伸展活性化イオンチャネルが，伸展刺激を感知して心肥大の形成に関わっていると考えられていた。そして近年，このイオンチャネルによるシグナル伝達経路上に存在するカルシニューリンが，心肥大の誘導に重要な役割を担っていると考えられるようになった[24]。カルシニューリンはCa^{2+}によって活性化される脱リン酸化酵素である。そのモデルを以下に紹介する。

細胞膜に伸展刺激が作用した結果，細胞膜に存在するCa^{2+}チャネルが開口し，細胞内にCa^{2+}が流入する。流入したCa^{2+}がさらに小胞体からCa^{2+}を放出させ，細胞内のCa^{2+}結合タンパク質カルモデュリンが活性化され，次いで脱リン酸化酵素であるカルシニューリンが活性化される。活性型カルシニューリンは転写因子NF-AT3 (nuclear factor of activated T cell 3) と結合して，NF-AT3を脱リン酸化して核内に移行させる。核内に移行したNF-AT3は，転写因子GATA4 (心臓の発生・分化において重要であり，恒常的に発現している) と協調して脳型ナトリウム利尿ペプチド (BNP) 遺伝子を制御し，心肥大を形成させる (図6)[17,25]。

以上の結果から，シグナル伝達系において重要な役割を担うカルシニューリンを抑制することで，心筋肥大が抑制され，心不全を防ぐことができると考えられている[17,25]。

2. 血管内皮細胞のシェアストレスに対する細胞応答

血管内皮細胞の機能はホルモン，サイトカインなどにより制御されているとされてきたが，さらにメカニカルストレスも血管内皮細胞の機能を制御していることが明らかとなってきた。血流にさらされている血管内皮細胞は，血流が引き起こすシェアストレスの負荷を常に受けている。その血流のシェアストレスを鋭敏に感知して，その変化に応じた細胞応答を行うことで，血管および循環器全体の恒常性を維持している[16]。

シェアストレスを感知する受容体についても，現在は完全な理解は得られていないが，その候補としては細胞膜に存在するイオンチャネルや，接着分子であるインテグリンおよびPECAM-1や細胞骨格が考えられている。インテグリンについては心肥大とともに述べたのでここでは省略する。

2.1 PECAM-1

PECAM-1 (platelet endothelial cell adhesion molecule-1, CD31) は分子量130 kDaの膜タンパ

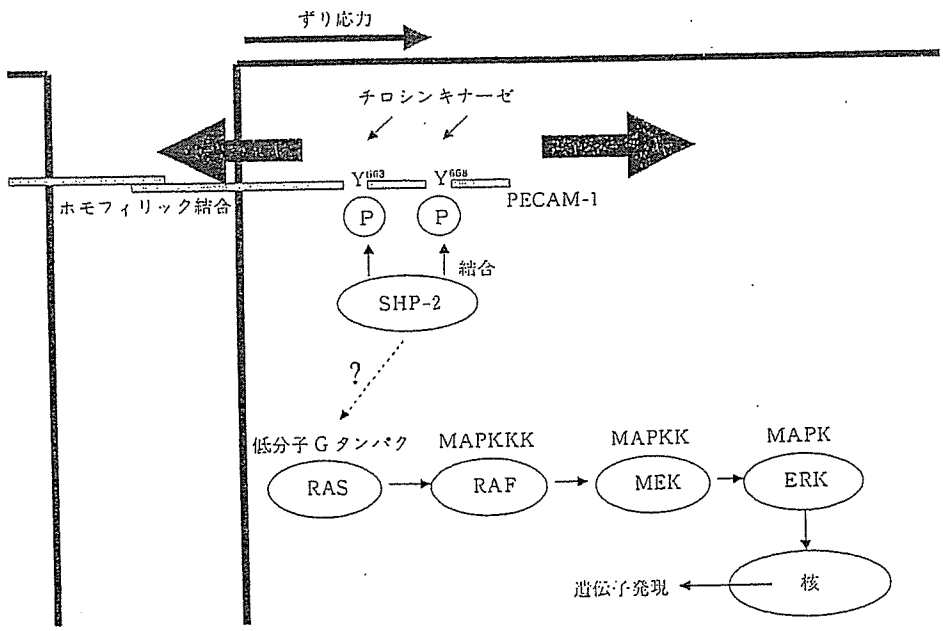

図7　内皮細胞におけるPECAM-1を介した細胞内情報伝達
(増田道隆，他：医学のあゆみ，Vol. 192, No. 13, pp. 1228, 図1，2000を引用)

ク質である。複数の血球細胞に存在するが，とくに血管内皮細胞で発現量が高い[26]。PECAM-1は隣接する細胞間を結合させているが，情報伝達分子としても機能し，リン酸化により制御されていると考えられている。

増田らは，その制御機構は以下のようなものであると推定している[27]。血管内皮細胞にシェアストレスが作用し，PECAM-1に張力がかかるとPECAM-1の立体構造が変化する。この構造の変化がPECAM-1のC末端側に存在するチロシン残基のリン酸化を引き起こし，さらにSHP-2が結合する[27,28]。SHP-2の脱リン酸化酵素活性の賦活化により，低分子GタンパクであるRASが活性化される。次いでRASの下流に存在する3段階のMAPKカスケードシステムにシグナルが伝わる(図7)[27]。

2.2　イオンチャネルを介したシェアストレス感知機構

安藤らは，血管内皮細胞に流れ刺激を加えると，Ca^{2+}の濃度が上昇することから，流れ刺激に伴うシェアストレスを感知する機構に，細胞内へのCa^{2+}の流入が関与すると考えている[29,30]。さらに，細胞外にCa^{2+}が存在しないと流れ刺激に対する反応が起こらないことから，細胞外Ca^{2+}の細胞内への流入が不可欠であると考えられている[16,29]。

ATPによって機能する陽イオンチャネルのプリノセプターP2Xのサブタイプである P2X4が，ATP存在下の流れ刺激で引き起こされるカルシウムイオン流入反応に関わる分子として特定されている[16,31,32]。P2X4が欠損するとシェアストレスが作用しても細胞内Ca^{2+}濃度の上昇が発生しなくなり，シェアストレスに無反応の細胞にP2X4を発現させると細胞内Ca^{2+}濃度の上昇が発生することが確認されている[31]。

また，内皮細胞に流れ刺激をかけてシェアストレスを作用させることで，K^+チャネルが開口して細胞膜の過分極が発生し，Ca^{2+}の細胞内への流入を引き起こすという現象も報告されている[31]。

第5節　骨組織における機械刺激に対する細胞応答

メカニカルストレスが骨組織に作用すると，骨組織は負荷に対して適切な骨形成を行うことが知られている。その作用機序はいまだ明らかにはなっていない。メカニカルストレスが骨組織にかかると骨芽細胞が骨形成を促し，破骨細胞による骨吸収を抑制することで骨量が増加する[33]。

