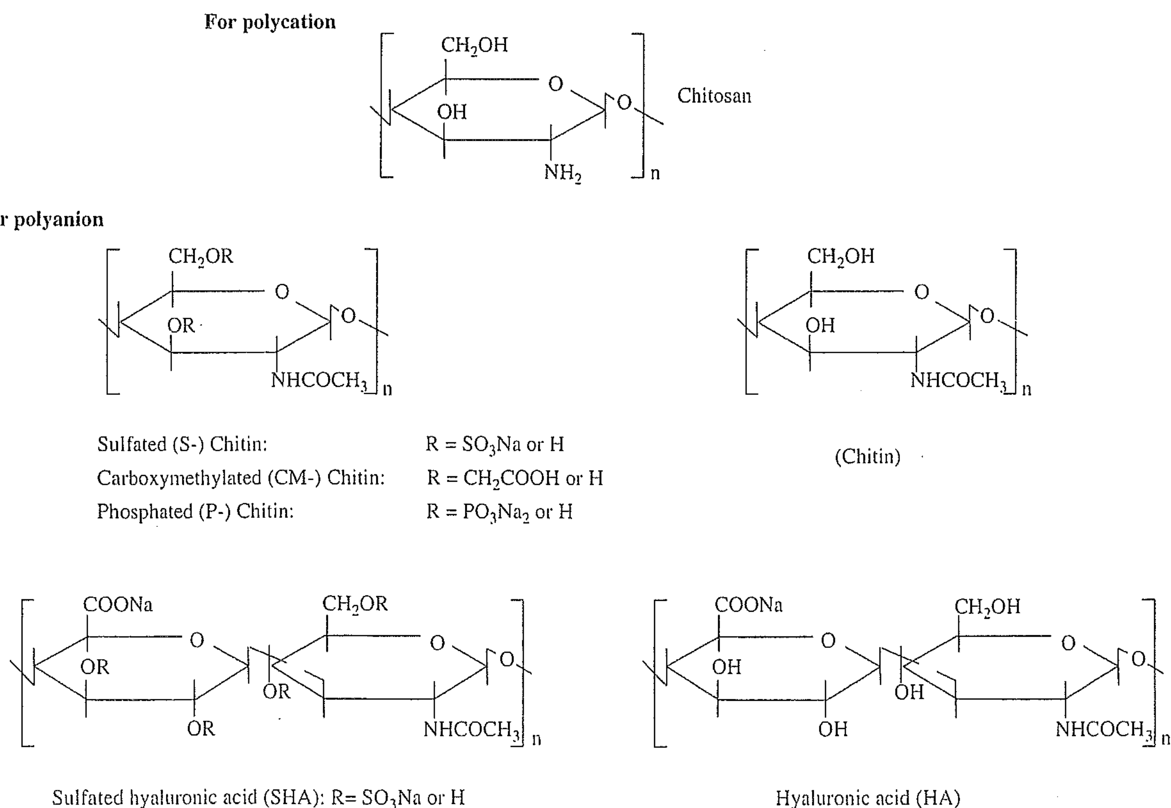

Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

2.2. Preparation of PEC and PEC-coated dishes

Polyanions were dissolved individually in distilled water (final concentration = 5×10^{-4} mol of ionic sites/l), and the pH of the solutions was adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 0.5% acetic acid solution and the pH adjusted to 6.0. The ratio of the solutions of polyanions and polycation was adjusted in each combination to neutralize the charge balance of PEC. This mixed solution (1 ml/35 mm tissue culture dish) was allowed to stand overnight at room temperature. After removing the supernatant solution, the dish was dried and annealed at 65 °C in an oven. Then, the dishes were washed with distilled water and oven-dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven. Water contact angles of PEC films were measured with the sessile drop method [23], and their zeta potentials were measured by Otsuka Electronics Co., Ltd. (Osaka, Japan).

2.3. Cell culture

NHOst were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan). The cells were maintained in incubators under standard conditions (37 °C, 5%-CO_2–95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst cells (1×10^5 cells/dish/2.5 ml medium) were cultured on PEC-coated dishes to evaluate the effects of their interaction with PEC. In each experiment, the medium was changed three times before GJIC of the cells was measured and their differentiation level was evaluated after a 1-week incubation.

2.4. Estimation of differentiation level of NHOst cultured on PEC films

The proliferation of NHOst cells cultured on PEC films was estimated by Tetracolor One assay (Seikagaku Co., Tokyo, Japan), which incorporates an oxidation-reduction indicator based on detection of metabolic activity. After a 1-week incubation, 20 μl of Tetracolor One solution was added to each test dish, followed by a further 2 h incubation. The absorbance of the supernatant at 450 nm was estimated by μQuant spectrophotometer (Bio-tek Instruments, Inc., Winooski, VT). Estimation of alkaline phosphatase (ALP) activity was performed according to an original procedure by Ohyama et al. [24]. After estimating the proliferation of the NHOst cells cultured on PEC films, the cells were washed by phosphate-buffered saline (PBS(−)), followed by addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 4 mM *p*-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the glycine buffer was detected at 405 nm using μQuant to evaluate the ALP activity of the test cells. The amounts of calcium deposited by the cell during a 1-week incubation were evaluated as follows: after fixing the cells in PBS(−) containing 3% formaldehyde and washing the cells with PBS(−), 0.5 ml of 0.1 M HCl was added to each well. The amounts of calcium dissolved in HCl were estimated using a calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to manufacturer's instruction.

