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Coding SNP in tenascin-C Fn-III-D domain associates with adult asthma

Akira Matsuda[1,*], Tomomitsu Hirota[1], Mitsuteru Akahoshi[1], Makiko Shimizu[1], Mayumi Tamari[1], Akihiko Miyatake[3], Atsushi Takahashi[2], Kazuko Nakashima[1,4], Naomi Takahashi[1], Kazuhiko Obara[1], Noriko Yuyama[5], Satoru Doi[6], Yumiko Kamogawa[7], Tadao Enomoto[9], Koichi Ohshima[10], Tatsuhiko Tsunoda[2], Shoichiro Miyatake[7], Kimie Fujita[11], Moriaki Kusakabe[12], Kenji Izuhara[13], Yusuke Nakamura[8], Julian Hopkin[14] and Taro Shirakawa[1,4]

[1]Laboratory for Genetics of Allergic Diseases and [2]Laboratory for Medical Informatics, SNP Research Center, RIKEN, Yokohama, Japan, [3]Miyatake Asthma Clinic, Osaka, Japan, [4]Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto, Japan, [5]Genox Research Inc., Kawasaki, Japan, [6]Osaka Prefectural Habikino Hospital, Osaka, Japan, [7]Department of Molecular and Developmental Biology and [8]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [9]Department of Otolaryngology, Japanese Red Cross Society, Wakayama Medical Center, Wakayama, Japan, [10]Department of Pathology, School of Medicine, Fukuoka University, Fukuoka, Japan, [11]College of Nursing, University of Shiga, Shiga, Japan, [12]Experimental Animal Research Center, Institute for Animal Reproduction, Ibaraki, Japan, [13]Department of Biomolecular Sciences, Saga Medical School, Saga, Japan and [14]Experimental Medicine Unit, University of Wales Swansea, Swansea, UK

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The extracellular matrix glycoprotein tenascin-C (TNC) has been accepted as a valuable histopathological subepithelial marker for evaluating the severity of asthmatic disease and the therapeutic response to drugs. We found an association between an adult asthma and an SNP encoding TNC fibronectin type III-D (Fn-III-D) domain in a case–control study between a Japanese population including 446 adult asthmatic patients and 658 normal healthy controls. The SNP (44513A/T in exon 17) strongly associates with adult bronchial asthma (χ^2 test, $P = 0.00019$, Odds ratio = 1.76, 95% confidence interval = 1.31–2.36). This coding SNP induces an amino acid substitution (Leu1677Ile) within the Fn-III-D domain of the alternative splicing region. Computer-assisted protein structure modeling suggests that the substituted amino acid locates at the outer edge of the beta-sheet in Fn-III-D domain and causes instability of this beta-sheet. As the TNC fibronectin-III domain has molecular elasticity, the structural change may affect the integrity and stiffness of asthmatic airways. In addition, TNC expression in lung fibroblasts increases with Th2 immune cytokine stimulation. Thus, Leu1677Ile may be valuable marker for evaluating the risk for developing asthma and plays a role in its pathogenesis.

INTRODUCTION

Asthma is a chronic inflammatory disease characterized by smooth muscle hypertrophy, excess mucus secretion and increased deposition of extracellular matrix (ECM) around the basement membrane (1–3). Many asthmatic patients also have an atopic tendency characterized by a Th2 dominant cytokine profile including interleukin (IL)-4 and IL-13 (4). Several studies showed genetic associations between asthma and proteinases like ADAM33 (5) or Th2 cytokine receptors (4,6), but to the best of our knowledge, there is no report of an association between asthma and ECM genes. The hexametric

*To whom correspondence should be addressed at: Laboratory for Genetics of Allergic Diseases, SNP Research Center, RIKEN, Suehiro 1-7-22, Tsurumi-KU, Yokohama 230-0045, Japan. Tel: +81 455039616; Fax: +81 455039615; Email: akimatsu@src.riken.go.jp

HiCEPを用いたシスプラチン耐性ガン細胞の網羅的発現プロファイリング

佐々木 直一[1),2)]・青塚 聡[2),3)]・松葉 恭一[3)]・井上 循[3)]
礒西 成治[4)]・石川 博[5)]・日下部 守昭[3),5),6)]

[1)]メッセンジャー・スケープ社 [2)]日清紡 [3)]アロカ㈱ANB筑波研究所
[4)]慈恵医大・産婦人科 [5)]慈恵医大・解剖 [6)]動繁研

【要 約】

卵巣癌には、シスプラチンに易感受性を示す症例が多く見られる。そのため、シスプラチンを用いた化学抗がん療法が標準治療として用いられているが、残念なことに長期間の投薬により耐性を示す症例がしばしば見られる。しかし、シスプラチン感受性や耐性獲得に関する分子機構は依然不明な点が多いのが事実である。そこで筆者らは、癌の薬剤耐性変化の分子機構を解明するため、シスプラチン耐性卵巣細胞株（C13株）とシスプラチン感受性卵巣細胞株（2008株）において、夫々特異的に発現している遺伝子群を、HiCEP法を用いた網羅的遺伝子発現プロファイル（Gene-EP）解析によって同定し、両細胞間で発現遺伝子群の相違を比較検討した。その結果、C13株で、2008株では見られない特徴的な47個のESTsを検出した。塩基配列を同定したところ、これまでに癌関連遺伝子（ESTs含む）とされている転写物が27個あり、残り20個はこれまで癌関連転写物として扱われていないものであった。その内、転写物として過去に報告が無い9個の新規なESTsも含まれていた。

【はじめに】

シスプラチン耐性に関与するDNA修復機構として、T. Taniguchiらはファンコニ貧血-BRCA1癌制御タンパク質経路が関与していることを示唆しているが、一方でC13株に対しては、これとは別の機構が関与していることも示唆している[1)]。このことから、他にシスプラチン耐性に関与している分子機構が存在している可能性を無視することは出来ない。この為、ゲノムワイドなアプローチにより多数の関連候補因子を見出すことが大変重要な問題と考え、現在、多くの研究グループが候補因子の抽出を検討している。

網羅的な発現頻度解析方法には、DNAチップ、SAGE法等があるが、DNAチップには塩基配列情報が必要であり、既知あるいは予測転写産物配列に限定されてしまう欠点がある。この意味では、SAGE法があるが、得られる塩基配列情報が少なく、更なる解析に進むには、時間的および経費的負担が懸念され、現実的ではないと思われる。一方、HiCEP法は、発現遺伝子プロフィールを網羅的に捉えることのできる方法であり、特に今回の様なGene Findingに対しては効果が発揮されることが期待できるので本法を用いることにした。また、材料としてのC13株は、今回比較した2008株からシスプラチン耐性細胞株として樹立されたものであり、染色体の損傷（ゲノム配列の欠損や遺伝子組換えなど）は懸念させるものの、細胞のもつ遺伝的バックグラウンドは同じであるので、Gene-EP比較において耐性に関与する転写産物が直接検出できる可能性が高いと考えられる。

連絡先：〒267-0056 千葉県千葉市緑区大野台1-2-3
日清紡研究開発センター
Tel: 043-205-0789 Fax: 043-205-0844
E-mail: sasaki@nisshinbo.co.jp

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All-*trans* retinoic acid down-regulates human albumin gene expression through the induction of C/EBPβ-LIP

Takahiro MASAKI*†‡, Tomokazu MATSUURA§, Kiyoshi OHKAWA†, Tatsuo MIYAMURA*, Isao OKAZAKI‡, Tetsu WATANABE‡ and Tetsuro SUZUKI*[1]

*Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, †Department of Biochemistry, The Jikei University School of Medicine, Tokyo 105-8461, Japan, ‡Department of Community Health, Tokai University School of Medicine, Kanagawa 259-1193, Japan, and §Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo 105-8461, Japan

ATRA (all-*trans* retinoic acid), which is a major bioactive metabolite of vitamin A and a potent regulator of development and differentiation, mediates down-regulation of the human albumin gene. However, the mechanism of ATRA-mediated down-regulation is not well understood. In the present study, deletion analysis and luciferase assays demonstrate that ATRA causes a marked decrease in the activity of the albumin promoter, the region between nt -367 and -167 from the transcription start site, where C/EBP (CCAAT/enhancer-binding protein)-binding sites are tightly packed, is indispensable for ATRA-mediated down-regulation. ChIP (chromatin immunoprecipitation) assays revealed that *in vivo* binding of C/EBPα to the region markedly decreases upon incubation with ATRA, whereas ATRA treatment marginally increases the recruitment of C/EBPβ. We found that ATRA has the ability to differentially and directly induce expression of a truncated isoform of C/EBPβ, which is an LIP (liver-enriched transcriptional inhibitory protein) that lacks a transactivation domain, and to increase the binding activity of C/EBPβ-LIP to its response element. Overexpression of C/EBPβ-LIP negatively regulates the endogenous expression of albumin, as well as the activity of the albumin promoter induced by C/EBP transactivators such as C/EBPα and full-length C/EBPβ. In conclusion, we propose a novel model for down-regulation of the albumin gene, in which ATRA triggers an increase in the translation of C/EBPβ-LIP that antagonizes C/EBP transactivators by interacting with their binding sites in the albumin promoter.

Key words: all-*trans* retinoic acid (ATRA), liver-enriched transcription factor, CCAAT/enhancer-binding protein (C/EBP), dominant-negative factor, FLC-4 cell, liver-enriched transcriptional inhibitory protein (LIP).

INTRODUCTION

Serum albumin is the most abundant and characteristic protein that is produced by the mature liver; albumin functions as a transporter of various substances and is a prime regulator of colloid osmotic pressure [1]. Albumin is exclusively synthesized in the liver, approx. 200 mg/kg per day [2], leading to its high steady-state concentration in plasma (35–50 g/l in humans). It has been reported that the albumin level in plasma is decreased as a result of reduced albumin synthesis in clinical disorders such as liver disease [2], infectious disease [3], and cancer [4]. Serum albumin can be used as a reliable indicator for the prognosis and severity of these diseases [5,6]. Therefore it is probable that albumin synthesis is regulated accurately and dramatically in a variety of physiological and pathophysiological conditions.