骨には軟骨細胞，骨芽細胞，骨細胞，破骨細胞などが存在し，増殖因子やサイトカインに応答して，各細胞の機能を発揮する。骨芽細胞は骨形成を，破骨細胞は骨吸収を担っている。

オステオポンチンは骨基質中に存在する分泌性のリン酸化糖タンパク質で，細胞間伝達物質としても機能し，さらに細胞の接着にも関わり，骨に対して多様な作用を示すと考えられている。オステオポンチンは骨芽細胞，破骨細胞，骨細胞において発現しているが[33]，その発現はメカニカルストレスにより影響を受ける。オステオポンチンは，骨芽細胞におけるメカニカルストレスを感知するシグナル伝達機構を担う因子の一つとして機能していると考えられ，骨形成がメカニカルストレスにより維持されることに関与していると考えられている[34]。

オステオポンチンは，細胞膜に存在するインテグリンを介して骨芽細胞を刺激すると考えられているが，骨の再構成において具体的にどのような影響及ぼしているかはいまだ明らかにされていない[35]。Duncanらは以下のようなモデルを提唱している[36]。

骨細管や骨小腔には細胞外液が存在しているが，メカニカルストレスが骨に作用すると，その細胞外液に流れが発生する。この細胞外液の流れ刺激が，骨細胞や骨芽細胞に複数の物質を分泌させる。それらの物質により，骨芽細胞はⅠ型コラーゲンやオステオポンチンをさらに放出する(図8)[33,36]。メカニカルストレスが作用してからオステオポンチンの発現が増加するまで時間がかかることから，メカニカルストレスが直接オステオポンチンの発現を制御している可能性は低く，メカニカルストレスの作用により迅速に発現が促進される物質が，オステオポンチンを増加させると考えられている[33]。すなわち，

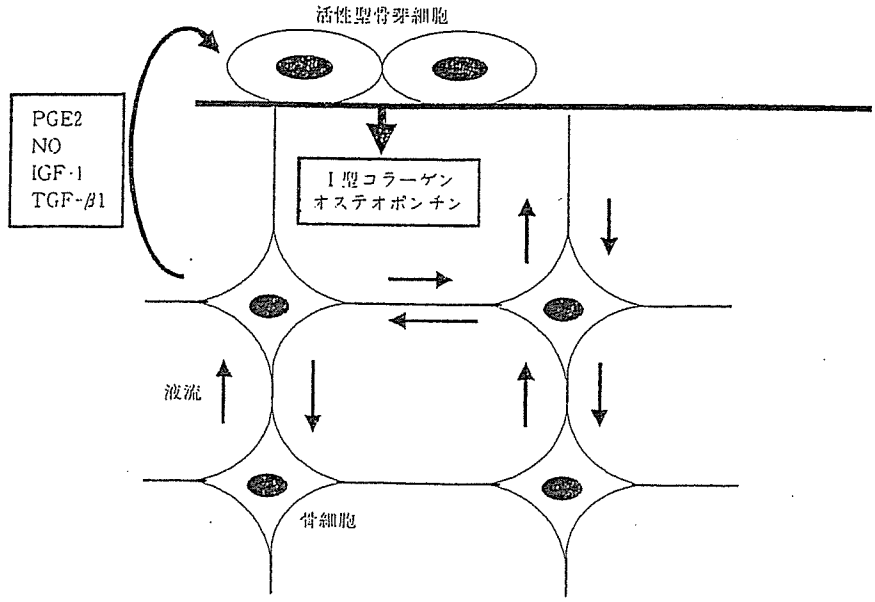

図8 骨におけるメカニカルストレスの働き
(大湾一郎：医学のあゆみ, Vol. 192, No. 13, pp. 1231-1232, 図1, 2000 を改変)

メカニカルストレスは間接的にオステオポンチンの産生を制御していることになる。オステオポンチンの発現を促す物質は，プロスタグランディン(PG)E_2や一酸化窒素(NO)，インスリン様増殖因子(IGF)−1，トランスフォーミング増殖因子(TGF)−β1などだと考えられている[33,36]。

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参考・引用文献

1) Y. C. Fung : "Where are the sources of fresh water to irrigate our field? — Message from Prof. Y. C. Fung." 日本機械学会誌, Vol. 98, pp. 885-887 (1995).
2) 林紘三郎，瀬口靖幸：バイオメカニクスとは．日本機械学会(編)，バイオメカニクス概説．オーム社，pp. 1-8 (1993).
3) 日本機械学会 編：生体機械工学．日本機械学会 (1997).
4) 林紘三郎：生体組織の・器官のバイオメカニクス—研究活動の現状．Jap. J. Sports Sci., Vol. 12, No. 1, pp. 25-31 (1993).
5) 林紘三郎：バイオメカニクス—現状と展望．BME, Vol. 9, No. 1, pp. 17-23 (1995).
6) 林紘三郎：バイオメカニクスの現状と将来．日本機械学会論文集(A編)，Vol. 61, pp. 1689-1694 (1995).
7) 林紘三郎：バイオメカニクス．コロナ社，pp. 50-51 (2000).
8) 曽我部正博，成瀬恵治，宮津/井上真寿美：内皮細胞における形態知覚と形態形成の分子機構医学のあゆみ，Vol. 192, No. 13, pp. 1201-1205 (2000).
9) F. Guharay and F. Sachs : "Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle." J. Physiol., Vol. 55, pp. 685-701 (1984).
10) H. Iida, et al. : "Mid1, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein — is required for Ca^{2+} influx and mating." Mol. Cell. Biol., Vol. 14, pp. 8259- 8271 (1994).
11) 飯田秀利，小島 至，佐藤主税，曽我部正博：伸展活性化 Ca^{2+} 透過チャネル Mid1 の遺伝子の単離と機能解析．医学のあゆみ，Vol. 192, No. 13, pp. 1211-1215 (2000).
12) 萩原誠久：伸展感受性 Cl-チャネル医学のあゆみ．Vol. 192, No. 13, pp. 1229-1230 (2000).
13) 萩原誠久，他：心筋細胞の容積調節に伴う膜電流変化．心臓，Vol. 26, pp. 886-894 (1994).
14) E. K. Hoffmann, et al. :"Membrane mechanisms in volume and pH regulation in vertebrate cells." Physiol. Rev., Vol. 69, pp. 315-377 (1989).