2.5. Measurements of GJIC activity

NHOst cultured on PEC films were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these films on the GJIC. FRAP analysis was carried out according to the procedure of Wade et al. [25] with some modifications [21]. Briefly, NHOst were plated on PEC-coated dishes and incubated for 1 or 7 days. The cells were incubated for 5 min at room temperature in PBS(−) containing Ca^{2+} and Mg^{2+} (PBS(+)) and a fluorescent dye, 5,6-carboxyfluorescein diacetate. After washing off excess extracellular dye with PBS(+), the cells in PBS(+) contacting at least two other cells were subjected to FRAP analysis under a Ultima-Z confocal microscope (Meridian Instruments, Okemos, MI) with a 10 × objective lens at room temperature. The cells were photobleached with a 488 nm beam, and recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total of 4 min. The data obtained from more than seven independent cells were expressed as the average ratio of the fluorescence recovery rate to the rate obtained from NHOst cultured on a collagen-coated dish.

2.6. Statistic analysis

All data were expressed as mean values ± standard deviation of the obtained data. The Fisher–Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

3. Results and discussion

When NHOst were cultured on five kinds of PEC films, their morphology and attachment to the film differed with the composition of the PEC. Fig. 2 shows the morphologies of the NHOst adhering to PEC films.

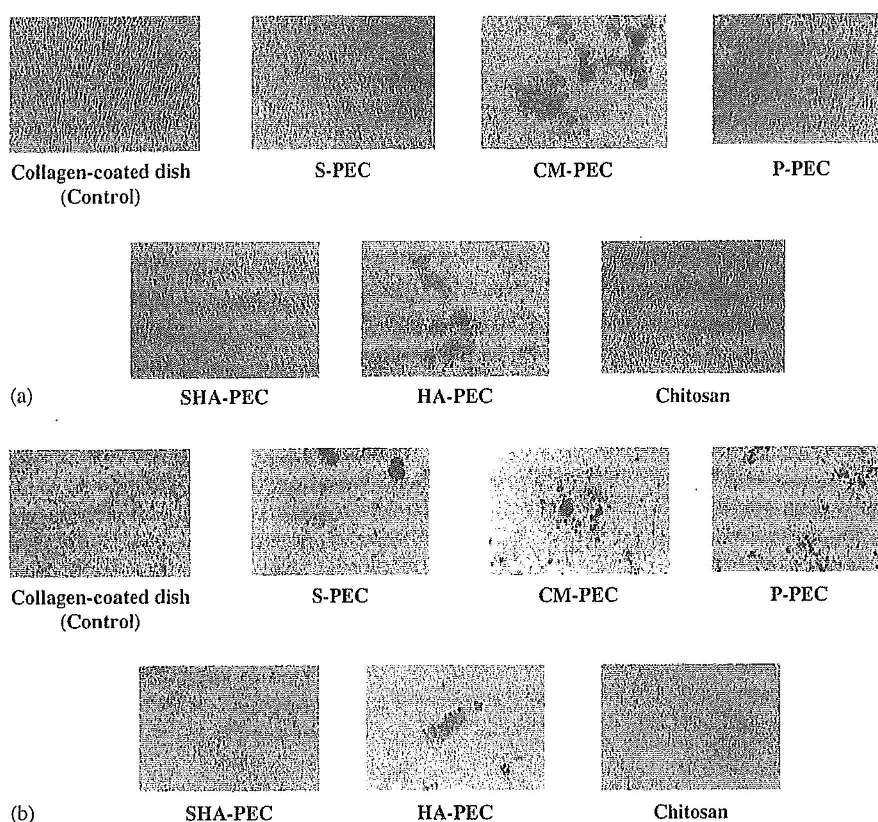

Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: × 100).

After 2-day incubation, the NHOst on PEC composed of chitosan and either sulfated chitin (S-PEC) or sulfated hyaluronan (SHA-PEC) showed morphologies similar to those on a normal culture plate. When cells were cultured on PEC of chitosan and phosphated chitin (P-PEC), some of them formed small aggregates, while the rest showed morphologies similar to those on S-PEC and SHA-PEC. On the other hand, NHOst cultured on PEC from chitosan and either carboxymethyl chitin (CM-PEC) or hyaluronan (HA-PEC) did not adhere well and showed aggregation. Similar morphologies of the cells on the PEC were observed after 1 day of incubation (data not shown). Even after 1 week of incubation, the morphologies and attachment of the cells on the PEC films did not change (Fig. 2). Only cells grown on cationic polysaccharide chitosan-coated culture dishes preserved morphology of very similar to NHOst grown on collagen-coated cultured dishes, indicating that these morphological differences are ascribable to differences in the anionic polysaccharides of which the PEC is composed.