Albumin synthesis is regulated mainly at the transcriptional level through tissue-specific transcription factors such as HNF (hepatocyte nuclear factor)-1 and C/EBP (CCAAT/enhancer-binding protein) [7,8]. The transcription of the albumin gene is down-regulated by a number of factors, including cytokines [9–11], vitamins [12–14], colloid osmotic pressure [15,16] and amino acid limitation [17]. ATRA (all-*trans* retinoic acid), a major bioactive metabolite of vitamin A, plays a crucial role in hepatocyte differentiation, proliferation and apoptosis [18,19]. ATRA has been shown to down-regulate albumin gene expression in rat hepatocytes [20] and human hepatoma cell lines [12,13]. In animal experiments, it has been reported that a decrease in serum albumin concentration is observed after the administration of ATRA to rodents [21]. Furthermore, in clinical studies of fenretinide (4-hydroxyphenyl-retinamide), a synthetic derivative of ATRA that posesses inhibitory activity against various types of malignant cells [22–24], administration of the drug caused hypoalbuminemia as an adverse effect [25]. Nevertheless, little is known about the ATRA-mediated down-regulation of albumin expression either in experimental or clinical research fields.

In the present study, we have examined the molecular mechanism by which ATRA down-regulates albumin expression in human HCC (hepatocellular carcinoma) cells, with special attention to the transcription factors involved. We present evidence that ATRA preferentially induces the expression of a truncated isoform of C/EBPβ: 20 kDa LIP (liver-enriched transcriptional inhibitory protein). We also present evidence that C/EBPβ-LIP functions as an antagonist of C/EBP transactivators in the expression of the albumin gene.

EXPERIMENTAL

Plasmids

The promoter fragment of the human albumin gene between nt -1867 and $+39$ was obtained by PCR amplification using the genomic DNA of human HCC FLC-4 cells [26,27] as a

Abbreviations used: ATRA, all-*trans* retinoic acid; C/EBP, CCAAT/enhancer-binding protein; C/EBPβ-FL, C/EBPβ-full-length; ChIP, chromatin immunoprecipitation; CUG-BP1, CUG triplet-repeat binding protein 1; DR, direct repeat; eIF, eukaryotic translation initiation factor; EMSA, electrophoretic mobility-shift assay; HCC, hepatocellular carcinoma; HNF, hepatocyte nuclear factor; LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein; RARE, retinoic acid response element; RT, reverse transcriptase.

[1] To whom correspondence should be addressed (email tesuzuki@nih.go.jp).

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Thoughts and Progress

Extracorporeal Bioartificial Liver Using the Radial-flow Bioreactor in Treatment of Fatal Experimental Hepatic Encephalopathy

*Hideki Kanai, *¶Hideki Marushima, †Naofumi Kimura, ‡Takamasa Iwaki, §Masaya Saito, ¶Haruka Maehashi, ¶Keiko Shimizu, ¶Makiko Muto, ¶Takahiro Masaki, ¶Kiyoshi Ohkawa, §Keitaro Yokoyama, §Masaaki Nakayama, **Tohru Harada, **Hiroshi Hano, ††Yoshiaki Hataba, ‡‡Takahiro Fukuda, ¶¶Masahiko Nakamura, ¶¶Naoto Totsuka, ¶¶Shutaro Ishikawa, *Yasuki Unemura, *Yuji Ishii, *Katsuhiko Yanaga, and §§Tomokazu Matsuura

*Department of Surgery, †Department of Pharmacology II, ‡Laboratory Animal Facilities, §Department of Internal Medicine, ¶Department of Biochemistry I, **Department of Pathology, ††Institute of DNA Medicine, ‡‡Department of Neuroscience, §§Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo, and ¶¶Biott Co. Ltd., Tokyo, Japan

Abstract: An extracorporeal bioartificial liver (BAL) that could prevent death from hepatic encephalopathy in acute hepatic insufficiency was aimed to develop. A functional human hepatocellular carcinoma cell line (FLC-4) was cultured in a radial-flow bioreactor. The function of the BAL was tested in mini-pigs with acute hepatic failure induced by α-amanitin and lipopolysaccharide. When the BAL system was connected with cultured FLC-4 to three pigs with hepatic dysfunction, all demonstrated electroencephalographic improvement and survived. Relatively low plasma concentrations of S-100 β protein, as a marker of astrocytic damage, from pigs with hepatic failure during BAL therapy were noted. BAL therapy can prevent irreversible brain damage from hepatic encephalopathy in experimental acute hepatic failure. **Key Words:** Acute hepatic failure—Radial-flow bioreactor—Cerebral edema—Astrocytes—α-Amanitin.

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Address correspondence and reprint requests to Dr. Tomokazu Matsuura, Department of Laboratory Medicine, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan. E-mail: matsuurat@jikei.ac.jp

A clinically effective bioartificial liver (BAL) requires development of a high-density cell culture module and a highly functioning liver cell line. We sought to develop a high-performance BAL to avoid lethal hepatic encephalopathy in acute hepatic insufficiency and establish the BAL as an extracorporeal circulation therapy able to surpass conventional blood purification procedures. Our extracorporeal BAL support system used a highly functional human hepatocellular carcinoma (HCC) cell line (FLC-4) cultured in a radial-flow bioreactor (RFB) (1,2). The RFB is packed with cell-adhesion scaffolds in a cylindrical array (Fig. 1A,B). The culture medium or the plasma flows from the periphery of the cylindrical module to the center. One important problem when cells are cultured densely is delivery of sufficient oxygen and nutrients even when these are plentiful at the inflow site. As a result, we could culture cells successfully at a density of 10^8/mL.

We have currently tested the BAL in mini-pigs with acute hepatic failure induced by α-amanitin, a mushroom-derived poison, and lipopolysaccharide (LPS), while monitoring with electroencephalography (EEG) to assess the effectiveness of BAL against hepatic encephalopathy. We measured the plasma levels of S-100 β protein, a marker of damage to astrocytes (3).

MATERIALS AND METHODS

Mini-pigs and monitoring

Male mini-pigs (CSK-MS) weighing 10–15 kg were a generous gift from Chugai Pharmaceutical (Tokyo, Japan). Prior to the experiments, they were maintained for 1–4 weeks at the Laboratory Animal Facilities of the Jikei University School of Medicine, receiving standard chow and water ad libitum. The study was approved by the institution's committee concerning animal experimentation.

Acute hepatic failure model

During inhalation anesthesia with 3–4% isoflurane, 0.05 mg/kg of α-amanitin (Calbiochem, Darmstadt, Germany) and 1 μg/kg of LPS (Sigma, St. Louis, MO, USA) dissolved in 10 mL of saline was administered via the splenic vein. Fifty percent

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BASIC STUDIES

A comprehensive gene expression analysis of human hepatocellular carcinoma cell lines as components of a bioartificial liver using a radial flow bioreactor

Makoto Kosuge[1,2], Hiroko Takizawa[2], Haruka Maehashi[3], Tomokazu Matsuura[4] and Senya Matsufuji[2]

1 Department of Surgery, The Jikei University School of Medicine, Tokyo, Japan
2 Department of Biochemistry II, The Jikei University School of Medicine, Tokyo, Japan
3 Department of Biochemistry I, The Jikei University School of Medicine, Tokyo, Japan
4 Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo, Japan

Keywords
bioartificial liver – gene expression analysis – hepatocellular carcinoma – human cell line – microarray – radial flow bioreactor

Correspondence
S. Matsufuji, MD, PhD, Department of Biochemistry II, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan.
Tel: +81 3 3433 1111
Fax: +81 3 3436 3897
e-mail: senya@jikei.ac.jp

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Abstract

Background/Aims: The cells constituting a bioartificial liver are crucial for an effective liver support system. We compared global gene expression profiles in a radial flow bioreactor or a monolayer culture of three functional liver cell lines previously established from human hepatocellular carcinoma. *Methods:* The expressions of 60 000 genes of the FLC-4, FLC-5, and FLC-7 cell lines were analyzed by the microarray technique with the Affymetrix GeneChip system. Global gene expression profiles were compared with two-way cluster analysis. Several liver function-related genes were compared between the bioreactor and culture conditions. *Results:* Cluster analysis revealed that gene expression profiles of bioreactor-grown cells resembled those of the normal liver. Genes related to cellular structure were highly expressed in the bioreactor-grown cells, while genes involved in proliferation or carcinogenesis were suppressed. In the bioreactor-grown cells, some genes for liver functions were expressed at a level similar to that in normal liver, although none of the cell lines expressed the complete set of genes encoding ammonium metabolism or cytochrome P450 species. *Conclusion:* The high-density three-dimensional culture in the radial flow bioreactor prompted differentiation of the cells. These data may be useful for improving the cells by genetic or pharmacological reinforcement and for monitoring bioartificial livers.

Liver transplantation has been the most effective therapy for hepatic failure, a major cause of death from liver diseases. However, insufficient numbers of equipped facilities and donor organs, together with the ethical issues involved, pose fundamental problems. It is therefore necessary to develop effective alternatives, and one promising approach is the temporary use of a bioartificial liver (BAL) that provides a substitute for the organ until tissue regeneration or a donor liver is obtained (1).

