Table III. *Clinicopathological factors and GNAS1 genotype in a Japanese population.*

Genotype	TT / CC	OR (95% CI)	TT / CT+CC	OR (95% CI)
Histology				
P	20/11	1.00 (referent)	20/39	1.00 (referent)
M	38/36	1.73 (0.73-4.12)	38/107	1.44 (0.75-2.76)
W	40/30	1.39 (0.58-3.36)	40/98	1.22 (0.64-2.36)
	p for trend=0.445		*p* for trend=0.550	
Stage				
A or B	57/51	1.00 (reference)	57/155	1.00 (referent)
C	29/14	0.53 (0.25-1.12)	29/56	0.72 (0.42-1.25)
D	14/15	1.16 (0.50-2.68)	14/38	1.06 (0.53-2.12)
	p for trend=0.175		*p* for trend=0.462	
GS				
8-10	14/6	1.00 (referent)	14/27	1.00 (referent)
7	42/36	2.05 (0.71-5.93)	42/107	1.27 (0.61-2.67)
6	12/8	1.60 (0.43-6.01)	12/35	1.44 (0.57-3.64)
<5	2/2	2.33 (0.26-20.67)	2/7	1.85 (0.34-10.13)
	p for trend= 0.578		*p* for trend=0.456	
PSA				
<4	4/1	1.00 (referent)	4/3	1.00 (referent)
4-10	17/16	3.85 (0.37-40.38)	17/55	3.87 (0.77-19.46)
10-20	13/9	2.85 (0.25-32.32)	13/32	2.96 (0.57-15.42)
20-50	5/5	4.08 (0.32-52.50)	5/15	3.62 (0.58-22.47)
50<	3/0	-	3/0	-
	p for trend=0.238		*p* for trend=0.048	

P: Poorly differentiated adenocarcinoma; M: moderately differentiated adenocarcinoma; W: well-differentiated adenocarcinoma; GS: Gleason score; PSA: prostatic-specific antigen; Stage: A (T_{1a-b}, N_0, M_0), B (T_{1c-2}, N_0, M_0), C (T_{3-4}, N_0, M_0), D ($T_{1-4}N_1M_{0-1}$ or $T_{1-4}N_{0-1}M_1$).

cAMP has been shown to induce a mitogenic response or increase *in vitro* invasiveness in LNCaP prostate cancer cells (31, 32). It has also been reported that persistent activation of Gsα-mediated signaling stimulates the proliferation of certain cell types and can contribute to invasive tumor development in humans (33), also changing the proliferative, invasive and tumorigenic properties of PC-3M prostate cancer cells (34, 35). In the clinic, progression of prostate cancer to a hormone-refractory state is a critical problem. The mechanisms are complex and include both the selection and outgrowth of pre-exisiting clones of androgen-independent cells as well as adaptive up-regulation of genes that predispose cancer cells to survive and grow after androgen ablation, but are still not completely understood. Kasbohm *et al.* demonstrated that the up-regulation of a subunit of heterotrimeric guanine nucleotide-binding Gs protein activated the AR in prostate cancer cells, synergizing with low concentration of androgen (36). Bagchi *et al.* detected cAMP-dependent protein kinase A (PKA) activation in both androgen-dependent (LNCaP and LAPC4) and androgen-independent (PC3M) prostate cancer cells and established generality for the pathway (37). They concluded that androgen might evoke a nongenomic signaling pathway to activate cAMP-dependent PKA which is needed for the genomic functioning of nuclear AR. Thus, persistant stimulation of $G_{s\alpha}$-mediated signaling could accelerate prostate tumor growth and the formation of metastases and offer a target for treatment. However, no correlations between clinicopathological factors and this polymorphism were detected in the present study. Further investigation of clinical data including relapse period and clinical outcome should however be performed.