It has been reported that cell attachment, morphology, and response are influenced by physico-chemical properties of the material surface [23,26]. To clarify what properties of PEC control the attachment and morphology of the cell, the contact angle and zeta

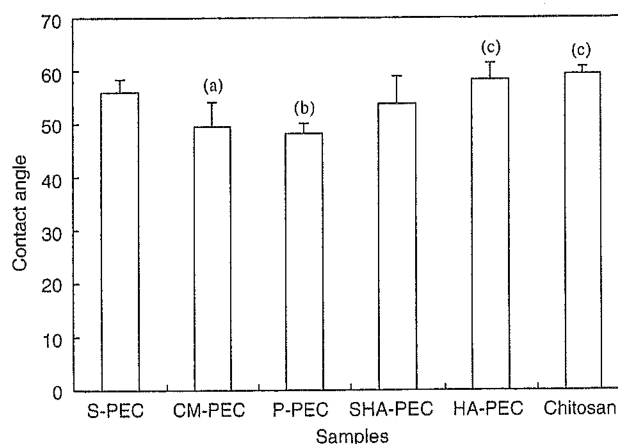

Fig. 3. Contact angles of PEC films studied: (a) $p<0.05$ against S-PEC, (b) $p<0.01$ against S-PEC, (c) $p<0.01$ against both CM-PEC and P-PEC.

potential of PEC films were estimated. Although their compositions are different, large differences in their contact angles were not observed (Fig. 3). On the other hand, a measurement of zeta potentials of the PEC showed interesting results (Table 1). The measurement revealed that S-PEC and SHA-PEC have negative zeta potentials, whereas PEC films made of polysaccharides

Table 1
Zeta potentials of various PEC prepared on a culture dish

	Culture	S-PEC	CM-PEC	P-PEC	SHA-PEC	HA-PEC
Zeta potential (mV)	−58.7	−28.0	34.5	24.9	−5.7	29.5

Table 2
The cell number and differentiation of NHOst cultured on various PEC films after 1 week

Samples	The cell number (percent against control)	ALP activity / The cell number (ratio)	Ca amount / The cell number (μg/ratio)
Collagen-coated dish	100.0±17.0	1.00±0.15	3.4±0.5
S-PEC	82.2±6.1	0.98±0.11	10.7±3.6
CM-PEC	6.0±2.6*	0.05±0.08*	27.4±3.0*
P-PEC	130.4±6.3	0.02±0.01*	2.5±0.8
SHA-PEC	71.4±22.1	1.35±0.48	2.1±1.0
HA-PEC	8.1±3.0*	0.52±0.31	38.3±12.3*
Chitosan	79.5±25.0	0.93±0.13	2.7±2.0

*$p<0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from −30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in simulated body fluid [27]. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP

activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC does not affect NHOst functions. Actually, there were no statistical differences in the amounts of calcium deposited between NHOst on the PEC and the collagen-coated dish although NHOst on S-PEC showed higher average calcium deposition. Thus, it is suggested that the PEC films made from sulfated polysaccharides are comparable substrates to a collagen-coated dish for cell culture. When compared to a normal culture dish, it has been reported that S-PEC can induce aggregation of cultured human fibroblasts and enhance their DNA synthesis in an earlier stage of cell culture by activation of the ERK pathway [28]. Since we used a collagen-coated dish as a control in this study, it is expected that the pathway of NHOst on the dish may be already activated through integrin molecules on the NHOst membrane. Therefore, the results in this study suggest the PEC from sulfated polysaccharides have a potential to proliferate and differentiate NHOst very similar to that of collagen.

To assess the effects of PEC films on cell function, gap junctional intercellular communication (GJIC), which is an important function of cells for maintenance of homeostasis [17], of NHOst on the films were measured. As shown in Fig. 4, GJIC of NHOst on PEC films did not show statistically significant differences compared to those grown on a collagen-coated dish. Although the GJIC of NHOst on CM-PEC showed a decrease after 1 day of incubation, it had recovered after 1 week. This result suggests that most PEC films have the potential to maintain homeostasis of attached cells although they showed different influences on the number and the

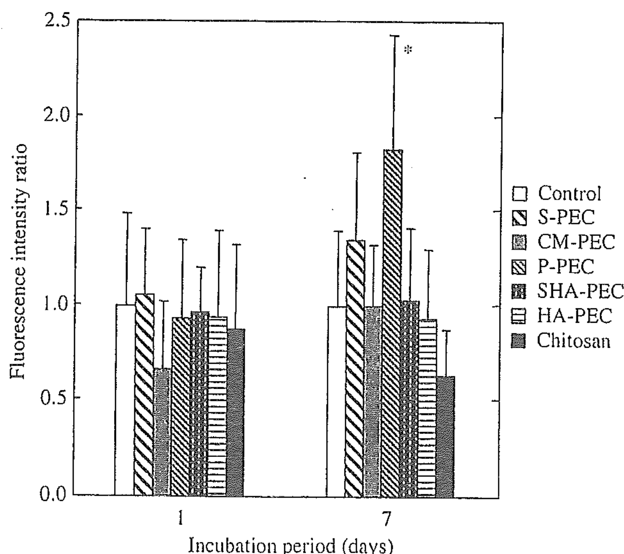

Fig. 4. Gap junctional intercellular communication activity of NHOst on various PEC films estimated by FRAP analysis technique. (*$p<0.01$ against control).