From a practical perspective, cells constituting a BAL are crucial (2). Cells most widely and successfully used are primary hepatocytes from human embryos or other mammalian organisms (3, 4), as well as cell lines established from human hepatocellular carcinoma. Rozga and colleagues (5, 6) reported that porcine hepatocytes cultured in a hollow fiber-type reactor improved clinical conditions in patients with liver failure. Sussman et al. (7) and Yamashita et al. (8) demonstrated that a BAL with human hepatoblastoma cell culture is effective in hepatitis treatment. On the other hand, several problems have been found in the application of BAL, including (i) difficulty in maintaining the cell cultures for a long period of time without losing physiological liver functions, (ii) insufficiency in cell supply, (iii) difficulty in scaling up BAL, (iv) secretion of proteins in unknown quantities and inappropriate profiles, and (v) the presence of various known and unknown infectious agents. Thus, a standard choice for cells constituting BAL has not been established (9, 10). Previously, Fujise et al. (11) reported the use of several cell lines derived from human hepatocellular carcinoma. Some of these have been further characterized and named FLC (functional liver cells). Because these cell lines preserve many physiological liver functions, we regarded them as a

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Syntheses of calcium-deficient apatite fibres by a homogeneous precipitation method and their characterizations

Mamoru Aizawa [a,*], Hiroko Ueno [b], Kiyoshi Itatani [b], Isao Okada [b]

[a] *Department of Industrial Chemistry, School of Science and Technology, Meiji University, Kanagawa 214-8571, Japan*
[b] *Department of Chemistry, Faculty of Science and Engineering, Sophia University, Chiyoda-ku, Tokyo 102-8554, Japan*

Available online 10 August 2005

Abstract

Calcium-deficient apatite fibres were successfully synthesized by a homogeneous precipitation method using starting solutions with a Ca/P ratio of 1.00–1.67. In the case of the Ca/P ratio of 1.67, the resulting apatite fibre had long-axes of about 60–100 μm and contained 5.2 mass % of carbonate ions. The Ca/P ratio of apatite fibres could be controlled in the range of 1.53–1.68 by changing the Ca/P ratio of the starting solutions from 1.00 to 1.67. The long-axes and the carbonate contents of the resulting calcium-deficient apatite fibres increased with Ca/P ratio of the starting solutions. These apatite fibres were of single crystal and had a preferred orientation in the *c*-axis direction.

Keywords: Powder-chemical preparation; Fibres; Apatite; Biomedical applications; Calcium-deficient hydroxyapatite

1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) has been widely applied as a biomaterial for substituting human hard tissues,[1,2] and as an adsorbent for chromatography.[3] The HAp crystal has two types of crystal planes, bearing different charges: positive on the *a*-planes and negative on the *c*-planes.[3] Thus, novel properties may be produced by controlling the orientation of the crystal planes. Controlled orientation may be achieved by modifying the morphology of HAp crystals. For example, in order to increase the positive charge on the surface of the HAp fibres, one can grow hexagonal-shaped HAp fibres which are oriented along the *c*-axis so that the *a*(*b*)-plane is wider than the *c*-plane. These apatite fibres have specific adsorptions to negatively charged acidic proteins.

The morphological control of HAp crystals has been reported previously by some researchers. For example, Ioku et al.[4] synthesized apatite whiskers with a long-axis of several micrometers in length by a hydrothermal process and demonstrated by transmission electron microscopy (TEM) that the whiskers were of single crystal. On the other hand, Yokogawa et al.[5] synthesized the plate-shaped apatites by a hydrothermal process in the presence of organic solvents.

We have also successfully synthesized apatite fibres with long axes of 60–100 μm by homogeneous precipitation method.[6,7] It was confirmed from the results of high-resolution transmission electron microscopy (HR-TEM) using a shadow imaging technique that the apatite fibres were of single crystals with the *c*-axis orientation parallel to the long axis of the fibre.[8,9]

Using the above fibres, we have promoted the development of (i) porous HAp ceramics with well-controlled pore sizes[7,10] and (ii) HAp/polymer hybrids possessing mechanical properties similar to those of living cortical bone by in situ bulk polymerization of the monomer in the pores of the ceramic.[11] This hybrid, with mechanical properties similar to those of cortical bone, has been shown to have excellent biocompatibility both in vitro and in vivo.[12,13] In addition, we have developed a three-dimensional scaffold with large interconnected pores of 100–250 μm in diameter and high porosities of 98–99% for tissue engineering of bone from the above-mentioned apatite fibres.[14–16] We have already clarified that the apatite-fibre scaffold has an excellent cellular response, such as enhanced differentiation to osteoblasts.

* Corresponding author. Tel.: +81 44 934 7237; fax: +81 44 934 7906.
E-mail address: mamorua@isc.meiji.ac.jp (M. Aizawa).

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Syntheses of silicon-containing apatite fibres by a homogeneous precipitation method and their characterization

Mamoru Aizawa[1,a], Nelesh Patel[2,b], Alexandra E. Porter[2,c], Serena M. Best[2,d] and William Bonfield[2,e]

[1]Department of Industrial Chemistry, School of Science and Technology, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Japan, 214-8571

[2]The Cambridge Centre for Medical Materials, Department of Materials Science and Metallurgy, University of Cambridge, Pembroke Street, Cambridge, CB2 3QZ, UK

[a]mamorua@isc.meiji.ac.jp, [b]ez8046@yahoo.co.uk, [c]aep30@cam.ac.uk, [d]smb51@cam.ac.uk, [e]wb210@cam.ac.uk

Keywords: Hydroxyapatite, Apatite fibre, Silicon-containing apatite, Homogeneous precipitation method

Abstract. Silicon-containing apatite (Si-HAp) fibres were successfully synthesized by a homogeneous precipitation method. The resulting Si-HAp fibres were composed of carbonate-containing apatite fibres with preferred orientation along the (h00) planes. The Si contents in the Si-HAp fibres could be controlled by the Si concentration of the starting solutions. TEM observation indicated that the Si-HAp fibres were single crystals. The Si-HAp fibres have potential as novel materials for high-performance biomedical devices.

Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) is used in biomedical applications, such as bone grafts and scaffolds for bone tissue engineering [1]. The composition of synthetic HAp is a similar to bone mineral; however, there are a number of distinct differences between the two materials in terms of their trace ion contents. In recent investigations, the bioactivity of the HAp has been shown to be enhanced by the substitution of suitable ions into its crystal lattice. For example, substitution of low levels of silicon into the HAp lattice has been found to dramatically improve the rate at which bone bonding occurs with the implant materials [2, 3].

HAp crystals belong to a hexagonal crystal system and possess a positive charge in their *a*-planes and the negative charge in their *c*-planes [4]. If one can control the morphology of the HAp crystal, the products can be applied as novel materials for biomedical devices using surface charges [1]. The authors have reported that apatite fibres can be synthesized by a homogeneous precipitation method (HPM) [5], and that the fibre was single-crystal and highly strained [6].

Our aims in the present investigation are to create novel high-performance apatite fibres with a trace level of silicon and to examine some properties of the resulting fibres.

DEVELOPMENT OF APATITE-FIBER SCAFFOLDS PROMOTOING HARD-TISSUE REGENERATION AND THEIR APPLICATION TO BIOMEDICAL DEVICES

Mamoru Aizawa[1], Hiroshi Uchida[2], Takahiko J. Fujimi[2], Nobuyuki Kanzawa[2], Morio Matsumoto[3], Hikaru Morisue[3], and Yoshiaki Toyama[3]

[1]Department of Applied Chemistry, School of Science and Technology, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, 214-8571, Japan
[2]Department of Chemistry, Faculty of Science and Engineering, Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo, 102-8554, Japan
[3]Department of Orthopedic Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan

* Corresponding author: mamorua@isc.meiji.ac.jp

Abstract: There are a rapidly increasing number of patients with diseases of their hard-tissue on the current demographic trends for old people to increase. In order to provide the aged with a high quality of life (QOL), we have developed novel scaffolds which will promote hard-tissue regeneration. We have developed porous scaffold for tissue engineering of bone using the single-crystal apatite fibers. The resulting apatite-fiber scaffolds have large pores with diameters of 110-250 μm and high porosities of 98-99%. The scaffolds were biologically evaluated using two kinds of cells, osteoblastic cells (MC3T3-E1) and rat bone marrow cells. In both cases, the cells cultured in the scaffolds showed excellent cellular response, such as good cell proliferation and enhanced differentiation into osteoblasts. We conclude that such scaffolds with high porosity and large pore size may be effective as the matrix of tissue engineered structures for promoting regeneration of bone.

Introduction

Tissue engineering is an important technology that encourages regeneration of the defecting tissue utilizing scaffolds, cells and growth factors. In the case of tissue engineering for bone, porous calcium-phosphate ceramics are generally used as scaffolds, together with bone marrow cells and rhBMP-2 or TGF-β, as reported by Ogushi and co-workers in detail [1].

Among the above three factors, the scaffold play a role of three-dimensional (3D) matrices for cells. In general, porous bioceramcis, such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) and tricalcium phosphate ($Ca_3(PO_4)_2$; TCP) have been used as a matrix for bone regeneration. Many researchers are trying to develop the high-performance scaffolds with high porosity, interconnected pores, and excellent biocompatibility.

We have also developed novel scaffolds using the apatite fibers which are synthesized by a homogeneous precipitation method [2,3]. The current apatite fibers were of single crystals with the *c*-axis orientation parallel to the long axis of the fiber [4].

Utilizing the sintering of individual fibers, we fabricated sheet-shaped scaffolds for tissue engineering of bone [5]. It has already been found that the apatite-fiber scaffold (AFS) has an excellent cellular response, such as enhanced allkaline phosphatase (ALP) activity. However, the pore size was too small to culture the cells three-dimensionally.

We have partly modified the fabrication process using carbon beads of about 150 μm in diameter as pore forming agents, in order to enlarge the pore size of the AFS. As a result of the trial, we could be successfully fabricated the AFS with large interconnected pores of 100-250 μm in diameter and high porosities of 98-99% [2,3].

In addition, we have clarified the interactions of the 3D AFSs with osteoblasts using two kinds of cells, that is, MC3T3-E1 of an osteblastic cell line and the rat bone morrow cell (RBMC) as a mesenchymal stem cell model. Actually, we examined the cellular responses to AFSs: cell attachment, proliferation, differentiation (assays and gene expression of differentiation makers of the osteoblasts), and morphology.