The G_{12} subfamily of heterotrimeric G proteins has been of interest to cancer researchers because its members were found to promote the growth and oncogenic transformation of murine fibroblasts (38). The G_{12} subfamily has been shown to promote prostate and breast cancer cell invasion *in vitro* (39, 40) and were also reported to be regulators of lysophosphatidic acid-induced ovarian cancer cell migration *in vitro* (41). These studies suggested that G_{12} subfamily-induced Rho activation may be critical for cancer cell behavior. Further studies of other G protein subfamilies including these polymorphisms should be conducted.

In conclusion, the present investigation did not provide support for a contribution of the *GNAS1* polymorphism to the risk of prostate cancer in this Japanese population.

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総　説

組織病理学用の切片を動物細胞の培養担体に利用した先端研究

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Advanced research utilizing histopathological sections as a culture substratum of animal cells

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Variqus culture substrata reflecting the microenvironment of cells *in vivo* have been developed to regulate multicellular structure and function. The substratum made of tissue/organ sections for histopathology (TOSHI), conserving microarchitecture and components *in vivo*, possessed the potential for inducing cell behavior in response to its microenvironmental signaling cues. Taking advantage of the TOSHI-substratum, we recently succeeded in the following two studies. Mouse embryonic stem cells were efficiently differentiated into hepatocyte-like cells when cultured on the TOSHI-substratum prepared from regenerating mouse livers after carbon tetrachloride intoxication. Also, the time-course cell behavior of two different cell lines on various TOSHI-substrata prepared from rat mature organs was converted into a three-dimensional graph chart, i.e. a mathematical model. Therefore, we hope that the former could be applied to the preparation of tailor-made hepatocytes *in vitro* and the latter will contribute to investigate the unknown characteristics of cells and/or tissues, i.e. cellomics and/or histomics, respectively. This review also describes visions for the future application of TOSHI-substrata to medical and pharmaceutical researches.

【キーワード】
細胞挙動，創薬，再生医療，セロミクス，ヒストミクス

1．はじめに

生体内の組織を構成する細胞は，経時的に変化している組織特異的な微小環境からのシグナ

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ルを認識して増殖や分化をはじめとする細胞挙動を決定している。この微小環境は，「多細胞間の間隙を流れている細胞外液」と「接着依存性細胞の足場となる細胞外マトリックス」である。これまでに，この微小環境の役割を模倣して様々な培養担体が開発され，多くの細胞培養システムが誕生してきた。例えば，前者の役割を再現する目的で開発された限外濾過膜製ホロファイバー（中空糸）束から成る担体は，生理活性物質の生産などに有用な細胞の高密度大量培養を達成できる通液培養システムを創出した。また，後者の役割を再現するために開発された各種細胞外マトリックス成分を加工した担体，真皮や小腸粘膜より細胞を除去して調製した無細胞性組織の担体，あるいは生体親和性の人工材料を加工した担体は，組織の再構築や薬剤に対する細胞応答の解析などに有用な数々の三次元培養システムを創出してきた[1]。

本稿では，生体組織の微細構造と構成成分が部域特異的に残存している組織病理学用の切片に着眼して開発した切片担体と，その担体を利用した培養システムについて紹介する。さらに，切片担体に用いる組織と切片担体上で培養する細胞の組合せ方を工夫することで創薬あるいは再生医療などへの応用が期待できる研究構想についても言及する。

2．切片担体の開発

細胞挙動を制御する様々な培養担体が開発されてきたが，生体組織の複雑な構造と成分の双方を反映した培養担体は未開発であった[2-4]。そこで，組織病理学の分野では染色による形態観察を目的として日常的に作製される「組織を薄切した切片」に着眼した。何故なら，切片には生体組織の微細構造のみならず抗体や核酸プローブで検出されるように様々な生体分子が部域特異的に残存しているので，細胞の培養担体に応用することで切片に介在している部域特異的な微細構造や生体分子に依存したシグナルを培養細胞に伝達できるのではないかと考えた。このような観点から，切片を動物細胞の培養担体に活用する培養新技術を開発し，原著論文では新しい概念で作製した「組織病理学用の組織／器官切片（Tissue/Organ Sections for HIstopathology）」担体なのでTOSHI担体と命名した[5,6]。