differentiation of NHOst. On the other hand, NHOst on chitosan, which was used as the polycation for all PEC, showed suppression of GJIC after 1 week. This suggests that chitosan disturbs homeostasis maintenance of NHOst, but improve its biocompatibility by forming PEC films with other anionic polysaccharides. Therefore, PEC might be used as a biocompatible material for medical devices and tissue engineering scaffolds.

4. Conclusion

PEC films composed of various polysaccharides were prepared, and their effects on NHOst functions were evaluated. Attachment, morphology, growth and differentiation of NHOst were influenced by the composition of the PEC on which they were grown. NHOst attachment decreased and their aggregates were observed on PEC prepared from polysaccharides containing a carboxyl group (CM- and HA-PEC). ALP activity of NHOst was suppressed on these PEC films although calcium deposition was observed more frequently than on other PEC films. In addition, these PEC films strongly suppressed proliferation of NHOst. PEC prepared from phosphated chitin and chitosan (P-PEC) showed low ALP activity and calcium deposition, although the number of NHOst was highest after 1-week incubation. These indicate unsuitability of these three PEC for usage in tissue engineering. On the other hand, NHOst adhered to and proliferated well on PEC films when sulfated polysaccharides were used as the polyanion (S- and SHA-PEC). Moreover, these PEC films showed almost the same suitability as the collagen-coated dish in all cell functions studied, indicating that these PEC films, especially S-PEC can be used as a scaffold for bone regeneration. Further studies, especially in vivo studies, are needed to clarify the usefulness of PEC films for tissue engineering.

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Hydroxy apatite microspheres enhance gap junctional intercellular communication of human osteoblasts composed of connexin 43 and 45

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Abstract: The aseptic loosening of artificial joints with associated periprosthetic bone resorption may be partly due to the suppression of osteoblast function to form new bone by wear debris from the joint. To assess the effect of wear debris on osteoblasts, effects of model wear debris on gap junctional intercellular communication (GJIC) of normal human osteoblasts were estimated. The GJIC activity of the osteoblasts after a 1-day incubation with the microspheres was similar to that of normal osteoblasts. However, hydroxy apatite particles, which have been reported to enhance the differentiation of osteoblasts in contact with them, enhanced the GJIC function of the osteoblasts. From RT-PCR studies, not only connexin 43 but also connexin 45 is suggested to play a role in the GJIC of the osteoblasts in an early stage of coculture with the microspheres, although it is still unclear how these connexins work and are regulated in the GJIC and differentiation. However, this study suggests that there is a relationship between the early levels of GJIC and the differentiation of the cells. Therefore, estimating the effect of biomaterials, even in the microsphere form, on the GJIC of model cells, with which the biomaterials may be in contact *in vivo*, can provide important information about their biocompatibility. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 74A: 181–186, 2005

Key words: gap junctional intercellular communication; human osteoblasts; microspheres; hydroxy apatite; connexin

INTRODUCTION

Biomaterials implanted into the harsh environment of the body cannot maintain their original shape, or even their desired function, sometimes resulting in undesirable side effects. One well-known example is the aseptic loosening of artificial joints observed in many patients who underwent a total joint replacement 5 to 25 years ago. It has already been reported that aseptic loosening with associated periprosthetic bone resorption is partly due to the activation of macrophages and osteoclasts by wear debris from the artificial joint.[1–14] Macrophages stimulated by wear debris *in vitro* release significant amounts of inflammatory mediators such as interleukin-1, interleukin-6, prostaglandin E2, collagenase, and tumor necrosis factor.[6–14] In addition, the biological effects of wear debris may depend on the type of material used as well as the shape, size, and amount of the debris.[4–11] Therefore, it is important to estimate the biocompatibility of biomaterials with not only their original shape but also possible transformed shapes after their usage.

During the last decade, we have been researching the inhibitory potential of many kinds of biomaterials on gap junctional intercellular communication (GJIC) as an index for their biocompatibility.[15–18] GJIC is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules, which results in regulating cell growth, development, and differentiation of cells.[19,20] Therefore, it is reasonable that disruption of this function is the cause of many kinds of diseases. In a previous report,[18] we examined the inhibitory activity of polymer microspheres, which were used as model wear debris from biomedical polymer *in vivo*, on the GJIC of rodent-derived fibroblasts. We concluded that estimating the inhibitory activity of the microspheres on the GJIC might be useful for considering their side effects in the body. In other words, it may be possible to predict whether wear debris causes aseptic loosening of artificial joints by estimating their effect on GJIC function.