In this paper for key note lecture in ABC2006, we will review i) fabrication of AFS and its characterization and ii) biological properties on the basis of *in vitro* evaluation using osteoblasts.

Materials and Methods

Fabrication process of apatite-fibre scaffold and its characterisation.

The AFSs were fabricated on the basis of previous reports [2,3]. The process is briefly described as

OSTEOGENIC DIFFERENTIATION IN A THREE-DIMENSIONAL APATITE-FIBER SCAFFOLD

Michiyo Honda[1*], Takahiko J. Fujimi[1], Nobuyuki Kanzawa[1], Koji Izawa[1], Takahide Tsuchiya[1], Mamoru Aizawa[2],

[1] Department of Chemistry, Faculty of Science and Engineering, Sophia University, Tokyo, Japan
[2] Department of Applied Chemistry, School of Science and Technology, Meiji University, Kanagawa, Japan

* Corresponding Author E-Mail Address: honda-m@sophia.ac.jp

Abstract: Osteogenic differentiation of MC3T3-E1 cells in a three-dimensional (3-D) scaffold has been studied in this work. We examined the expression of bone-related genes during differentiation of MC3T3-E1 cells in a 3-D culture system. To evaluate the relevance of 3-D culture environment to osteogenic differentiation, we compared two distinct carriers. We used the tissue culture plate for 2-D culture and Apatite-Fiber Scaffold (AFS) for 3-D culture. The AFS-formed 3-D network of fibers had a high porosity and two different sizes of pores.
Culturing cells in the AFS resulted in increases in mRNA expression level of type I collagen, osteocalcin and osteopontin in the absence of dexamethasone at 4, 7 , 14 and 21 days compared with those in the 2-D culture. Additionally, in the AFS those bone-related genes expressed earlier than in 2-D culture and started osteogenic differentiation. These results demonstrated that AFS had the 3-D culture system enhanced osteogenic differentiation. We thought AFS facilitated the cell proliferation and the cell differentiation by using two different pores properly. We concluded that this characteristic structure of AFS was one of the essential factors for osteogenic differentiation.

Introduction

The need to repair defects in bone is a significant problem faced by orthopaedic medicine. Tissue engineering and regenerative medicine offer solutions to a number of clinical problems that have not been adequately addressed through the use of permanent replacement devices [1]. At present, the cell tissue devices that function as an alternative organization and internal organs are developed by using the cell culture technology to achieve new management that takes the place of the organ transplantation. Tissue engineering has been used to enhance the utility of biomaterials for clinical bone repair by the incorporation of an osteogenic cell source into a scaffold followed by the *in vitro* promotion of osteogenic differentiation before host implantation [2]. As the first step, it is necessary to culture a large amount of target cells for an anagenesis. However, the organization with the function cannot be regenerated by a conventional, two-dimensional culture. The culture space arranged in three dimensions is necessary for the ideal cell culture, because cells are proliferated and differentiated *in vivo* in a three-dimensional environment. Appropriate pore size and porosity are necessary for the three-dimensional cell extension; in this context, most studies were carried out in the 2-D culture system to understand the changes in molecular events observed during the cell differentiation. However, 3-D cellular development is essential for *in vitro* bone formation [3, 4]. *In vitro* osteoblast differentiation in the 3-D culture system may be more closely relevant to the *in vivo* bone formation process. Apatite-fiber scaffold (AFS) with high porosity and excellent biocompatibility has been developed based on such clinical demand [5].

The aim of this study is to investigate the cell compatibility and the ability of high-density cell culture in the AFS and to evaluate the relevance of 3-D culture environment to osteogenic differentiation. The present study revealed that 3-D culture system was effective to regulate cell proliferation and differentiation similar to the situation *in vivo*.

Materials and Methods

Fabrication process of apatite-fiber scaffold and its characterization

The hydroxyapatite (HAp) fibers were prepared from aqueous solutions in the $Ca(NO_3)_2$-$(NH_4)_2HPO_4$-$(NH_2)_2CO$-HNO_3 system through a homogeneous precipitation method using urea, as previously reported [5,6]. The HAp fibers were suspended with spherical carbon beads (Nika beads; Nihon Carbon Company) with a diameter of ~150 μm in the mixed solvent (ethanol/water=1/1(v/v)). The carbon beads were added to the HAp fiber at a ratio of carbon/HAp (w/w): 20/1. The green compacts for the scaffold were fabricated by pouring and vacuum pumping the above mixed suspension containing ~1 mass % of the HAp (5 cm^3) into a vinylchloride mould of 16.5 mm in internal diameter. The resulting compacts were fired at 1300°C for 5 h in a steam atmosphere to develop the structure of the scaffold. The resulting scaffolds were characterized as follows: i) phase identification by X-ray diffractometry (XRD) and infrared spectroscopy (IR),

APATITE-FIBER SCAFFOLD PROVIDES THREE-DIMENSIONAL CULTURE ENVIRONMENT FOR OSTEOBLAST-LIKE CELLS

Shigeki Izumi[1], Michiyo Honda[1], Nobuyuki Kanzawa[1*], Takahiko J. Fujimi[1], Hiroshi Uchida[1], Takahide Tsuchiya[1], Hikaru Morisue[2], Morio Matsumoto[2], Mamoru Aizawa[3]

[1] Department of Chemistry, Faculty of Science and Technology, Sophia University, Tokyo, Japan
[2] Department of Orthopaedic Surgery, School of Medicine, Keio University, Tokyo, Japan
[3] Department of Applied Chemistry, School of Science and Technology, Meiji University, Kanagawa, Japan

[*] Corresponding Author E-Mail Address: n-kanza@sophia.ac.jp

Abstract: Tissue engineering has been used to enhance the utility of biomaterials for clinical bone repair by the incorporation of an osteogenic cell into a scaffold. In this study, we investigated whether apatite-fiber scaffold (AFS) can be used as a three-dimensional scaffold for cells. AFS has two different types of pores, micro and macro pores. Micro-pores, which arise from interspaces of AFS at random distribution, provide 10-μm wide spaces for cells. The other wide pores (~250-μm width), designated macro-pores; are formed after baking carbon beads, which are embedded during a preparation of AFS/beads mixed compacts. These pores formed inter-pore connections, and the pore sizes were regarded as appropriate for trapping cells. Cell-seeded scaffolds were cultured for 2 or 7 days. We evaluated the morphological changes of cultured cells such as cell attachment with optical and confocal laser scanning microscopes. These observations revealed that the seeded cells extended in Apatite-Fibers three-dimensionally, and that the cells were homogeneously distributed. These results demonstrated that seeded cells were spread equally in AFS by a simple method that involved putting some drops of cell suspension into scaffolds. Moreover, seeding efficiency was considerably higher than that of an existing commercially produced scaffold.

Introduction

Aging is one of the biggest concerns not only for Japan but also for the other countries, where the number of osteoporosis patients becomes a socially important health issue. To maintain the quality of life, autografting and allografting cancellous bone have been widely used for bone graft procedures. Moreover, synthetic grafting materials have been developed to use as a bone replacement material [1]. Hydroxyapatite (HAp) is an osteoconductive material that can be used in the bone replacement due to its chemical and crystallographic similarity to carbon-containing apatite in human bones and teeth [2]. Recently, HAp and other calcium phosphate materials are used as scaffolds that pre-embedded bone cells or other agents for the bone regeneration purposes [3]. Thus, bone tissue engineering is an emerging interdisciplinary field involving principles of the life sciences and engineering concerned with the formation of three-dimensional bone substitutes by culturing osteogenic cells on natural or synthetic scaffolds.

Our ultimate goal is to create not merely osteoconductive scaffolds, but substitutes that are able to regenerate the bones of the osteoporosis patients. In this context, we developed the apatite-fiber scaffold (AFS) as a novel scaffold for bone repair [4-6]. By changing mixture ratios of spherical carbon beads and HAp fibers, we developed scaffolds with an appropriate size of macro-pores and interconnected micro-pores. The median of the macro-pore size is ~250 μm (AFS2000), which is suitable for three-dimensional cell culture. Biological and mechanical properties of the scaffold are highly dependent on the fine structure of AFS, such as pore size, porosity, and interconnectivity [7, 8]. However, we have not yet clarified the biological effect of the AFS.

In the present study, we focused on the biological properties of the AFS; how cells are penetrated, extended and proliferated in the scaffold. Cells are expected to extend three dimensionally, similarly to the situation *in vivo*. Because cell attachment contributes to the regulation of most aspects of the cellular activity, we firstly examined the homogenous dispersion of cells into AFS. We further examined the fine structure of cells in AFS using a confocal laser scanning microscope.

Materials and Methods

Fabrication of AFS

A fibrous HAp was prepared as previously reported [4, 9]. The HAp fibers were suspended with spherical carbon beads (Nika beads; Nihon Carbon Company) with a diameter of ~150 μm in the mixed solvent (ethanol/water=1/1(v/v)) at carbon/HAp (w/w) ratio: 20/1 (AFS2000). Figure 1 shows the particular macroscopic (Fig. 1A) and scanning microscopic images (Fig. 1B) of AFS2000.