15) 青木浩樹：心筋肥大におけるRhoファミリーと筋線維形成の分子機構．医学のあゆみ，Vol. 201, No. 7, pp. 467-471 (2002).
16) 安藤譲二，山本希美子：内皮細胞のシェアストレス感知機構．医学のあゆみ，Vol. 192, No. 13, pp. 1217-1221 (2000).
17) 久藤純代，小室一成：心肥大形成時におけるメカニカルストレス受容機序．医学のあゆみ，Vol. 192, No. 13, pp. 1207-1210 (2000).
18) 永井敏雄：メカニカルストレス受容体としてのインテグリン．医学のあゆみ，Vol. 201, No. 7, pp. 485-487 (2002).
19) D. Schlaepfer et al.: "Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase." Nature, Vol. 372, pp. 786-791 (1994).
20) 相川竜一：メカニカルストレス受容機構としてのインテグリン．医学のあゆみ，Vol. 193, No. 13, pp. 1227 (2000).
21) R. S. Ross and T. K. Borg: "Integrins and the myocardium." Circ. Res., Vol. 88, No. 11, pp. 1112-1119 (2001).
22) 長谷川洋：心肥大にかかわるMAPKファミリー．医学のあゆみ，Vol. 201, No. 7, pp. 455-458 (2002).
23) C. Ruwhof and A. van der Laarse: "Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways." Cardiovasc. Res., Vol. 47, pp. 23-37 (2000).
24) J. D. Molkentin: "A calcineurin-dependent transcriptional pathway for cardiac hypertrophy." Cell, Vol. 93, pp. 215-228 (1998).
25) 大塚正史，小室一成：心肥大形成における遺伝子発現に関与するCa^{2+}．医学のあゆみ，Vol. 201, No. 7, pp. 463-466 (2002).
26) P. J. Newman et al.: "The biology of PECAM-1." J. Clin. Invest., Vol. 99, pp. 3-8 (1997).
27) 増田道隆，大澤正輝：シェアストレス受容伝達におけるPECAM-1の役割．医学のあゆみ，Vol. 192, No. 13, p. 1228 (2000).
28) M. Masuda, et al.: "Platelet endothelial cell adhesion molecule-1 is a major SH-PTP2 binding protein in vascular endothelial cells." FFBS Lett., Vol. 408, pp. 331-336 (1997).
29) J. Ando: "Cytoplasmic calcium respnse to fluid shear stress in cultured vascular endothelial cells." In vitro cell dev. Biol., Vol. 24, pp. 871-877 (1988).
30) J. Ando, et al.: "Effect of extracellular ATP level on flow-induced Ca^{++} response in cultured vascular endothelial cells." Biochem. Biophys. Res. Commun., Vol. 179, pp. 1192-1199 (1991).
31) K. Yamamoto, et al.: P2X2 receptors functions as the predominant ATP-gated cation channels in vascular endothelial cells. FASEB J., Vol. 13, pp. A861 (1999).
32) 安藤譲二：内皮細胞のメカニカルストレス感知・応答機構．実験医学，Vol. 20, No. 8, pp. 1200-1206 (2002).
33) 大湾一郎：メカニカルストレスによるオステオポンチンの発現．医学のあゆみ，Vol. 192, No. 13, pp. 1231-1232 (2000).
34) 野田政樹，他：骨代謝制御シグナルとしてのオステオポンチン．実験医学，Vol. 20, No. 17 (増刊), pp. 2465-2476 (2002).
35) 米田俊之：成長を続ける骨研究．実験医学，Vol. 20, No. 17 (増刊), pp. 2432-2440 (2002).
36) R. L. Duncan and C. H. Turner: "Mechanotransduction and the functional response of bone to mechanical strain." Calcif. Tissue Int., Vol. 57, pp. 344-358 (1995).

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FULLERENES, NANOTUBES, AND CARBON NANOSTRUCTURES
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[60]Fullerene as a Novel Photoinduced Antibiotic†

Yoko Kai,[1,#] Yuka Komazawa,[1] Atsuko Miyajima,[2] Naoki Miyata,[1] and Yoko Yamakoshi[1,*]

[1]Division of Organic Chemistry and [2]Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan

ABSTRACT

The antibacterial activity of [60]fullerene (C_{60}), dissolved with poly(vinylpyrrolidone) K30 (PVP), was studied. Under photo-irradiation, C_{60}/PVP aqueous solutions showed antibacterial activity, whereas PVP solution alone or fullerene solutions in the absence of light showed no activity. These results reveal that C_{60} is a potentially good device as a photoinduced antibacterial agent.

Key Words: Antibacterial activity; Photosensitivity; Poly(vinylpyrrolidone).

†Dedicated to Professor Akira Tanimura on the occasion of his 75th birthday.

#Bachelor of Science from Showa Women's University.
*Correspondence: Yoko Yamakoshi, Division of Organic Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan; E-mail: yamakosh@nihs.go.jp.

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1. INTRODUCTION

The photosensitivity of fullerenes[1–4] is well-known property with potential application as medicinal devices.[5–7] The Nakamura and Helene group and the Foote and Rubin group reported on water-soluble C_{60} derivatives, which have DNA-cleaving activity,[8–10] cytotoxicity[11,12] under photo-irradiation condition. We have reported that aqueous solution of C_{60} and C_{70}, solubilized with non-ionic detergent PVP,[13] have biological properties (supercoiled DNA cleavage,[14] hemolysis[15] and initiation in carcinogenesis[16]) under irradiation of visible light. These experiments show that fullerenes are potentially available as lead compounds for a new type of medicines utilizing photodynamic therapy.[17–19] Recently, we have reported that the active species responsible for the photoinduced bioactivities of fullerenes are reduced oxygen species such as superoxide radical anion and hydroxyl radical.[20] Such so-called "active oxygen species" can affect strongly biomolecules such as membrane lipids or DNA resulting in cell death. We now apply this activity of C_{60} to practical use, namely photodynamic antimicrobial activity.

Recently, multiple drug resistant bacteria have emerged as a considerable threat to public health, possibly due to overuse of traditional antibiotics. New types of the antibacterial compounds are desirable, particularly those whose activity may be modulated by specific activation such as by light. As described previous, C_{60} is reported as an effective photosensitizer excited by relatively long wavelength of visible-light (S–S absorption: 530, 920 nm, T–T absorption: 400, 740 nm),[21,22] which can penetrate through the cells of microbacteria, suggesting that the combination of fullerenes and light may give ideal effect as a novel class of antibiotics. In this study, we examined the antibacterial property of C_{60} in aqueous solution under visible-light irradiation.