No benefit of any kind will be received either directly or indirectly by the authors
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However, it must be noted that the effects of the microspheres may be different when the effects on the GJIC of human-derived cells are estimated. Osteoblasts have been reported to communicate with one another via GJIC function, and the function is believed to be critical to the coordinated cell behavior necessary in bone tissue development.[21,22] Therefore, the question is raised whether wear debris has an inhibitory effect on the GJIC and the GJIC inhibition has a relation with the aseptic loosening of artificial joints. Because we have already observed some precoated polymer microspheres around 5 μm in diameter showed the potential to inhibit GJIC of fibroblasts contacting with them,[23] we estimated effects of various microspheres around 5 μm in diameter on GJIC function using normal human osteoblasts to discuss the relationship between the GJIC and the differentiation of osteoblasts. In this study, we employed fluorescence recovery after photobleaching (FRAP) analysis for estimating the GJIC function,[17] and assessed the potential effect of many kinds of microspheres on the GJIC.

MATERIALS AND METHODS

Microspheres

Monodispersed polystyrene (PS) microspheres (5 μm in diameter) were purchased from Japan Synthetic Rubber Co., Ltd. (Tokyo, Japan). Low-density polyethylene (PE) microspheres were generously supplied by Sumitomo Seika chemicals Co., Ltd. (Tokyo, Japan). Alumina (Al_2O_3) microspheres were obtained from the Association of Powder Process Industry and Engineering. Sintered hydroxy apatite microspheres (HA, 7.2 μm in diameter) were prepared and supplied by Ube Material Industries, Ltd. A Multisizer II (Coulter Electronics Inc., Hialeah, FL) was used to determine the average diameter of PE and alumina microspheres: 6.4 and 5.1 μm, respectively. Microspheres were sterilized by dispersing them in a 70% ethanol solution, followed by centrifugation in sterile conditions to remove the ethanol solution. The microspheres were dispersed in sterile methanol for cell differentiation tests at specified concentrations. The suspension of microspheres in methanol was added to 35-mm type I collagen-coated cell culture dishes (Asahi techno glass, Chiba, Japan), and the plates dried overnight at room temperature. The obtained microsphere-coated dishes (100 μg/dish) were subjected to the assays.

Cell culture

Normal human osteoblasts (NHOst) were purchased from BioWhitteker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo, Japan). The cells were maintained in incubators under standard conditions (37°C, 5%-CO_2-95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 m*M* beta-glycerophosphate. NHOst (1 $\times 10^5$ cells/dish/2.5 mL medium) were cultured on microsphere-coated dishes for estimating the effect of the microspheres interacted from the bottom of the cells. To estimate the effect of microspheres on cells adhered to the culture plates, the NHOst cells were cultured with microsphere-containing medium (100 μg/2.5 mL medium) after they had adhered to the collagen-coated dishes. The test cells were cultured while changing the medium three times when the measurement of GJIC was performed after a 7-day incubation.

Measurement of GJIC activities

NHOst cultured with microspheres were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these microspheres toward the GJIC. FRAP analysis was carried out according to an original procedure by Wade et al.,[24] with some modifications.[17] Briefly, NHOst were plated on microsphere-coated dishes and incubated for 1 or 7 days. After a wash with phosphate buffer saline (PBS) containing $MgCl_2$ and $CaCl_2$ [PBS(+)], the cells were incubated for 5 min at room temperature in PBS(+) containing 5,6-carboxyfluorescein diacetate (7 μg/mL, excitation 488 nm and emission 515 nm). After the washing off of excess extracellular dye with PBS(+), the cells in the test dishes in PBS(+) were subjected to the FRAP analysis. In the control experiment, cells were inoculated on an untreated glass bottom dish and treated with the same procedure as the tested cells. Cells in contact with test microspheres and at least two other cells were subjected to FRAP analysis under an Ultima-Z confocal microscope (Meridian Instrument, Okemos, MI) with a 10× objective lens at room temperature. The cells were photobleached with a 488-nm beam and the recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total period of 4 min. The data obtained from more than seven independent cells were expressed as the average of fluorescence recovery rate in comparison to the rate obtained from NHOst cultured without microspheres.

Effect of microspheres on calcium deposition by NHOst

The amount of calcium deposited during a 7-day incubation of the cells were evaluated as follows: NHOst were cocultured with either precoated or added microspheres in 24-well collagen-coated culture plates (Asahi techno glass, Chiba, Japan) for 1 week (2 $\times 10^4$ cells/20 μg microspheres/well/500 μL medium). After the cells were fixed in formaldehyde, 0.5 mL of 0.1 *M* HCl was added to each well after washing the cells with PBS. The amounts of calcium dissolved in HCl were estimated using a Calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to the manufacturer's direction.