動物組織を薄切した切片担体は，ヒトを含む様々な動物のあらゆる組織より作製できる。具体的には，先ず組織病理学の実験手技に従って凍結組織，パラフィン包埋組織，あるいは樹脂包埋組織をミクロトームで薄切した切片をスライドグラス上に伸展し乾燥する。次に，前二者については凍結包埋剤あるいはパラフィンを除去した後（樹脂切片は脱樹脂できないのでそのまま），細胞培養に利用するため抗生物質を添加したＰＢＳあるいは70%エタノール等の処理により滅菌してから細胞培養液で平衡化して培養担体として使用する。以上が切片担体を作製する概略であるが，生体組織由来の構造や成分を切片担体にどのように取り入れるかは目的次第でいろいろと工夫する必要がある。例えば，新鮮な組織は未固定のまま凍結するのかホルマリン等で固定するのか，あるいは培養担体として使用する組織切片に対してタンパク変性を目的として熱や紫外線などで物理学的処理を施すのか，脱細胞化や脱脂を目的として界面活性剤や有機溶媒などで化学的処理を施すのか，または特定の抗原の露出やブロッキングを目的として酵素や抗体などで生物学的処理を施すのか，等々である。このようにして作製した切片担体上に細胞を播種すると，培養細胞は組織切片に介在する部域特異的なシグナルを認識して経時的に細胞挙動が誘導されるので，目的に応じた解析を進めることができる（図１）。

3．切片担体を利用した培養システム

当初の研究では，ウシ胎盤の凍結組織より作製した切片担体上で，異なる４種類の細胞（ヒト絨毛癌細胞株であるBeWo細胞，ウシ肺動脈血管内皮細胞株であるCPAE細胞，正常ヒト新生児包皮皮膚線維芽細胞であるNHDF細胞，およびラット褐色細胞腫であるPC-12細胞）を培養した。その結果，胎児側胎盤領域ではBeWo細胞のスフェロ