2. EXPERIMENTAL

Preparation of C_{60} and Aqueous Solution

The aqueous solution of C_{60} was prepared by the previously described poly(vinlylpyrrolidone) (PVP)-method.[13] This non-ionic detergent (Figure 1) is widely used as a suspension-reagent for the medicines, quasi-drugs and cosmetics because it is regarded as a non-toxic detergent ($LD_{50} > 100$ g/kg for mouse and rat). Important, it enables the dissolution of the hydrophobic C_{60}, which is generally completely insoluble in the aqueous or water-miscible solvents likely to be relevant to biological applications.[23–28]

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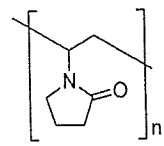

Figure 1. Structure of poly(vinylpyrrolidone) K30 (PVP). (n = abt. 360, MW = abt. 40,000, $LD_{50} > 100$ g/kg for mouse and rat).

This detergent-solubilization method was applied for the biological studies, according to the following typical procedure: To a solution of 10 mL of 0.31% poly(vinylpyrrolidone) K30 (Wako Pure Chemical Industry, Osaka, Japan) in chloroform (Wako Pure Chemical Industry, Osaka, Japan), a solution of C_{60} (>99.98%, Terms Co., USA) 2.5 mg in 20 mL of toluene (Wako Pure Chemical Industry, Osaka, Japan) was added and mixed well in 500 mL round-bottomed flask. The solvents were removed slowly *in vacuo* by an evaporator at room temperature and then dried by a vacuum pump for two days. The thin film-like residue, which covers the inside of the surface of the flask, was sterilized by autoclave and then 10 mL of sterilized nutrient broth (NB)[29] was added (3.5×10^{-2} mM C_{60} in 0.31% PVP). The other concentrations of C_{60}/PVP and the PVP in broth solution were prepared in the same manner. A 10 mL aliquot of each NB solution (C_{60}/PVP in NB, PVP in NB, and NB only) was placed in sterilized L-shape test tubes for assay.

Antibacterial Assay

The *Bacillus subtilis* PCI219 was used for the antibacterial tests. The bacteria were stored as a spore suspension and were pre-incubated to log-phase in NB before use. An aliquot of log phase-growth of cells was inoculated to each media (C_{60}/PVP in NB, PVP in NB, and NB only) and incubated in colorless transparent L-shape glass test tube in aerobic condition under photo-irradiation by 300-W photoreflector lamps (National REF 100 V, 300 W–W). The temperature of the water in the incubator was kept to be at 35°C by the water-circulating system in order to prevent increasing the temperature of the water caused by the photo-irradiation (Figure 2). For a control test, the cells in each broth were incubated without photo-irradiation. After each time of incubation, an aliquot of cell broth suspension the broth was taken from every incubation, diluted by PBS(−) (Dulbecco's Ca^{2+} and Mg^{2+}—free phosphate buffered saline) and OD_{595} was measured in order to detect the growth of the bacteria.

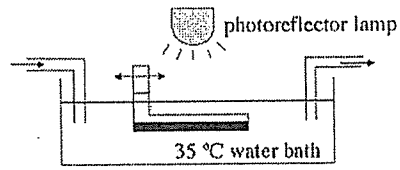

Figure 2. Incubation system for photoinduced antibacterial assay.

3. RESULTS AND DISCUSSION

The growth curve of the bacteria in the C_{60}/PVP in NB under photo-irradiation is shown in Fig. 3 (●). Compared to the growth curve of blank experiment (○), the growth of bacteria in C_{60}/NB solution under photo-irradiation is suppressed significantly. From the result of incubation in PVP/NB (■), no significant effect of PVP itself was shown. From these results, it was indicated that C_{60} is an effective photoinduced antibacterial compound. The growth curve in each broth without light irradiation are shown in Fig. 4, indicating that there is no significant effect of C_{60} or PVP on the bacteria growth without photo-irradiation.

In order to ensure the antibacterial effect of photoinduced C_{60}, dose-dependent test were carried out. Figure 5 shows clearly the dose-dependent effect of photoinduced C_{60} on bacteria growth. Even in the presence of low concentration of C_{60} (1.8×10^{-2} mM), the growth of bacteria was supressed efficiently until 6 hrs of incubation. Figure 6 shows that there is no significant

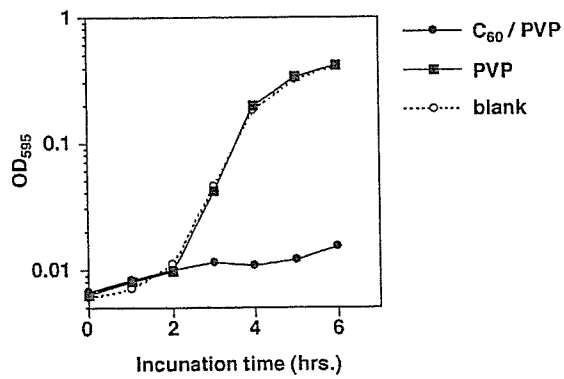

Figure 3. Growth curve of *B. subtilis* in the presence of C_{60} (0.035 mM) and PVP (0.31%) under the irradiation of visible light.

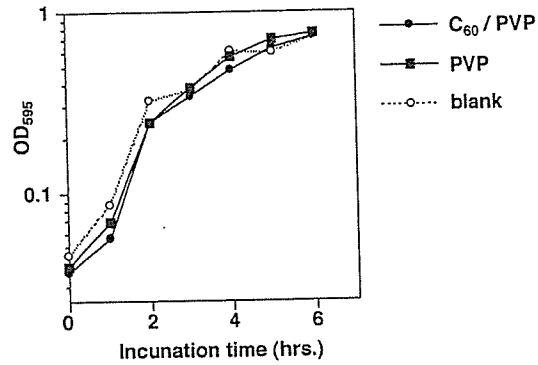

Figure 4. Growth curve of *B. subtilis* in the presence of C_{60} (0.035 mM) and PVP (0.31%) without the irradiation of visible light.

effect by PVP on the growth curve under photo-irradiation, demonstrating a critical role for C_{60} in effecting the antibacterial activity.

Antibacterial effect by ultraviolet irradiation is well known and UV light irradiation is quite common way of sterilizations. And also γ-ray irradiation is widely used as a sanitizer of foods. In the present report, we used visible light, which does not have sterilizations effect only by itself. The mechanism of this visible light-induced antibacterial activity was speculated that reduced type of active oxygen species (O_2.$^-$ and .OH), which were generated by electron

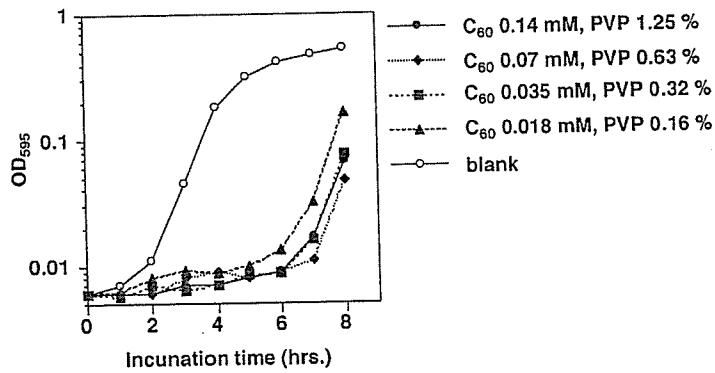

Figure 5. Dose-dependent effect of C_{60} on the growth of *B. subtilis* incubated under the irradiation of visible light.