RT-PCR for estimating expression of connexins

According to the method reported by Ichikawa et al.,[25] RT-PCR was performed to detect the expression of connexin mRNA in NHOst. After culturing NHOst with microspheres for a scheduled time, total RNA was extracted from the NHOst using TRIZOL® reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. After dissolving the RNA in diethylpyrocarbonate-treated water, the total RNA concentration was measured spectrophotometrically using Genequant (Amarsham Biosciences Corp., Piscataway, NJ). RNA samples were adjusted to a minimum concentration among collected samples in each experiment and reversibly transcribed to cDNA using Superscript™ II (Invitrogen Corp.). For PCR amplification of human connexin 45, Takara Ex-Taq™ (Takara Shuzo Co., Ltd., Shiga, Japan) was used with Ex-Taq™ buffer consisting of 20 pmol each of two human connexin-45 specific primers (forward 5′GTGGCAACTCCCTCTGTGAT3′ and reverse 5′GGATCCTCAAGTTCCCTCCT3′). For PCR amplification of human connexin 26, 32, and 43, Takara LA-Taq™ (Takara Shuzo Co., Ltd.) was used with Ex-Taq™ buffer consisting of 6 pmol each of the human connexin-specific primers (for connexin 26, forward 5′ATGGATTGGGGCACGC3′ and reverse 5′TTAAACTGGCTTTTTTGACTTCCC3′. For connexin 32, forward 5′ATGAACTGGACAGGTTTGTACACCTTGCTC3′ and reverse 5′TCAGCAGGCCGAGCAGCGG3′. For connexin 43, forward 5′ATGGGTGACTGGAGCGCCTTAGGC3′ and reverse 5′CTAGATCTCCAGGTCATCAGGCCG3′). The PCR profile for connexin 45 involved pretreatment at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 90 s. The PCR profile for connexin 26, 32, and 43 (35 times) was as follows: pretreatment at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 120 s. Reaction products were analyzed by electrophoresis in 1.5% (w/v) agarose gel, followed by staining of the products by SYBR® Green I (Takara Shuzo Co., Ltd.) and detection of a 566-bp (connexin 45), 671-bp (connexin 26), 852-bp (connexin 32), and 1149-bp (connexin 43) band, respectively. For the standardization of connexin cDNA, PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample was performed using GAPDH-specific primers (forward 5′CCCATCACCATCTTCCAGGAGCGAGA3′ and reverse 5′TAAGTAGGACAACAAGGAGGTCGTGACGACGC3′; product size 578-bp). All reactions included negative controls without cDNA.

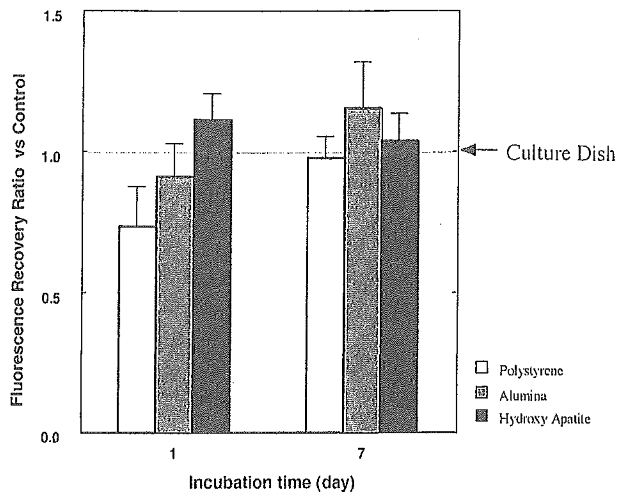

Figure 2. Effect of added microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively.

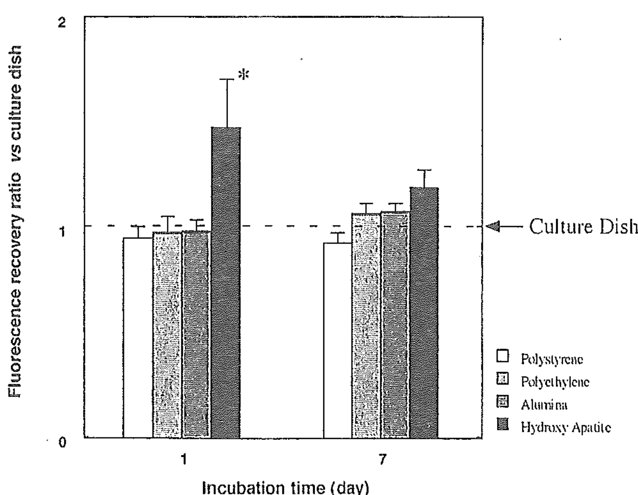

Figure 1. Effect of precoated microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively. (*$p < 0.01$ against culture dish).

Statistical analysis

All data were expressed as the mean value ± the standard error of the means of the obtained data and treated statistically with Student's *t* test.