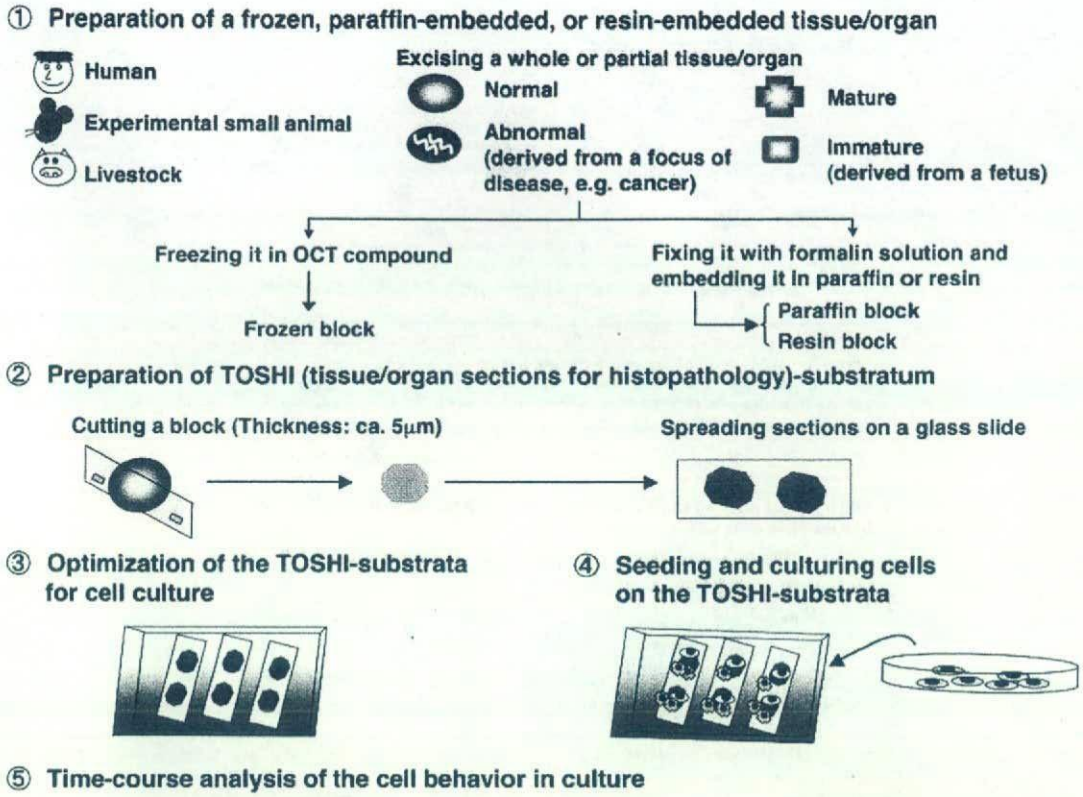

Fig. 1

Schematic procedure for preparing TOSHI (tissue/organ sections for histopathology) -substratum and culturing cells on it. Reproduced and partially-modified from Ref. 7 with permission of the publisher.

イド（多細胞性球状凝集塊），CPAE細胞の毛細血管網様構造，およびPC-12細胞の神経網様構造を形成する細胞の分化誘導が観察された。また切片担体には，無血清培養で誘導されるPC-12細胞のアポトーシスを阻害して細胞生存率を維持する活性があった。さらに，ドデシル硫酸ナトリウム（SDS）で脱細胞化処理した切片担体上にNHDF細胞を培養して，担体由来の細胞外マトリックス成分を巻き込んだ3次元組織を再構築する技術を確立した[5-8]。以上の結果は，1つの組織に由来する切片担体上で様々な細胞株の挙動を網羅的に解析するセロミクス研究が展開できることを示唆する[9]。

一方，四塩化炭素で軽度の肝障害を惹起した後の肝再生状態にあるマウスに，尾静脈よりマウス胚性幹細胞（以下，ES細胞）を投与すると，ES細胞は肝臓に移行した後に生着して肝細胞に分化することが報告されている[10]。そこで，この四塩化炭素を投与した後の障害あるいは再生の様々なステージにある肝組織より切片担体を作製した後，各切片担体上でES細胞を培養して接着，増殖および肝細胞への分化に関する細胞挙動を解析した。その結果，ES細胞の経時的な挙動は，切片担体に用いた肝組織の状態により異なることが分かった。具体的には，障害進行過程の肝組織より作製した切片担体上で培養したES細胞は丸い形態を示し，接着率と分化効率は共に低かった。これに対して，再生進行過程の肝組織より作製した切片担体上で培養したES細胞は敷石状に伸展し，接着率が高かった。特に，再生活性の強い四