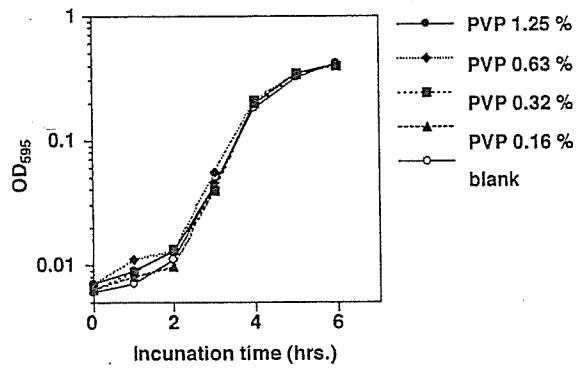

Figure 6. Dose-dependent effect of PVP on the growth of *B. subtilis* incubated under the irradiation of visible light.

transfer pathway, affect the cell membrane of bacteria causing suppress of its growth with a good agreement with the hemolytic effect. Importantly, visible light is much safer rather than other sterilizations light like UV and γ-ray indicating that it has less effect to the materials (medical devices, food packing material, etc.), which should be sterilized. In this study, we have studied on the antibacterial activity of C_{60} solubilized by detergent against common gram-positive bacteria, *B. subtilis*. This is the first finding of photoinduced antibacterial activity of non-modified C_{60} and further study on antibacterial spectra using other bacteria, fungi, and yeast would be interesting in the future for the practical applications.

4. CONCLUSION

A new photosensitizer C_{60} was shown to be a novel type of effective photoinduced antibacterial agent. Notably, C_{60} has effective quantum yield with longer excitation wavelength, therefore it is possible to switch antibacterial activity on or off by light in the context of biological system. Such properties may have a critical role in the development of novel, selective photodynamic therapies for control of multiple drug resistant bacterial infections.

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REFERENCES

1. Foote, C.S. Photophysical and photochemical properties of fullerenes. Top. Curr. Chem. **1994**, *169*, 347–363.
2. Foote, C.S. Photopysical, photochemical, and chemical reactions of fullerenes and dihydrofullerene derivatives. In *Physics and Chemistry of the Fullerene*; Prassodes, K., Ed.; Kluwer Academic Publishers: Dordrecht, 1994; 79–96.
3. Guldi, D.M.; Prato, M. Excited-state properties of C_{60} fullerene derivatives. Acc. Chem. Res. **2000**, *33*, 695–703.
4. Guldi, D.M.; Kamat, P.V. Photophysical properties of pristine fullerenes, functionalized fullerenes, and fullerene-containing donor-bridge acceptor systems. In *Fullerenes: Chemistry, Physics and Technology*; Kadish, K.M., Ruoff, R.S., Eds.; A John Wiley and Sons, Inc.: New York, 2000; 225–282.
5. Jensen, A.W.; Wilson, S.R.; Schuster, D.I. Biological aplications of fullerenes–A review. Bioorg. Med. Chem. **1996**, *4*, 767–786.
6. Da Ros, T.; Prato, M. Medicinal chemistry with fullerene and fullerene drivatives. Chem. Commun. **1999**, 663–669.
7. Wilson, S.R. Biological aspects of fullerenes. In *Fullerenes: Chemistry, Physics and Technology*; Kadish, K.M., Ruoff, R.S., Eds.; A John Wiley and Sons, Inc.: New York, 2000; 437–466.
8. Tokuyama H.; Yamago S.; Nakamura, E.; Shiraki, T.; Sugiura, Y. Photo-induced biochemical activity of fullerene carboxylic acid. J. Am. Chem. Soc. **1993**, *115*, 7918–7919.
9. Boutorine, A.S.; Yokuyama, H.; Takasugi, M.; Isobe, H.; Nakamura, E.; Helene, C. Fullerene-oligonucleotide conjugates: Photoinduced sequence-specific DNA cleavage. Angew. Chem. Int. Ed. Engl. **1994**, *33*, 2462–2465.
10. An, Y.-Z.; Chen, C.-H.B.; Anderson, J.L.; Sigman, D.S.; Foote, C.S.; Rubin, Y. Sequence-specific modification of guanosine in DNA by a

C_{60}-linked deoxyoligonucleotide: Evidence for a non-singlet oxygen mechanism. Tetrahedron **1996**, *52*, 5179–5189.

11. Irie, K.; Nakamura, Y.; Ohigashi, H.; Tokuyama, H.; Yamago, S.; Nayamura, E. Photocytotoxicity of water-soluble fullerene derivatives. Biosci. Biotech. Biochem. **1996**, *60*, 1359–1361.
12. Nakamura, E.; Tokuyama, H.; Yamago, S.; Shiraki, T.; Sugiura, Y. Biological activity of water-soluble fullerenes. Structural dependence of DNA cleavage, cytotoxicity, and enzyme inhibitory activities including HIV-protease inhibition. Bull. Chem. Soc. Jpn. **1996**, *69*, 2143–2151.
13. Yamakoshi, Y.; Yagami, T.; Fukuhara, K.; Sueyoshi, S.; Miyara, N. Solubilization of fullerenes into water with poly(vinylpyrrolidone) applicable to biological tests. J. Chem. Soc., Chem. Commun. **1994**, 517–518.
14. Yamakoshi, Y.; Yagami, T.; Sueyoshi, S.; Miyata, N. Acridine adduct of [60]fullerene with enhanced DNA-cleaving activity. J. Org. Chem. **1996**, *61*, 7236–7237.
15. Yamakoshi, Y.; Yamazaki, E.; Sueyoshi, S.; Miyata, N. Hemolytic effect of photoexcited [60]fullerene. In *Fullerenes, Recent Advances in the Chemistry and Physics of Fullrenes and Related Materials*; Ruoff, R.S., Kadish, K.S., Eds.; The Electrochem. Soc., Inc: Pennington, NJ, 1997; Vol. 5, 238–243.
16. Sakai, A.; Yamakoshi, Y.; Miyata, N. Visible light irradiation of [60]fullerene causes killing and initiation of transformation in BALB/3T3 cells. Fullerene Sci. Technol. **1999**, *7*, 743–746.
17. Nakajima, N.; Nishi, C.; Li, F.-M.; Ikada, Y. Photo-induced cytotoxicity of water-soluble fullerene. Fullrerene Sci. Technol. **1996**, *4*, 1–19.
18. Tabata, Y.; Murakami, Y.; Ikada, Y. Antitumor effect of poly(ethyl glycol)-modified fullerene. Fullerene Sci. Technol. **1997**, *5*, 989–1007.
19. Tabata, Y.; Murakami, Y.; Ikada, Y. Phorodynamic effect of phlyethylene glycol-modified Fullerene on Tumor. Jpn. J. Cancer Res. **1997**, *88*, 1108–1116.
20. Yamakoshi, Y.; Sueyoshi, S.; Fukuhara, K.; Miyata, N. .OH and $O_2\cdot^-$ generation in aqueous C_{60} and C_{70} solutions by photoirradiation: An EPR study. J. Am. Chem. Soc. **1998**, *120*, 12363–12364.
21. Arbogast, J.W.; Darmanyan, A.P.; Foote, C.S.; Rubin, Y.; Diederich, F.N.; Alvarez, M.M.; Anz, S.J.; Whetten, R.L. Photophysical properties of C_{60}. J. Phys. Chem. **1991**, *95*, 11–12.
22. Arbogast, J.W.; Foote, C.S.; Kao, M.J. Electron transfer to triplet fullerene C_{60}. J. Am. Chem. Soc. **1992**, *114*, 2277–2279.
23. Sivaraman, N.; Dhamodaran, R.; Kaliappan, I.; Srinivasan, T.G.; Vasudeva-Rao, P.R.; Matheews, C.K. Solubility of C_{60} in organic solvents. J. Org. Chem. **1992**, *57*, 6077–6079.