DEVELOPMENT OF POROUS CERAMICS WITH WELL-CONTROLLED PORE SIZES CREATED FROM SINGLE-CRYSTAL APATITE FIBERS AND ITS BIOLOGICAL EVALUATION

Y. Tanaka[1], H. Morisue[2], M. Matsumoto[2], Y. Toyama[2] and M. Aizawa[1*]

[1] School of Science and Technology, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa, Japan 214-8571

[2] School of Medicine, Keio University, 35 Shinanomachi, Shinjyuku-ku, Tokyo, Japan 160-8582

* mamorua@isc.meiji.ac.jp

Abstract: Porous hydroxyapatite (HAp) ceramics having well-controlled porosity and pore size could be fabricated by firing apatite-fiber compacts mixed with carbon beads of 150 μm in diameter. The resulting HAp ceramics were of about 70% in porosity, and the pores were distributed in two ranges of several micrometers originating from intertwining of individual fibers and of 100-200 μm from the carbon beads. Most of the pores were regarded as open pores. The MC3T3-E1 cells cultured on/in porous HAp ceramics showed good proliferation. The cylindrical porous HAp ceramics were implanted into a tibia of rabbit, together with a control of commercially available porous HAp ceramics with porosity of 60%. After 8 weeks, newly-formed bone was abundantly present inside implants originating from the apatite fibers, as compared with the ones from the commercially available porous HAp ceramics. These results demonstrate that the present porous HAp ceramics well-controlled for the pore structure have excellent biocompatibility.

Introduction

In orthopedic surgery, bone grafting has been performed to treat diseases and injuries, such as bone tumor and bone fracture. In general, bone implantation is classified into three types: i) auto-grafting, ii) allo-grafting and iii) artificial-bone grafting. Among these bone implantations, auto-grafting is well-known as a common practice and is harvested from the patient. However, there are two serious problems as easily expected, i.e., limitations in supply from the host's bone and subsequently, the takeout surgery from the normal part of the host. Alternatively, allo-grafting has been performed using donor bones obtained from bone banks; however, it also has problems of supply, immunogenic factors and quality. Thus, for artificial-bone grafting, which would have the least problem in the implantation, porous HAp ceramics, which can be integrated with newly-formed bone of the host, are generally used [1].

Porous HAp ceramics for bone-grafting are required to contain interconnected open pores whose sizes of over 100 μm in diameter [2], to lead to penetration and proliferation of osteoblasts, vascular ingrowths and integration of newly-formed bone into porous ceramics. Microstructure of porous ceramics with interconnected open pore is also effective for being saturated with a body fluid or as mediums for cell culture.

Previously, porous HAp ceramics have been fabricated via the following processes [1,3,4]: i) sintering of the HAp particle in the co-presence of naphthalene particles or hydrogen peroxide, ii) utilization of apatite cement prepared from tetracalcium phosphate ($Ca_4O(PO_4)_2$) and calcium hydrogen phosphate ($CaHPO_4$), and iii) HAp conversion by hydrothermal reaction of marine invertebrates with diammonium hydrogen phosphate ($(NH_4)_2HPO_4$).

Using single-crystal apatite fibers [5], we have developed the porous HAp ceramics with well-controlled porosity and interconnected open pores [6]. In addition, we have successfully prepared the porous HAp ceramics with pores of over 100 μm in diameter by firing the apatite-fiber compacts mixed with carbon beads [7].

Our aims of the present investigation were to fabricate porous HAp ceramics from apatite fiber and carbon beads, and then to examine *in vitro/vivo* the biocompatibilities of the resulting porous ceramics.

Materials and Methods

Fabrication of porous ceramics and their characterization

As previously reported [5], single-crystal apatite fibers were synthesized by a homogeneous precipitation method from $Ca(NO_3)_2$-$(NH_4)_2HPO_4$-$(NH_2)_2CO$-HNO_3-H_2O solution. The starting solution having a Ca/P ratio of 1.67 was prepared by mixing 0.167 mol· dm^{-3} $Ca(NO_3)_2$, 0.100 mol· dm^{-3} $(NH_4)_2HPO_4$, 0.500 mol· dm^{-3} $(NH_2)_2CO$ and 0.10 mol· dm^{-3} HNO_3 aqueous solutions. The solution was heated at 80 °C for 24 h and then at 90 °C for 72 h to synthesize apatite fibers.

Porous HAp ceramics were fabricated from the apatite fibers as follows [7]: the fibers were suspended into pure water to prepare the slurry with apatite contents of 2 mass%. Carbon beads with 20 or 150 μm

THREE-DIMENSIONAL CELL CULTURE OF HEPATOCYTES USING APATITE-FIBER SCAFFOLD AND APPLICATION TO A RADIAL-FLOW BIOREACTOR

A. Hiramoto[1], T. Matsuura[2] and M. Aizawa[1,*]

[1]Department of Applied Chemistry, School of Science and Technology, Meiji University,1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa, Japan 214-8571

[2]Division of Central Clinical Laboratory, Jikei University Hospital, 3-19-18 Nishi-shinbashi, Minato-ku, Tokyo, Japan 105-8461

*mamorua@isc. meiji. ac. jp

Abstract: We have successfully developed apatite-fiber scaffolds (AFSs) for bone tissue engineering using the single-crystal apatite fibers and carbon beads. In the present investigation, we examined the possibility of three-dimensional (3D) culture of hepatocytes using the AFSs, aiming to apply the scaffold as a matrix of the artificial liver model. FLC-4 cells were used as a model of hepatocyte. The results of cell proliferation showed that the FLC-4 cells 3D-cultured in the AFS have higher DNA contents than that on cell-culture polystyrene plate for control. The morphological observation showed that the FLC-4 cells adhered thoroughly to AFS. In order to assay the function as a hepatocyte, we determined the amount of albumin produced from FLC-4 cells during cell culture periods by an ELISA method; the albumin in the medium was used for assay. The amount of albumin production increased with cultivation time. In another experiment using a radial-flow bioreactor (RFB), the FLC-4 cells were viable in the RFB over a period for 21 days. The amounts of glucose and lactic acid were measured during 3D-cell culture using the RFB. The amounts of glucose decrease, while that of lactic acid increased. These changes indicate that the FLC-4 cells were fully grown in the RFB.

Introduction

Recently, a liver transplant increases year by year; however, it has a fatal problem of donor's lack. Thus, the development of the artificial liver that assists the liver function is being needed. Tissue engineering is noticed as one of the solutions to the above problem, and it regenerates the defected lacked tissue by combining three factors: cells, growth factors and scaffold.

We have succeeded in development of the scaffold with interconnected macro-pores, which enable three-dimensional cell culture, using single-crystal apatite fibers (AFs) and carbon beads [1, 2]. This scaffold is named as "apatite-fiber scaffold (AFS)". The AFS has an excellent biocompatibility to osteoblast *in vitro* and hard tissues *in vivo*.

In order to apply the above AFS to the regeneration of real organs (for example, liver), we performed the culture of FLC-4 cells [3] as a model of hepatocyte. Furthermore, we examined the proliferation and morphology of the hepatocytes in the AFS.

As for the evaluation of function as a hepatocyte, we determined the amount of albumin which one of the vital proteins produced from FLC-4 cells.

In order to realize three-dimensional cell culture, we used a radial-flow bioreactor (RFB). A RFB enables a highly functional three-dimensional culture as bio-artificial liver [4]. The capacity of bioreactors depends not only on their mechanistic structures but also on scaffolds packed in them. Thus, we carried out three dimensional (3D) cell culuture using the RFB settled with AFS over periods for 21 days, and assessed the viability of FLC-4 cells by monitoring pH, concentrations of glucose and lactic acid. Instead of the AFSs, cellulose beads were also settled into the RFB as a control in the present work.

Materials and Methods

Materials

The AF was synthesized via a homogeneous precipitation method using urea [5, 6]. The AF was mixed with the spherical carbon beads having a diameter of ~150 μm in the mixed solvent (ethanol/water=5/5(v/v)). As previously reported in Ref [1,2], the carbon beads were added to the AF in the following carbon/HAp (w/w) ratios: 20/1, 10/1 and 0/1. The resulting compacts were fired at 1300 °C for 5 h in a steam atmosphere to fabricate the AFS; hereafter, we named each scaffold derived from carbon/HAp=20/1, 10/1 and 0/1 (w/w) as "AFS2000", "AFS1000" and "AFS0", respectively.

The crystal phase of the AFS was identified with an X-ray diffractometer (XRD) and a Fourier transform infrared spectrometer (FT-IR), and the microstructure was observed by a scanning electron microscope (SEM).

Polymerizable Cationic Gemini Surfactant

Masahiko Abe,[†,‡] Kazuyuki Tsubone,*[,†] Takaaki Koike,[†] Koji Tsuchiya,[‡] Takahiro Ohkubo,[‡] and Hideki Sakai[†,‡]

Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, and Institute of Colloid and Interface Science, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku, Tokyo 162-8601, Japan

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A polymerizable cationic gemini surfactant, $[CH_2{=}C(CH_3)COO(CH_2)_{11}N^+CH_3)_2CH_2]_2{\cdot}2Br^-$, **1** has been synthesized and its basic interfacial properties were investigated (in water and in the presence of 0.05 M NaBr). For comparison, the properties of monomeric surfactant corresponding to **1**, $CH_2{=}C(CH_3)COO(CH_2)_{11}N^+(CH_3)_3{\cdot}Br^-$, **2**, were also investigated. Parameters studied include cmc (critical micelle concentration), C_{20} (required to reduce the surface tension of the solvent by 20 mN/m), γ_{cmc} (the surface tension at the cmc), Γ_{cmc} (the maximum surface excess concentration at the air/water interface), A_{min} (the minimum area per surfacatant molecule at the air/water interface), and cmc/C_{20} ratio (a measure of the tendency to form micelles relative to adsorb at the air/water interface). For the polymerizable gemini surfactant, **1**, the methacryloxy groups at the terminal of each hydrophobic group in a molecule have no contact with the air/water interface in the monolayer, whereas for the corresponding monomeric surfactant, **2**, the methacryloxy group contacts at the interface forming a looped configuration like a bolaamphiphile. Polymerized micelles of the gemini surfactant are fairly small monodisperse and spherical particles with a mean diameter of 3 nm.