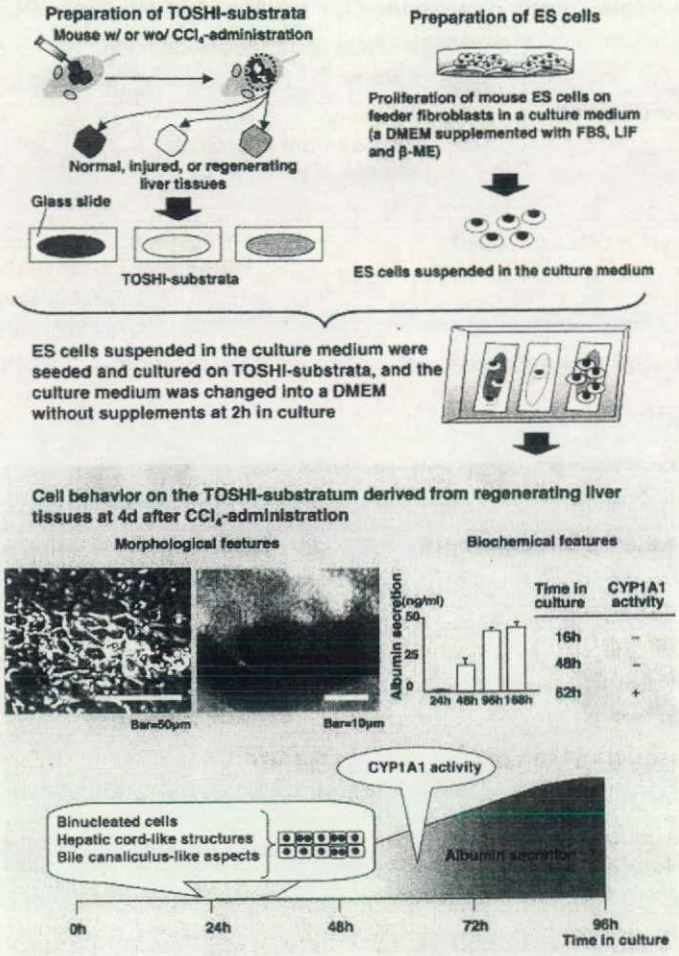

Fig. 2

Outline of the experiment for analyzing cell behavior of mouse embryonic stem (ES) cells cultured on the TOSHI-substrata derived from normal, injured, or regenerating mouse liver tissues.

塩化炭素投与後４日目の切片担体上では，培養24時間目までに約４倍に増殖する細胞集団が認められ，索状構造を形成した細胞集団や二核細胞も存在した。また，この４日目の切片担体上では，培養24時間目までに約70%の接着細胞がアルブミンを発現し，培養48時間目には培養液中へのアルブミン分泌，さらに培養62時間目にはCYP１A１活性も確認された（図２）。つまり，再生過程の肝組織より作製した切片担体を利用することで，ES細胞を短時間で効率よく肝細胞様細胞へ分化誘導できることが明らかとなった[7,8,11]。以上の結果は，様々な組織に由来する切片担体上で１つの細胞株の挙動を網羅的に解析するヒストミクス研究が展開できることを示唆する[9]。

上述の研究成果をもとに，切片担体と培養細胞の組み合わせは多種多様であるので，将来的には相互作用の解析結果を集積したデータベースを構築することが重要になると考えた[9]。そこで，最近の研究では，切片－細胞間の相互作用データベースを構築する第一段階として，数種類の臓器よ