RESULTS

Figure 1 shows effects of various microspheres on GJIC of NHOst in contact with the microspheres for 1 and 7 days. The microspheres were precoated on 35-mm culture dishes before cell seeding. When the NHOst were cultured with precoated PS, PE, and alumina microspheres, their GJIC level was similar to that in NHOst cultured on a normal culture dish. On the other hand, the GJIC level was 1.5 times that of NHOst on the normal dish when they were cultured with precoated hydroxy apatite microspheres. After 7 days, the GJIC of NHOst in contact with microspheres became similar to that of normal NHOst, irrespective of the type of microsphere. The change in GJIC of NHOst in contact with added microspheres is shown in Figure 2. As seen in Figure 1, hy-

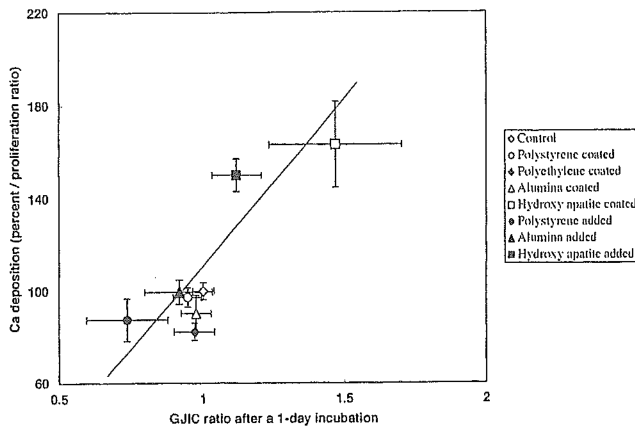

Figure 3. Relationship between GJIC on day 1 and calcium deposition ratio after 7-day coculture of NHOst with various microspheres ($r^2 = 0.74$).

droxy apatite microspheres enhanced their GJIC after a 1-day culture compared to cells on a normal plate. The degree of enhancement of GJIC is, however, smaller than that seen in NHOst in Figure 1, and no significant difference was observed between NHOst in contact with the hydroxy apatite microspheres and those cultured without microspheres. In addition, Figure 2 indicates that addition of PS microspheres into a culture of NHOst inhibited GJIC.

To consider the effects of tested microspheres on not only GJIC but also the differentiation of NHOst, changes in the amount of calcium deposited after a 1-week coculture of NHOst with various microspheres were estimated. From Figure 3, it is suggested that there is the possible relation between the GJIC of NHOst cocultured with microspheres for 1 day and

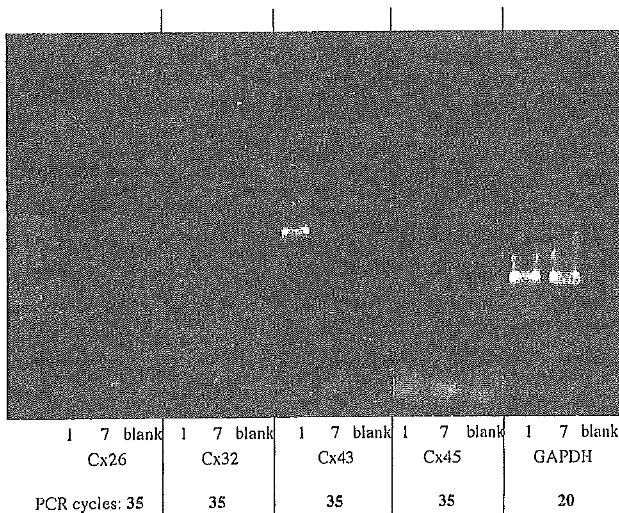

Figure 4. Expression of mRNA of various connexins (Cx) in NHOst cultured for 1 and 7 days. The number of NHOst cultured on 35-mm collagen-coated culture dishes was 2 × 10^5. RT-PCR cycles of each lane are expressed at the bottom of the figure.

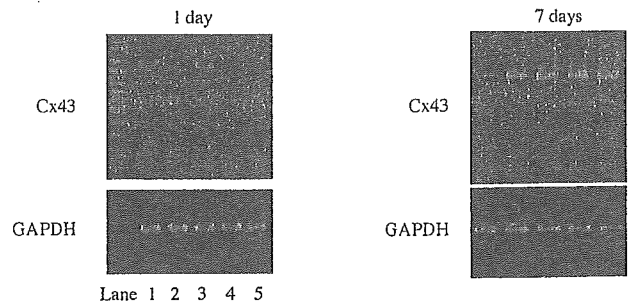

Figure 5. Expression of connexin 43 (Cx 43) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 43 and GAPDH is 35 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

the amount of calcium deposited after a 1-week coculture with the same microspheres.

To clarify which connexins exist in NHOst, we performed RT-PCR to detect mRNA of connexin 26, 32, 43, and 45 in NHOst cultured on a normal culture dish. Figure 4 shows the result of RT-PCR to amplify the mRNA from whole RNA collected from NHOst cultured for 1 and 7 days. As shown in the figure, only connexin 43 and 45 were detected in NHOst. When cells were cultured for 7 days, connexin 43 was detected at a lower level than that detected after the 1-day culture, while connexin 45 was not detected.

Figures 5 and 6 show the results of RT-PCR to amplify mRNA of connexin 43 and 45 in NHOst cultured with various precoated microspheres. The NHOst cultured with microspheres did not express mRNA of connexin 43, except those with PE microspheres. After 7 days, the expression was suppressed in the normal NHOst while the expression was observed in NHOst cultured with microspheres, irrespective of kind of the microsphere. On the other hand, mRNA expression of connexin 45 was suppressed after a 1-day culture of NHOst only with alumina microspheres, followed by a decrease in expression of the mRNA after their 7-day culture.