In the past decade, new types of surfactant called gemini surfactants (or sometimes called dimeric surfactants) having two hydrophobic tails and two hydrophilic headgroups connected through a linkage adjacent to the hydrophilic headgroups in a molecule have attracted considerable interest since these compounds have very much lower cmc values and much greater efficiency in reducing surface tension (pC_{20}) than expected.[1a] These properties result from the fact that the hydrophobic groups of gemini surfactant molecules can pack at the interface more closely than those of the corresponding monomeric surfactants, especially when the linkage consists of two methylene groups.[1a] Owing to their extraordinary surface activity, they are regarded as an outstanding new generation of surfactants with excellent solubilization, wetting, and rheological properties at low concentration.[2]

On the other hand, polymerizable surfactants have been used in a variety of application including capturing the structure of spherical micelles[3,4] and as a stabilizer in emulsion polymerization,[5,6] miniemulsion polymerization,[7] and microemulsion polymerization.[8] Kaler et al. showed that polymerizable anionic wormlike micelles were obtained upon mixing the hydrotropic salt *p*-toluidine hydrochloride with the polymerizable anionic surfactant sodium 4-(8-methacryloyloxyoctyl)oxybenzene sulfonate.[9] Polymerization captures the cross-sectional radius of the micelles (~2 nm), induces micellar growth, and leads to the formation of a stable single-phase dispersion of wormlike micelles polymers. Walker et al. synthesized a cationic surfactant of the form (C_nH_{2n+1}) trimethylammonium 4-vinylbenzoate (where n = 14−18) and investigated the parameters necessary to independently control the final radius and length of rodlike polymerized aggregates.[8] Sodium di(undecenyl)tartarate,[10] one of the polymerizable gemini surfactant, has recently been investigated as novel pseudostationary phases in micellar electrokinetic chromatography.[11] The lyotropic liquid-crystalline phase bahavior of cross-linkable and polymerizable gemini surfactants, bis(alkyl-1,3-diene)-based phosphonium amphiphiles, has been studied to design nanostructures.[12] Very little information is found in the literature on polymerizable gemini surfactants with the exception of these two species. We report in this paper the interfacial properties of a novel polymerizable cationic gemini surfactant with a polymerizable group at the terminal of each hydrophobic group, $[CH_2{=}C(CH_3)COO(CH_2)_{11}N^+(CH_3)_2CH_2]_2{\cdot}2Br^-$, **1**, while comparing with those of the monomeric surfactant corresponding to the gemini **1**, $CH_2{=}C(CH_3)COO(CH_2)_{11}N^+(CH_3)_3{\cdot}Br^-$, **2**,[13] conventional cationic surfactants, tetradecyltrimethylammonium bromide (TTAB), and cetyltrimethylammonium bromide (CTAB). The interfacial properies of studied include cmc, pC_{20} (negative logarithmic molar surfactant concentration required to reduce the surface tension of the solvent by 20 mN/m), γ_{cmc} (surface tension value at cmc), Γ_{max} (maximum surface excess concentration at air/water interface), A_{min} (minimum area occupied by one surfactant molecule at air/water interface), and cmc/C_{20} ratio (a measure of tendency to adsorb at the interface relative to that to form micelles in bulk phase).[1b] The chemical structures of **1** and **2** are shown in Figure 1, together with those of a comparable cationic gemini surfactant, ethanediyl-1,2-bis(dodecyldimethyl-

* Corresponding author. Tel: 04-7124-1501 (ext. 3663). Fax: 04-7122-1442. E-mail: ktsubone@rs.noda.tus.ac.jp.

† Faculty of Science and Technology.

‡ Institute of Colloid and Interface Science.

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www.wjgnet.com
wjg@wjgnet.com

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EDITORIAL

The hepatic sinusoidal endothelial lining and colorectal liver metastases

Filip Braet, Keissuke Nagatsuma, Masaya Saito, Lilian Soon, Eddie Wisse, Tomokazu Matsuura

Filip Braet, Lilian Soon, Eddie Wisse, Australian Key Centre for Microscopy and Microanalysis, Electron Microscopy Unit, The University of Sydney, NSW 2006, Australia
Keissuke Nagatsuma, Department of Pathology, The Jikei University School of Medicine, Tokyo, Japan
Masaya Saito, Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan
Tomokazu Matsuura, Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo, Japan
Correspondence to: A/Professor Filip Braet, Australian Key Centre for Microscopy and Microanalysis, Electron Microscope Unit, Madsen Building F09, University of Sydney, Sydney, NSW 2006, Australia. filip.bract@emu.usyd.edu.au
Telephone: +61-2-93517619 Fax: +61-2-93517682
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Abstract

Colorectal cancer (CRC) is a common malignant disease and the severe nature of cases in men and women who develop colorectal cancer makes this an important socio-economic health issue. Major challenges such as understanding and modeling colorectal cancer pathways rely on our understanding of simple models such as outlined in this paper. We discuss that the development of novel standardized approaches of multidimensional (correlative) biomolecular microscopy methods facilitates the collection of (sub) cellular tissue information in the early onset of colorectal liver metastasis and that this approach will be crucial in designing new effective strategies for CRC treatment. The application of X-ray micro-computed tomography and its potential in correlative imaging of the liver vasculature will be discussed.

Key words: Apoptosis; Australia; Correlative microscopy; Endothelial cells; Hepatic metastasis; Colorectal cancer; CC531; Gaps; Interferon gamma; Kupffer cells; Natural killer cells; Nitric oxide; Macrophages; Modeling; Phagocytosis; Plugging; Pit cells; Stellate cells; X-ray micro-computed tomography

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant disease, with the majority of deaths attributable to hepatic metastasis. In the Western world, it is the second cause of cancer death in women after breast cancer, and the third cause of cancer death in men after lung and prostate cancer, being responsible for about 492 000 deaths p.a. worldwide and 4500 deaths p.a. in Australia[1]. In addition, the statistical data reveal that the risk of developing CRC disproportionately strikes individuals in the age group 65 years and older, illustrating its health longevity impact on the ageing population. At the first diagnosis of CRC, 20% of the patients already have liver metastasis and 30% of the patients will develop metastasis afterwards. 80% of the patients who die of CRC have metastases in the liver and prognosis is generally poor[2].

It is obvious that once the tumour cells have immigrated to the liver, they cross the hepatic sinusoidal endothelial barrier and by the time liver metastases form, most steps in the metastatic cascade have been completed. Consequently, exploring the preceding stages of CRC metastasis, proliferation and new blood vessel formation as well as mechanisms to disturb cell survival are to date of main interest as they are largely unexplored. As discussed in the following sections, the availability of new reconstructing and modeling techniques provide liver cancer biologists with an invaluable tool to bridge the gap between bench science and the development of potential novel liver CRC (immuno) therapeutic strategies.

COLORECTAL CANCER AND THE HEPATIC SINUSOIDAL IMMUNE SYSTEM

When the tumour cells invade the vascular bed and metastasise to the liver, they encounter the liver specific immune defence mechanisms. This hepatic sinusoidal immune system involves the hepatic-specific natural killer cells (NK) (pit cells)[3], Kupffer cells (KC) (liver-associated macrophages)[4] and hepatic endothelial cells (HEC)[5], and is proven to play an important role in protecting the liver from invading colon carcinoma cells[6]. The conventional paradigm of CRC liver metastasis is based on a multi-step process characterized by a series of structural, cellular and molecular events, which give the tumour cells the ability to proceed through the many phases of liver metastasis. Based on literature survey the following common sequence of key-events within the liver sinusoids are involved in the

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Susceptibility to colon carcinogenesis in C3H↔C57BL/6 chimeric mice reflects both tissue microenvironment and genotype

Tetsuya Tsukamoto[a,*], Masami Yamamoto[a], Hiroko Fukami[a], Akemi Yoshikawa[a], Hiroki Sakai[a], Akihiro Hirata[a], Moriaki Kusakabe[b,c], Masae Tatematsu[a]

[a]*Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1, Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan*
[b]*Experimental Animal Research Center, Institute for Animal Reproduction, 1103, Fukaya, Kasumigaura, Ibaraki 300-0134, Japan*
[c]*Department of Anatomy, School of Medicine, Jikei University, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan*

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Abstract

Considerable rodent strain differences have been documented with regard to susceptibility to colon carcinogens. To clarify mechanisms, chimeras of susceptible strain C3H and relatively resistant strain C57BL/6N (B6) mice were exposed to a colonotropic carcinogen, 1,2-dimethylhydrazine (DMH) and tumor incidence and multiplicity were assessed. In the chimeras, incidence was as high as the C3H level. Multiplicity of lesions of B6 cells was also increased ($P<0.001$), but maintenance of the strain difference. When tumor localization was analyzed, tumors of B6 genotype in chimeras demonstrated a greater spread of distribution than in the parental case. The chimeric environment may thus stimulate tumor initiation but cell autonomous suppressive factors may be retained.

Keywords: Chimeric mouse; Colon carcinogenesis; Susceptibility; 1,2-dimethylhydrazine; Rodent strain differences

1. Introduction

Colorectal cancer is a leading cause of cancer death in Western world and has recently demonstrated sharp increase in Eastern Europe and Japan [1]. Recent developments in genetic analysis have increased our understanding of the roles of genetic factors and animal models are now available to generate precise information. Inbred mice lines show various susceptibilities to the colon carcinogen, 1,2-dimethylhydrazine (DMH) [2,3], high yields of tumors being observed in SWR/J, P/J, C3H, BALB/c, and ICR animals, whereas AKR/J, DBA/2, and C57BL/6 are classified as resistant [2–4]. Factors controlling the susceptibility may include cell autonomous genetic determinants, the colonic microenvironment, and systemic parameters, including systems for activation and detoxification of carcinogens. To elucidate the roles of individual

* Corresponding author. Tel.: +81 52 762 6111x7062; fax: +81 52 764 2972.
E-mail address: ttsukamt@aichi-cc.jp (T. Tsukamoto).