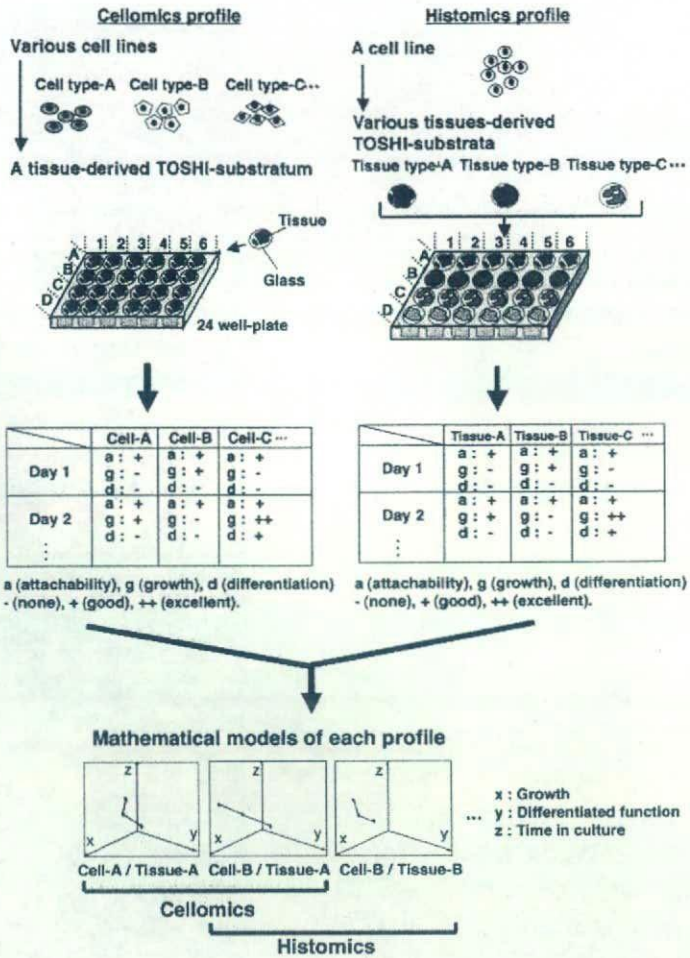

Fig. 3

Novel research concept for cellomics and histomics. Reproduced and partially-modified from Ref. 7 with permission of the publisher.

り作製した切片担体上で異なる2種類の細胞の挙動を解析した後に，解析データの数理モデル化を試みた。具体的には，ラットより摘出した各臓器（大脳，胸腺，心臓，腎臓，精巣，肝臓，など）より切片担体を作製した後，ラットインスリノーマ細胞株であるRIN5F細胞およびヒト肝癌細胞株であるHepG2細胞を培養した。それぞれの切片担体と培養細胞の組み合わせごとに経時的な細胞増殖および分化機能（培養液中に分泌されるインスリンまたはアルブミン量）を解析し，得られたデータを3次元グラフとして表記した。その結果，数理モデル化した細胞挙動のプロファイルは，細胞が同じでも切片に用いた組織に依存して異なること，また切片に用いた組織が同じでも細胞に依存して異なることを実証した（投稿論文準備中）。このことは，1つの組織に由来する切片担体を利用して異なる多数の細胞株を網羅的に解析したセロミクスデータと，1つの細胞株を利用して異なる多数の組織に由来する切片担体を網羅的に解析したヒストミクスデータの数理モデル化が可能であることを示唆する（図3）。今後，組織切片と細胞の相互作用プロファイルを集積したデータベ

ースが構築できれば，「特性を診断したい細胞と特性既知の組織切片」または「特性を診断したい組織切片と特性既知の細胞」を組み合せた培養から得られる細胞挙動プロファイルをデータベースへフィードバックすることで新しい診断システムが創出できると考えている[7-9]。

また，最近，ヒト前立腺がん細胞株であるDU145細胞をラット各種臓器より調製した切片担体上で培養して挙動変化を観察する実験を開始した。その結果，切片担体に依存して細胞の接着増殖性が異なるのみならず，細胞形態も大きく変化することが分かってきた（図4）。