24. Ruoff, R.S.; Malhotra, R.; Tse, D.S.; Lorents, D.C. Solubility of fullerene (C_{60}) in a variety of solvents. J. Phys. Chem. **1993**, *97*, 3379–3383.
25. Sivaraman, W.A.; Tour, J.M. Potent solvents for C_{60} and their utility for the rapid acquisition of ^{13}C NMR data for fullerenes. J. Chem. Soc., Chem. Commun. **1993**, 1207–1209.
26. Heyamann, D. Solubility of fullerenes C_{60} and C_{70} in seven normal alcohols and their deduced solubility in water. Fullerene Sci. Technol. **1996**, *4*, 509–515.
27. Beck, M.T.; Mandi, G. Solubility of C_{60}. Fullerene Sci. Technol. **1997**, *5*, 291–310.
28. Manauskas, S.M.; Jurs, P.C. Prediction of C_{60} solubility from solvent molecular structure. J. Chem. Inf. Comput. Sci. **2001**, *41*, 419–424.
29. To bacto beef extract 3 g and bacto peptone 5 g, 1 L of water was added and sterified by autocleave.

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EFFECTS OF BIOMATERIALS AND NUTRIENT FACTORS ON CHONDROGENESIS OF HUMAN CHONDROCYTES.

MUHAMMAD SHAHIDUR RAHMAN, TOSHIE TSUCHIYA.
Division of Medical Devices, National Institute of Health Sciences. 1-18-1, Kamiyoga, Setagaya ku, Tokyo 158-8501, Japan.

Abstract

The biocompatibility of poly-L-lactic acid (PLLA) and copolymer of poly(DL-lactic-co-glycolic acid) 50:50 (PLGA), and the effects of basic fibroblast growth factor (bFGF) and ferrous sulfate ($FeSO_4$) on human articular chondrocytes (HAC) proliferation and differentiation were investigated in a 4 weeks micromass culture system. Aliquots of 20 µl (per well) containing $4x10^5$ cells were spotted onto 24-well tissue culture plates and supplemented with the media containing dimethyl sulphoxide (as a vehicle), PLLA, PLGA, bFGF, or $FeSO_4$, respectively. Cells cultured with medium only was used as a control. Alamar blue and alcian blue staining were done to determine the chondrocyte proliferation and differentiation, respectively. All cultures showed sufficient cell proliferation close to control level. The cultures exposed to PLLA, PLGA, bFGF and $FeSO_4$ increased HAC differentiations up to the levels of 1.2-, 1.6-, 1.3- and 2-fold of the control, respectively. These results suggest that both the PLLA and PLGA are suitable as scaffold for tissue-engineered cartilage, and bFGF and $FeSO_4$ also possessed the stimulatory activities on HAC chondrogenesis.

1. Introduction

The limited availability of autologous chondrocytes has encouraged many researchers to investigate tissue engineering approaches to repair injured articular cartilage. A suitable material for tissue engineering must be biocompatible to the body. Among the biodegradable polymers, poly-L-lactic acid (PLLA), copolymer poly(DL-lactic-co-glycolic acid) 50:50 (PLGA) and its relating polymers have been extensively investigated, using mainly animal cells, and some side effects of these polymers have also been reported. Further investigations with these materials using human articular

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chondrocytes (HAC) are of great interest with respect to successful clinical application. To promote adequate cell proliferation and differentiation, it is important to add nutrients in the early stage of chondrocyte culture. Among the various nutrients and growth factors examined, basic fibroblast growth factor (bFGF) was extensively used but played a controversial role.[1,2] No study yet has reported the effects of ferrous sulfate ($FeSO_4$) on human chondrocyte culture.

In this study, the biocompatibility of PLLA and PLGA, and the influences of bFGF and $FeSO_4$ were investigated using HAC in a micromass culture system.

2. Materials and Methods

2.1. Samples and Solutions Used for Cell Culture

PLLA (Mw = 5,000) and PLGA (Mw = 5,000) were obtained from Nakalai Tesque, Inc. (Kyoto, Japan) and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical Co. Irvine, UK). Sterile bFGF was purchased from Promega (Madison, WI, USA) and $FeSO_4$ was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Both bFGF and $FeSO_4$ were dissolved in distilled Milli-Q water, and the solution of $FeSO_4$ was purified by filtration through a 0.22 μM Millipore filter.

2.2. Cells and Culture Methods

HAC of knee joint were commercially obtained from BioWhittaker, Inc., (Walkersville, MD, USA). High-density micromass cultures were initiated by spotting $4x10^5$ cells in 20 μl of medium onto Costar 24-well microplates for tissue culture (Costar ® Type 3513, Corning Co. Ltd., NY, USA). Following 2 h attachment period in a 5 % CO_2 incubator at 37°C, the wells were flooded with chondrocyte culture media (1 ml/well). Media were supplemented with DMSO (0.8 μl/ml), PLLA (50 μg/ml), PLGA (50 μg/ml), bFGF (10 ng/ml) and $FeSO_4$ (50 μg/ml), respectively. HAC cultured without any supplements was used as a control. The control and sample media were changed in every 2-3 days and the cultures were continued for 4 weeks. Four to five cultures were run for each case.