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RESEARCH ARTICLE

Fates of Cdh23/CDH23 With Mutations Affecting the Cytoplasmic Region

Satoshi Yonezawa,[1]* Norio Yoshizaki,[2] Takashi Kageyama,[3] Takayuki Takahashi,[4] Mamoru Sano,[5] Yoshihito Tokita,[1] Shigeo Masaki,[1] Yutaka Inaguma,[1] Atsuko Hanai,[1] Nobuhiko Sakurai,[6] Atsushi Yoshiki,[7] Moriaki Kusakabe,[8,9] Akihiko Moriyama,[6] and Atsuo Nakayama[1]

[1]*Institute for Developmental Research, Aichi Human Service Center, Kasugai, Japan;* [2]*Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan;* [3]*Institute of Primate Research, Kyoto University, Inuyama, Japan;* [4]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Japan;* [5]*Department of Biology, Faculty of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan;* [6]*Graduate School of Natural Sciences, Nagoya City University, Nagoya, Japan;* [7]*Experimental Animal Division, BioResource Center, RIKEN Tsukuba Institute, Tsukuba, Japan;* [8]*ANB Tsukuba Institute, ALOKA Co. Ltd. and Institute for Animal Reproduction, Kasumigaura, Japan;* [9]*Department of Anatomy, Jikei University School of Medicine, Tokyo, Japan*

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BUS/Idr mice carrying a mutant *waltzer* allele (v^{bus}) are characterized by splayed hair bundles in inner ear sensory cells, providing a mouse homolog of USH1D/DFNB12. RT-PCR-based screening for the presence of mutations in mouse *Cdh23*, the gene responsible for the *waltzer* phenotype, has identified a G > A mutation in the donor splice site of intron 67 (Cdh23:c.9633+1G > A: GenBank AF308939.1), indicating that two altered Cdh23 molecules having intron-derived COOH-terminal structures could be generated in BUS mouse tissues. Immunochemical analyses with anti-Cdh23 antibodies showed, however, no clear Cdh23-related proteins in v^{bus}/v^{bus} tissues, while the antibodies immunoreacted with ~350 kDa proteins in control mice. Immunofluorescent experiments revealed considerable weakening of Cdh23 signals in sensory hair cell stereocilia and Reissner's membrane in the v^{bus}/v^{bus} inner ear, and transmission electron microscopy demonstrated abundant autophagosome/autolysosome vesicles, suggesting aberrant Cdh23:c.9633+1G > A-derived protein-induced acceleration of lysosomal bulk degradation of proteins. In transfection experiments, signal sequence-preceded FLAG-tagged transmembrane plus cytoplasmic regions (TMCy) of tissue-specific Cdh23(±68) isoforms were localized to filamentous actin-rich protrusions and the plasma membrane of cultured cells, whereas FLAG-TMCy:c.9633+1G > A proteins were highly insoluble and retained in the cytoplasm. In contrast, FLAG-tagged TMCy:p.Arg3175His and human TMCy:c.9625_9626insC forms were both localized to the plasma membrane in cultured cells, allowing prediction that USH1D-associated CDH23:p.Arg3175His and CDH23:c.9625_9626insC proteins could be transported to the plasma membrane in vivo. The present results thus suggest different fates of CDH23/Cdh23 with mutations affecting the cytoplasmic region. Hum Mutat 27(1), 88–97, 2006. © 2005 Wiley-Liss, Inc.

KEY WORDS: cadherin 23; CDH23; Usher syndrome; USH1D; *waltzer* mouse; splicing variants; autophagy

INTRODUCTION

Our knowledge about normal and pathologic processes relevant to auditory function has been rapidly increasing through recent progress in identification of deafness genes [Holme and Steel, 1999; Steel and Kros, 2001; Müller and Littlewood-Evans, 2001; Frolenkov et al., 2004]. The Usher syndrome, the most frequent cause of recessive deafness, accompanied by blindness and occasional vestibular dysfunction, is clinically classified into USH1, USH2, and USH3 forms. USH1, the most severe, is genetically heterogeneous, with at least seven responsible loci, *USH1A-1G* [Petit, 2001; Ahmed et al., 2003; Keats and Savas, 2004]. The utility of deaf mouse models for identifying *USH1* genes is now apparent from research on shaker-1 [Gibson et al., 1995; Weil et al., 1995], waltzer [Di Palma et al., 2001a; Bolz et al., 2001], Ames waltzer [Alagramam et al., 2001a,b], deaf circler [Verpy et al., 2000; Johnson et al., 2003], and Jackson shaker [Kikkawa et al., 2003; Weil et al., 2003] strains. Analyses of a variety of alleles in these models can be expected to provide further valuable information about the molecular mechanisms underlying normal inner ear function and development, as well as genotype-phenotype relationships.

The BUS/Idr mouse carrying a *waltzer* allele (v^{bus}) is characterized by severely deranged stereocilia and a decreased number of hairlets in sensory cells of the inner ear [Yonezawa et al., 1996, 1999; Moriyama et al., 1997]. Alteration in cadherin

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*Correspondence to: S. Yonezawa, Department of Embryology, Institute for Developmental Research, Aichi Human Service Center, Kamiya-cho 713-8, Kasugai 480-0392, Japan.
E-mail: yonez@inst-hsc.jp

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Association of extracellular matrix metalloproteinase inducer in endometrial carcinoma with patient outcomes and clinicopathogenesis using monoclonal antibody 12C3

KAZU UEDA[2], KYOSUKE YAMADA[2], MITSUYOSHI URASHIMA[1], YOSHIO ISHIBASHI[3], MISAKO SHIRAI[4], TAKASHI NIKAIDO[4], HIROYUKI TAKAHASHI[4], AIKOU OKAMOTO[2], MISATO SAITO[2], MAKOTO YASUDA[2], KIYOSHI OHKAWA[5] and TADAO TANAKA[2]

[1]Division of Clinical Research and Development; Departments of [2]Obstetrics and Gynecology, [3]Surgery, [4]Pathology and [5]Biochemistry, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan

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Abstract. Extracellular matrix metalloproteinase inducer (EMMPRIN) is a member of the immunoglobulin superfamily of adhesion molecules and has a role in the activation of several matrix metalloproteinases (MMPs). We evaluated whether EMMPRIN expression is related to tumor progression and patient outcome in human endometrial carcinoma. Paraffin-embedded surgical tissue samples from 112 patients with endometrial carcinoma were stained with anti-EMMPRIN antibody (monoclonal antibody 12C3:MoAb 12C3) for immunohistochemical analysis. EMMPRIN protein was expressed in cancerous lesions with the incidence of 97.3% (109 of 112 cases), but not in normal lesions. The scores determined by the combination of intensity and pattern of EMMPRIN staining in cancer cells correlated significantly with various histopathological risk factors: advanced stage, P=0.001; poorly differentiated carcinoma, P<0.001; lymph node metastasis, P=0.002; and lymphatic vessel infiltration, P=0.027. More importantly, recurrence-free survival was shortened in patients with higher EMMPRIN scores (HR, 3.08; 95% CI, 1.32-7.19; P=0.01). These results suggest that measurement of EMMPRIN expression with simple immunohistochemical staining may enhance the understanding of the pathophysiology of endometrial carcinoma.

Introduction

Matrix metalloproteinases (MMPs) are endopeptidases that play critical roles in promoting tumor disease progression, including tumor angiogenesis. In many solid tumors, MMP expression could be attributed to tumor stromal cells and is partially regulated by tumor-stroma interactions by means of tumor cell-associated extracellular matrix metalloproteinase inducer (EMMPRIN) (1). The roles of EMMPRIN and MMPs in tumor invasiveness have been confirmed immunohistochemically in several types of cancer cells (2-4). Moreover, research on EMMPRIN in malignant disease has recently attracted attention, and the expression of EMMPRIN has been reported to correlate with clinical prognosis of patients with breast carcinoma (5,6), ovarian carcinoma (7) and other types of cancer (8-11).

The prognosis for endometrial carcinoma patients with early clinical stage and well-differentiated carcinoma is generally satisfactory, but advanced stage and/or poorly differentiated carcinoma is an aggressive tumor with a poor prognosis (12-14). It would be beneficial to elucidate the pathophysiology of endometrial carcinoma concerning tumor invasiveness and differentiation.

We have established a murine monoclonal antibody (MoAb) 12C3 (15) that specifically binds to EMMPRIN protein (8). In the current study, EMMPRIN protein-expression patterns in endometrial carcinoma were examined immunohistochemically using MoAb 12C3 to determine their relation to clinicopathologic findings and recurrence-free survival.

Materials and methods

Tumor specimens. The Jikei University School of Medicine Ethics Review Committee approved the study protocol. A total of 112 endometrial carcinoma operative specimens were retrospectively obtained at the Jikei University Hospital (Tokyo, Japan) between January 1998 and March 2003. Tumors were histologically classified according to the WHO international system and the clinical cancer staging and the histological grade were defined according to the International Federation of Gynecology and Obstetrics (Table I). All of the 112 cases underwent hysterectomy, bilateral salpingo-oophorectomy and lymphadenectomy (or pelvic lymph node sampling). No cases received chemotherapy, radiotherapy or hormone therapy before they underwent operation.