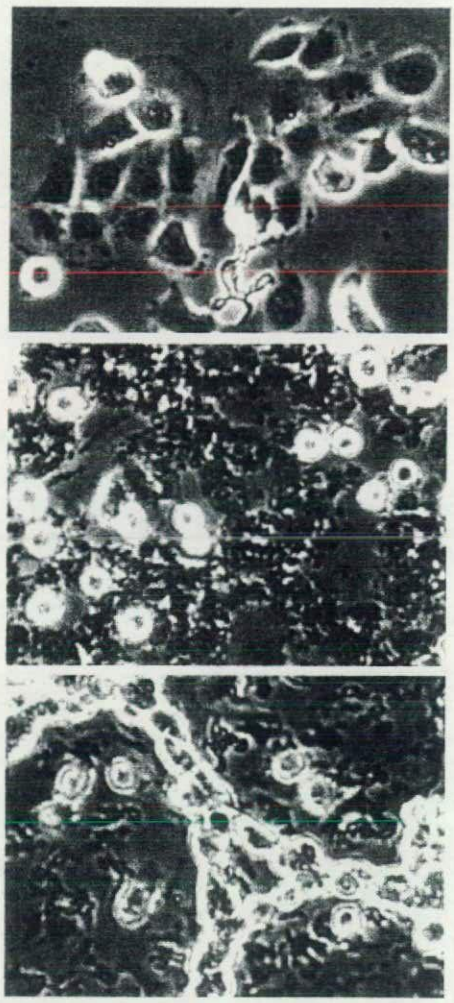

Fig. 4

Phase-contrast microphotographs of DU145 cells cultured for 2days on plastic (a), TOSHI-substrata derived from rat cerebellum (b) and kidney (C).

4．創薬あるいは再生医療などへの応用構想

「動物組織を薄切した切片担体」は，培養細胞と切片担体の組み合わせ方を工夫することで，生命科学の基盤研究のみならず創薬や再生医療への応用研究が展開できると考えている（図5）。

創薬への応用研究としては，生理活性物質の探索，あるいは薬効および毒性の評価などが展開できると考えている。生理活性物質の探索では，特定の細胞が切片担体の特定部域で接着，増殖（阻害），分化などの細胞応答特性を示す培養モデルの特徴を利用して，特定部域からリガンド，増殖（阻害）因子，分化誘導因子などの生理活性物質を単離する研究が展開できる。また，薬効および毒性の評価では，動物実験代替法として有用な培養モデルを以下のように構築できる。通常用いる切片担体は厚みが5μmなので，厚みが10mmの組織であれば2,000枚の切片担体を作製することができる。また，実験動物一個体に化学物質を投与すれば，化学物質の薬効あるいは毒性を反映した切片担体は標的器官のみならず全身の諸器官からも作製することができる。従って，実験動物一個体を有効に活用して多数の切片担体を作製し，切片担体に介在する薬効あるいは毒性を様々な培養細胞の挙動で解析する新しい組織培養システムの構築が期待できる。ひいては特定の化学物質を投与した同じ実験動物に由来する切片担体を，複数の異なる研究機関でバリデーションに利用することも期待できる。さらに，切片担体は実験動物のみならずヒトの様々な生検材料からも作製できる。つまり，切片担体を利用した薬効および毒性の試験モデルは，実験動物の削減，あるいは置き換えの観点から有用な代替法になると考えられる[4,7]。