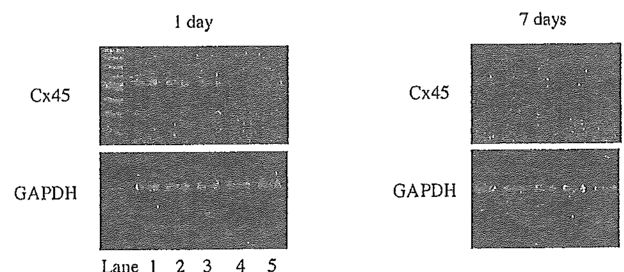

Figure 6. Expression of connexin 45 (Cx 45) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 45 and GAPDH is 40 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

DISCUSSION

As shown in Figure 3, normal human osteoblasts (NHOst) in contact with microspheres showed different levels of calcium deposition only after a 1-week culture, suggesting composition of the microspheres affects NHOst differentiation level. The differentiation was suppressed by the contact with PS, PE, and alumina microspheres, while HA microspheres showed the potential to enhance the differentiation. It has been reported that GJIC plays an important role in not only the homeostasis of cells but also their differentiation.[19–22] In addition, GJIC is affected by the microsphere's composition, as has been reported using a fibroblast cell line.[18] Therefore, the results shown in Figures 1 and 2 suggest that the enhanced differentiation of NHOst relates to GJIC enhancement on a 1-day culture in contact with HA microspheres, especially the precoated microspheres. In addition, on coculture with other microspheres, GJIC was slightly suppressed at 1 day, although no significant difference compared to control NHOst was observed. We have already studied effects of the microspheres on NHOst differentiation, and enhancement of calcium deposition by coculture with the hydroxy apatite microspheres was observed. Figure 3 suggests a relationship between the calcium deposition and GJIC on day 1. This also indicates that GJIC of the NHOst, in contact with materials in the microsphere form, in the early stage may be one factor affecting their differentiation.

It has been reported that GJIC of cells derived from human osteoblasts is mainly composed of connexin 43 and 45.[22,26,27] In this study, it is also indicated that GJIC of NHOst is composed of connexin 43 and 45 (Fig. 4). Therefore, it is possible that changes in the level of their GJIC is ascribed to the change in mRNA expression level of connexin 43 and 45 and their expression ratio. From Figures 5 and 6, mRNA of connexin 43 was expressed only in normal NHOst and those cultured with PE microspheres, while it was slightly expressed in NHOst cocultured with HA. On the other hand, mRNA of connexin 45 was expressed in NHOst in all conditions, except those cocultured with alumina microspheres. Because HA was observed to enhance GJIC of NHOst, this suggests that connexin 45 may play a role in GJIC at an early stage. This also suggests that a higher level of connexin 45 than that of connexin 43 may be important in the enhancement of GJIC. However, although the mRNA expression of neither connexin 43 nor 45 was observed in NHOst cocultured with alumina microspheres, their GJIC was similar to that of normal NHOst. Moreover, it has reported that gap junctions formed by connexin 43 are more permeable to negatively charged dyes such as lucifer yellow, calcein, and carboxyfluorescein used in this study, more than those formed by connexin 45, and an increase of connexin 43 expression and GJIC function parallel osteoblast differentiation.[22,28] These are inconsistent with our findings and indicate that not high expression, but a rapid decrease of connexin 45 mRNA is probably very important for GJIC change and differentiation of the osteoblasts. Therefore, even though connexin 45 may play an important role in the early stage of GJIC in NHOst, it is probable that another connexins or other mechanisms of GJIC play a role in the GJIC of NHOst.

Because many proteins are involved in GJIC formation,[28] other mechanisms or proteins may be important in the GJIC change induced by the contact with the microspheres. It has reported that cadherins, which are important proteins for form tight junction between cells, control connexin 43-mediated GJIC.[29,30] In addition, a microtubule network inside a cell has been reported to play an important role as guidance for delivery of connexons, which are composed of six connexin molecules, to the cell membrane to make gap junctions.[31] Usually, surface characteristics of materials affect cell attachment as well as cell morphology, suggesting signal cascades of cell attachment and cytoskelton rearrangement in the cell were influenced by the characteristics. Therefore, it is probable that a surface characteristic of the microspheres affect these molecules in NHOst, resulting in changes of GJIC activities. Further studies on changes in not only connexin molecules but also other molecules such as cadherin, actin, and microtubule in NHOst, is necessary to clarify the mechanism of GJIC. In the future, we will study the above, and find another molecules participating in the GJIC of NHOst and the mechanisms regulating the connexins in NHOst.

In conclusion, the GJIC level of NHOst changes on contact with microspheres, and is affected by the composition of the microspheres. The GJIC level in the early stage might be important in the differentiation control of NHOst and the level may be controlled partly by expression of connexin 43, connexin 45, and unclarified connexins in addition to other mechanisms regulating GJIC function. Detecting a biomaterial's effect on the GJIC of human cells may be one useful method for estimating its biocompatibility.

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