2.3. Cell Morphology Assay

Cell morphology was determined by inverted light microscopy. Alternate day observations were done.

2.4. Cell Proliferation Studies

Cell proliferation was quantitatively measured by alamar blue (Biosource,

International, Inc, Camarillo, CA, USA) assay. After the culture period, the medium from all the wells was discarded, and the wells were filled with 1 ml/well of 20-fold diluted alamar blue solution with the medium. After 4 h of incubation period at 37°C, two aliquots of 100 μl of solution from each well were transferred into new 96-well tissue culture plate (Costar ® Type 3595, Corning Co. Ltd., NY, USA). Equal volume of fresh medium per well (total three wells) served as blank. Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Flamingham, MA, USA) quantitated the extent of cell proliferation at 535 nm excitation and 590 nm emission. Blank values were subtracted from experimental values to eliminate background readings.

2.5. Alcian Blue Staining

After the alamar blue assay, medium was discarded and the micromass cultures were washed once with 0.5 ml/well of PBS (-) at room temperature. Cultures were then stained with 1 % alcian blue solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), pH 1.0, and rinsed with 3% acetic acid solution. The cartilage proteoglycans were extracted with 4-M guanidine hydrochloride (GH) and the bound dye was measured at a wavelength of 600 nm using an ELISA reader (Bio-Tek Instruments, Inc., Winooski, Vt., USA). Fresh 4-M GH served as blank and was deducted from experimental values to eliminate background readings.

2.6. Statistical Study

Student's *t*-test was used to compare the samples. Statistically significance was accepted at $p < 0.05$. Data were presented as the mean ± s.d.

3. Results

3.1. Cell Morphology

The micromass cultures predominantly formed a uniform sheet of chondrogeneic cells and maintained a rounded shape. The control cells showed few poorly defined cluster formations. However, more nodules were observed in PLLA and PLGA containing cultures than the control. The cultures exposed to bFGF and $FeSO_4$ showed a denser extracellular matrix (ECM) production than the control.

3.2. Cell Proliferation

Chondrocyte proliferation was quantitatively determined by alamar blue assay and expressed as percentage of the control average value (Figure 1). The control average value was 100 %. The values of cell proliferation in the samples exposed to DMSO,

PLLA, PLGA, bFGF, and $FeSO_4$ were in the range from 99 to 108 %.

3.3. Cell chondrogenesis

The bound form of proteoglycans with the alcian blue was extracted with 4-M GH and calculated in percentage of the control average value (Figure 2). The sample exposed to DMSO had negligible impact on cell differentiation (97 %). All samples, either exposed to biomaterials or nutrients, revealed significantly higher cell differentiation as compared to control ($p > 0.05$), with $FeSO_4$ elicited the highest cell differentiation (203 %). Cell differentiation of the samples exposed to PLLA, PLGA, and bFGF were 124 %, 159 % and 125 % of the control, respectively.

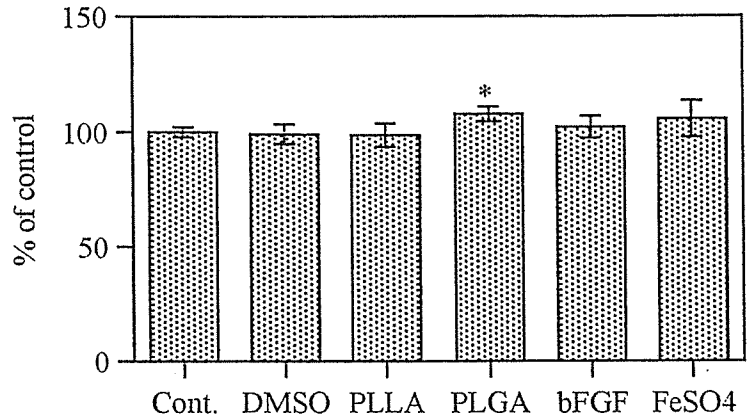

Figure 1. Cell proliferation of human articular chondrocytes after 4 weeks of culture period. * $p < 0.05$.

3.4. Differentiation index

Cell differentiation index (cell differentiation/cell proliferation) of control, DMSO, PLLA, PLGA, bFGF, and $FeSO_4$ were 1, 0.98, 1.3, 1.5, 1.3, 1.9, respectively. HAC exposed to these biomaterials and nutrients were significantly differentiated in comparison with the control ($p < 0.05$).

4. Discussion

Optimal repair of chondral defects is likely to require a suitable population of chondrogenic cells, a biodegradable matrix and effective nutrients to provide a space-filling structure during the early stages of cartilage formation. In this work, we analyzed the biocompatibility of PLLA and PLGA for cartilage tissue engineering. Experiments were also carried out to determine the role of bFGF and $FeSO_4$ on chondrocyte proliferation and differentiation. Notably, neither significant inhibition nor stimulation of chondrocyte proliferation was observed by the addition of these biomaterials or nutrients to the cultures (Fig. 1). The increased cell proliferation and differentiation of the sample

exposed to PLGA in this study is in accord with the result of marrow stromal osteoblasts.[3] Here, in this article we used human chondrocytes, while animal cells were

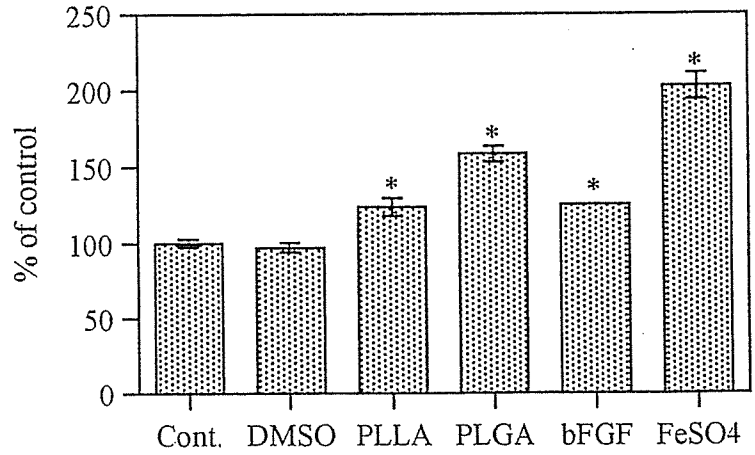

Figure 2. Cell differentiation of human articular chondrocytes after 4 weeks of culture period. * $p < 0.05$.

used in the previous reports.[1-4] Moreover, no report investigated the biocompatibility of the biodegradable polymers using HAC in a micromass culture system. Both bFGF and $FeSO_4$ enhanced the differentiation of HAC (Fig. 2). The different findings of chondrocyte proliferation and differentiation by bFGF between the present and earlier studies, may be contributed to the different origin sites of chondrocytes, as already suggested.[4] We were unable to compare our findings of $FeSO_4$ with any other study, as this study is the first report on the role of $FeSO_4$ in chondrogenesis. We conclude that both PLLA and PLGA are suitable substrates for preparing tissue engineered cartilage, and $FeSO_4$ when added to the culture for tissue engineering might potentially enhance tissue regenerative ability.