Correspondence to: Dr T. Tanaka, Department of Obstetrics and Gynecology, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan
E-mail: tanaka3520@jikei.ac.jp

Key words: EMMPRIN, endometrial carcinoma, monoclonal antibody, recurrence-free survival

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The Functional Interrelationship between Gap Junctions and Fenestrae in Endothelial Cells of the Liver Organoid

Masaya Saito · Tomokazu Matsuura · Keisuke Nagatsuma · Ken Tanaka · Haruka Maehashi · Keiko Shimizu · Yoshiaki Hataba · Fumitaka Kato · Isao Kashimori · Hisao Tajiri · Filip Braet

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Abstract Functional intact liver organoid can be reconstructed in a radial-flow bioreactor when human hepatocellular carcinoma (FLC-5), mouse immortalized sinusoidal endothelial M1 (SEC) and A7 (HSC) hepatic stellate cell lines are cocultured. The structural and functional characteristics of the reconstructed organoid closely resemble the *in vivo* liver situation. Previous liver organoid studies indicated that cell-to-cell communications might be an important factor for the functional and structural integrity of the reconstructed organoid, including the expression of fenestrae. Therefore, we examined the possible relationship between functional intact gap junctional intercellular communication (GJIC) and fenestrae dynamics in M1-SEC cells. The fine morphology of liver organoid was studied in the presence of (1) irsogladine maleate (IM), (2) oleamide and (3) oleamide followed by IM treatment. Fine ultrastructural changes were studied by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and compared with control liver organoid data. TEM revealed that oleamide affected the integrity of cell-to-cell contacts predominantly in FLC-5 hepatocytes. SEM observation showed the presence of fenestrae on M1-SEC cells; however, oleamide inhibited fenestrae expression on the surface of endothelial cells. Interestingly, fenestrae reappeared when IM was added after initial oleamide exposure. GJIC mediates the number of fenestrae in endothelial cells of the liver organoid.

Keywords Electron Microscopy · Defenestration · Fenestra · Fenestrae formation · Liver sieve · Pore · Porosity · Sinusoidal endothelial cell · Transendothelial channel · Transendothelial transport

M. Saito (✉) · T. Matsuura · K. Nagatsuma · K. Tanaka · H. Tajiri
Division of Gastroenterology and Hepatology,
Department of Internal Medicine,
Jikei University School of Medicine,
3-25-8 Nishi-shinbashi, Minato-ku
Tokyo 105-8461, Japan
e-mail: masayas@jikei.ac.jp

T. Matsuura
Department of Laboratory Medicine,
Jikei University School of Medicine, Tokyo, Japan

H. Maehashi · K. Shimizu
Department of Biochemistry I,
Jikei University School of Medicine, Tokyo, Japan

Y. Hataba
Integrated Imaging Research Support, Tokyo, Japan

F. Kato · I. Kashimori
Pharmacology Research Group, Discovery Research Laboratories, Nippon Shinyaku, Kyoto, Japan

F. Braet
Australian Key Centre for Microscopy and Microanalysis,
University of Sydney, Sydney, NSW 2006, Australia

Introduction

Previously, we reported that functional liver organoid was able to reassemble when human hepatocellular carcinoma cells (FLC-5), mouse immortalized sinusoidal endothelial cells (M1-SEC) and mouse immortalized hepatic stellate cells (A7 HSC) were cocultured in a radial-flow bioreactor (RFB). The intricate structural arrangement of the reconstructed liver organoid was proven to be successful as urea production in function of time could be measured as a biochemical functional marker in the supernatant. Furthermore, fenestrae were clearly detected as a structural indicator on the surface of M1-SEC cells (Saito et al.,

A Novel Method for Three-Dimensional Observation of the Vascular Networks in the Whole Mouse Brain

HISASHI HASHIMOTO,[1]* MORIAKI KUSAKABE,[2,3] AND HIROSHI ISHIKAWA[1]
[1]*Department of Anatomy, The Jikei University School of Medicine, Minatoku, Tokyo 105-8461, Japan*
[2]*Institute for Animal Reproduction, Experimental Animal Research Center, Ibaraki 300-0134, Japan*
[3]*Matrix Cell Research Institute, Inc, Ibaraki 300-0134, Japan*

KEY WORDS three-dimensional image; blood vessels; brain; mouse; paraffin

ABSTRACT A novel method for acquiring serial images suitable for three-dimensional reconstruction of vascular networks in the whole brain of mouse was developed. The brain infused with a White India ink-gelatin solution was fixed and embedded in paraffin containing Sudan Black B through xylene also containing Sudan Black B. Each sliced surface of the paraffin block was coated with liquid paraffin and its image was serially acquired. Coating with liquid paraffin extremely improved the quality of the image. The series of serial images was free of distortion and a three-dimensional image was reconstructed without the problem of the alignment and registration of adjacent images. The volume-rendered image indicated three-dimensional distribution of blood vessels in a whole brain. No ghost or shadow was observed on a volume-rendered image of the White India ink-gelatin infused brain. The *z*-axial resolution examined on the orthogonal sections reconstituted from serial images obtained at an interval of 5 μm showed no cross talk, indicating that the *z*-axial resolution was no larger than 5 μm. A proper understanding of the vascular system in a whole brain is indispensable to reveal the development of the vascular system in the brain of normal and genetically manipulated mouse and vascular alterations in pathological situation, such as stroke and neurodegenerative disease. Although simple and inexpensive, this method will provide fundamental information on the vascular system in a whole brain. *Microsc. Res. Tech. 71:51–59, 2008.*

INTRODUCTION

Three-dimensional observation of the biological object is effective and essential to a proper understanding its structure. The confocal laser scanning microscope (CLSM) has become popular in recent years and made it possible to observe three-dimensional structures of a thick object. A series of serial images is easily obtained by optical sectioning of the thick object with CLSM and is easily reconstructed into three-dimensional images. However, an application of confocal laser scanning microscopy has some week points, such as a limitation on the thickness of the specimen and low axial resolution.

Three-dimensional distribution of blood vessels in the brain of the mouse and the rat has widely studied on a thick section of the brain infused with Indian ink or fluorochrome-labeled gelatin, and on a corrosion cast of vessels in the brain (Coyle, 1975, 1978; Coyle and Jokelainen, 1982; Hashimoto et al., 1998b; Hashimoto et al., 1999; He et al., 2000; Koppel 1982; Rieke, 1987; Rieke et al., 1981; Scremin, 2004; Ward et al., 1990). However, manual manipulation of the focusing plan of a microscope is required to reveal the three-dimensional distribution of blood vessels in a thick section of the Indian ink infused brain. Scanning electron microscopy can produce two-dimensional images of the surface of the corrosion cast of blood vessels from various viewpoints and provide a three-dimensional appearance, though the superficial casts must be removed to observe the deeper vessels. The CLSM observation of a fluorochrome-labeled gelatin infused specimen can provide a series of serial images by optical sectioning and a real three-dimensional image of the blood vessels can be produced only in a limited depth. In the last decade, a microcomputer tomography (μCT) has been developed and applied to the observation of vascular networks (Bentley et al., 2002; Jorgensen et al., 1998; Krucker et al., 2006) and, furthermore, synchrotron radiation μCT was introduced (Plouraboué et al., 2004). The recent development and advances in synchrotron radiation μCT technologies enabled to observe the three-dimensional distribution of the vascular networks at increasing levels of resolution (Heinzer et al., 2006). However, the μCT is not generally available yet.

To make a three-dimensionally reconstructed image of a comparatively large specimen, such as a vascular networks in a whole mouse brain, serial sectioning may be the only way to be adopted. However, the problem of the distortion of a section and the difficulties in fitting the position of adjacent sections are inevitable to reconstruct a three-dimensional image from serial sections. In this study, we have tried to acquire serial

*Correspondence to: Hisashi Hashimoto, Department of Anatomy, The Jikei University School of Medicine, 3-25-8 Nishishinbashi, Minatoku, Tokyo 105-8461, Japan. E-mail: hashimoto.anat@jikei.ac.jp

H. Ishikawa is currently at the Laboratory of Regenerative Medical Science, The Nippon Dental University School of Life Dentistry at Tokyo, 1-9-20, Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan.

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Development of the Mucosal Vascular System in the Distal Colon of the Fetal Mouse

TAKAYASU ITO,[1] SATOSHI OHI,[1] TOSHIAKI TACHIBANA,[1] MASASHI TAKAHARA,[1] TETSUYA HIRABAYASHI,[1] HIROSHI ISHIKAWA,[1,2] MORIAKI KUSAKABE,[3,4,5] AND HISASHI HASHIMOTO[1]*

[1]Department of Anatomy, The Jikei University School of Medicine, Minato-ku, Tokyo, Japan

[2]Laboratory of Regenerative Medical Science, The Nippon Dental University School of Life Dentistry at Tokyo Chiyoda-ku, Tokyo, Japan

[3]Research Center for Food Safety, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

[4]Matrix Cell Research Institute, Inc., Ibaraki, Japan

[5]Institute for Animal Reproduction (IAR), Experimental Animal Research Center, Kasumigaura, Ibaraki, Japan

ABSTRACT

The formation of the crypt in the distal colon of the mouse was investigated in association with the development of vascular networks. For histological observation, 1-μm cross-sections were made from the distal colon of fetal mice in 13 to 18 days of gestation. Three-dimensional distributions of vascular networks in the organ were observed after perfusing fetuses with rhodamine isothiocyanate-labeled gelatin and immunostaining for laminin to examine the boundary between the epithelium and the mesenchyme. At 13 days of gestation, the distal colon and its epithelium formed a cylindrical tube and a loose primary plexus of vessels was built in the mesenchyme. In the distal colon of 15 days of gestation, the caudal portion began to form the crypt and the vascular plexus built up from a few layers was situated apart from the boundary between the epithelium and the mesenchyme. As the development proceeded, the formation of the crypt occurred in the caudorostral direction. The developing crypt advanced into the vascular plexus, so that a few vessels situated in the mesenchyme between crypts. As the crypt elongated, these vessels formed a small plexus situated perpendicular to the primary plexus, while the primary plexus became monolayered and loosened. The new plexus was composed of ascending vessels and traversing ones, but the regular honeycomb-like plexuses around openings of crypts have not established yet even in 18 days of gestation. The vascular system as well as the crypt in the distal colon will take further a few postnatal weeks to be completed. Anat Rec, 291:65–73, 2007.

Key words: mouse; distal colon; crypt; fetus; vascular networks; three-dimensional reconstruction

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*Correspondence to: Hisashi Hashimoto, Department of Anatomy, The Jikei University School of Medicine, 3-25-8 Nishishinbashi, Minato-ku, Tokyo, 105-8461, Japan. Fax: 81-3-3433-2065. E-mail: hashimoto.anat@jikei.ac.jp

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