5. References

1. Toolan BC, Frenkel SR, Pachence JM, Yalowitz L, Alexander H. (1996) Effects of growth-factor-enhanced culture on a chondrocyte-collagen implant for cartilage repair. J Biomed Mater Res 31,273-280.
2. Matsusaki M, Ochi M, Uchio Y, Shu N, Kurioka H, Kawasaki K, Adachi N. (1998) Effects of basic fibroblast growth factor on proliferation and phenotype expression of chondrocytes embedded in collagen gel. Gen Pharmacol. 31, 759-764.
3. Ishaug SL, Crane GM, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. (1997) Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. J Biomed Mater Res 36, 17-28.
4. Lee JD, Hwang O, Kim SW, Han S. (1997) Primary cultured chondrocytes of different origins respond differently to bFGF and TGF-beta. Life Sci. 61, 293-299.

STUDIES ON THE BIOCOMPATIBILITY OF ARTIFICIAL ORGANS AND TISSUE ENGINEERED PRODUCTS:

EMBRYONIC NEURONAL CELL DIFFERENTIATION ON THE VARIOUS KINDS OF BIODEGRADABLE POLYMERS

TOSHIE TSUCHIYA, YUKA ITAHASHI, TOMOKO ICHIKAWA AND AKIRA ICHIKAWA.

Division of Medical Devices, National Institute of Health Sciences, Kamiyoga, 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan.

Abstract

Representative biodegradable-polymers are assayed to clarify the property when used as the materials for the nerve regeneration using the midbrain cell differentiation systems. As for polyglycolic acid [PGA], both the proliferation and differentiation of the midbrain cells were dose-dependently inhibited by PGA3000(Mw=3000). Poly(L-lactic acid)[PLLA]5000 (Mw=5000)showed slightly inhibitory effects on the proliferation and differentiation. In the case of the poly (L-lactic acid-co- ε -caprolactone)$_{25}$ 10000[P(LA-CL)$_{25}$ 10000] (Mw=10000), the differentiation was inhibited at 7.5 μg/ml to the levels of about 50 % of the controls. But, P(LA-CA)$_{50}$ 18000 (Mw=18000) showed weakly inhibitory action on the differentiation of midbrain cells in comparison with P(LA-CA)$_{25}$10000 . The inhibitory activity of these polymers on neuronal cell differentiation were in the following order: P(LA-CA)$_{25}$10000 > PGA3000> P(LA-CA)$_{50}$ 18000= PLLA5000. The catalyst of $SnCl_2$ showed the strongest inhibitory action on the midbrain cell differentiation among test chemicals.

1. Introduction

One of the first synthetic biodegradable polymers studied as a material to guide nerve regeneration over a significant defect was poly(lactic-co-glycolic acid)(PLGA) or polygalactin. Specifically, polygalactin or Vicryl suture mesh was used to bridge 7-9 mm defects in the tibial nerves of rabbits [1]. Kiyotani et al. [2] reported that the PGA-collagen composite tube is a promising tool for use as a nerve guide tube in peripheral nerve regeneration. Another polyesters, polylactic acid (PLA) and poly(lactide-co-caprolactone)[P(LA-CL)] supported nerve regeneration [3,4]. Although the regenerated nerve cable had a slightly different morphology from normal nerve, these biodegradable polymers were considered to be low

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irritants to the regenerating nerve [1-4]. In the present study,the effects of the oligomers derived from biodegradable polymers were investigated using rat embryonic midbrain (MB) cell differentiation system.

2.Materials and Methods

PLLA Mw=5,000 and PGA (Mw=3,000) were obtained from Nakalai Tesque. Inc. (Kyoto, Japan). $P(LA\text{-}CL)_{25}$ 10000 (L-lactic acid: ε -caprolactone=75:25, Mw=10,000), $P(LA\text{-}CL)_{50}$18000(L-lactic acid: ε -caprolactone=50:50, Mw=18,000) and $SnCl_2$ were the gifts from UBE INDUSTRY L.T.D.. Four polymers were dissolved in dimethyl sulphoxide (DMSO). $SnCl_2$ was dissolved in water and sterilized by filtration. MB micromass culture was carried out according to the procedure reported previously [5]. MB tissues were removed from the embryos of pregnant Wistar-imamichi rats on day 13 of gestation. MB cell suspensions were prepared in culture medium consisting of Ham's F12 plus 10 % fetal calf serum (F12-10 % FCS) and were adjusted to give $5x10^6$ MB cells /ml. A 20 μl aliquot of each cell suspension was delivered to a well of 24-well tissue culture plates. The next day, polymer solution in the medium was incorporated into the MB culture plates, then further cultured for another 6 days. MB cells grew and differentiated in vitro. The extent of neural cell differentiation was assessed by counting the differentiated foci [5]. Cell proliferation was determined by alamar Blue assay [6].

2. Results

Figure 1 shows the differentiated foci stained with hematoxylin. In proportion to the concentrations of PGA3000, the numbers of differentiated foci in the cell island decreased (Fig. 1, upper stand x7.5) and the sizes of the neuronal cell foci were smaller than those treated with none (=control) and DMSO (Fig. 1, lower stand x40). Table 1 compiles the inhibitory activities of four polymers and its catalyst, $SnCl_2$. $SnCl_2$ showed the strongest inhibitory-action on the MB cell differentiation and proliferation among five substances (Table 1). On the contrary, PLLA5000 showed the lowest inhibitory activities in the MB cell differentiation and proliferation. In the case of $P(LA\text{-}CL)_{50}$ 18000, the cell proliferation was inhibited more than the cell differentiation. While, in the case of $P(LA\text{-}CL)_{25}$ 10000, cell differentiation was inhibited more than the cell proliferation (Table 1). PGA3000, PLLA 5000 and $SnCl_2$ showed the similar activities in both cell differentiation and cell proliferation, respectively (Table 1). The order of the inhibitory potentials as follows: $SnCl_2$ > $P(LA\text{-}CL)_{25}$ 10000 > PGA3000 > $P(LA\text{-}CL)_{50}$ 18000 > PLLA5000.

3. Discussion

The inhibitory activities of these four polymers on the embryonic neuronal cell differentiation were detected and evaluated for the first time. Not only the molecular size of the oligomers but also the chemical composition affects the neuronal cell proliferation and differentiation.