また，再生医療への応用研究としては，オーダーメイド成熟細胞の調製や自家移植用組織の再構

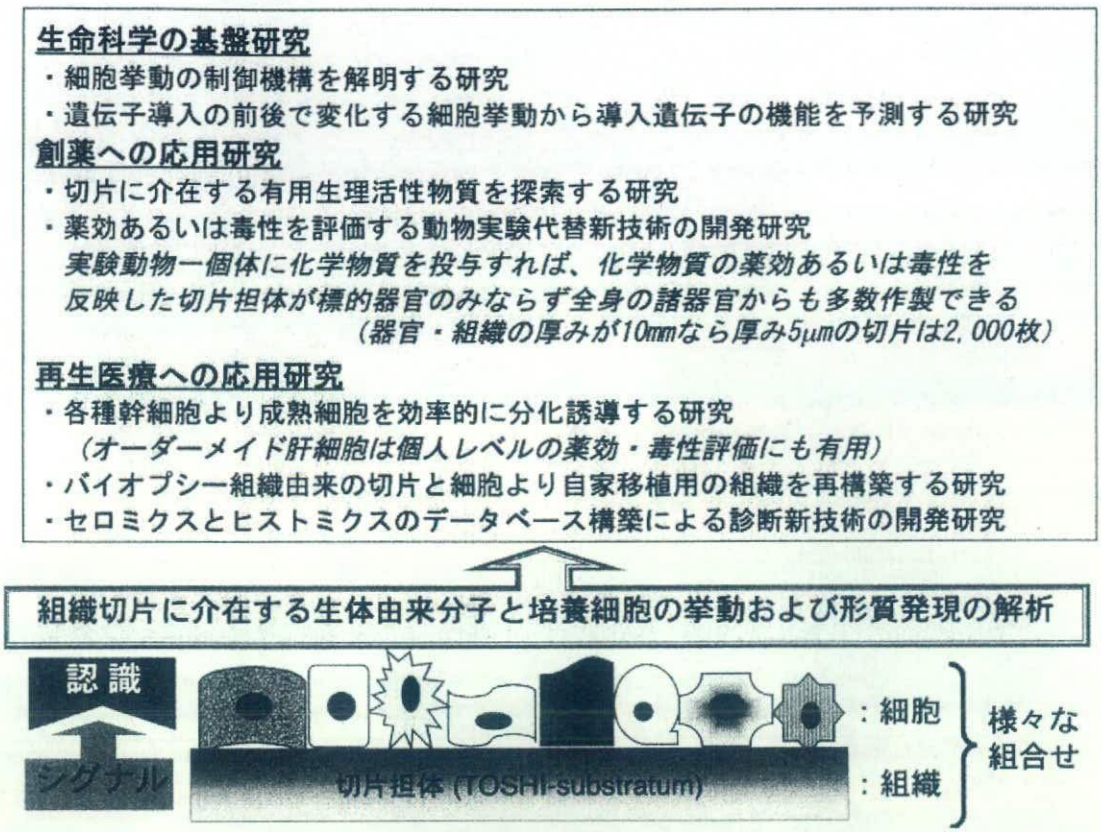

Fig. 5

Advantages of the culture system utilizing TOSHI-substratum.

築などに展開できると考えている。その他，上述のセロミクスおよびヒストミクスのデータベースを構築する研究が発展すれば，患者より分離した細胞や組織を診断する全く新しい技術を創出できると考えている[6,8,9]。

5．おわりに

創薬あるいは再生医療の研究発展には，今後どのような培養システムの開発が重要となってくるのであろうか。例えば，細胞挙動の制御，生理活性物質の探索，あるいは動物実験の代替に有用な培養システムの開発ではないかと考えている。具体的には，幹細胞の分化系譜を制御するとともに安全性を評価できるような培養システム，今までに注目されていなかった資源の宝庫から生理活性物質を探索できるような培養システム，あるいは薬効・毒性を評価するための動物実験を削減できるような培養システムである。このような培養システムの開発には，本稿で紹介した切片担体の活用構想が役立つと期待している。

謝辞

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Application of a New Bioassay Technique Using Goldfish for Assessment of Water Toxicity

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ABSTRACT: There are a variety of chemicals in aquatic environment, so it is important to assess the toxicity. The biomarkers such as induction of DNA damage, micronuclei, vitellogenin, and hepatic P450 in fish are known to be effective for monitoring genotoxic and/or estrogenic chemicals. However, there is little study to use these biomarkers in same fish. Goldfish (*Carassius auratus*) is widely used and is suitable in size to collect blood or organs. In this study, validity of multiple-biomarkers in goldfish was checked using standard chemicals and applied in the river water. Ho River, which flows through the textile dyeing factory in Shizuoka Prefecture, Japan, was reported to show genotoxicity toward *Salmonella typhimurium* TA98 and YG1024. When the goldfish were exposed to Ho River, DNA damage, estrogenic activity, and CYP1A induction were observed. Through the study, it was assumed that not only mutagens/carcinogens but also endocrine disrupting chemicals and poly aromatic hydrocarbons were present in Ho River. Therefore, chemical identification should be required. We could evaluate both genotoxicity and estrogenic activity simultaneously, so goldfish might be a good experimental model for estimation of chemical contamination levels in aquatic environment. © 2008 Wiley Periodicals, Inc. Environ Toxicol 23: 720–727, 2008.

Keywords: goldfish; genotoxicity; estrogenic activity; P450 enzyme activity; biomonitoring

INTRODUCTION

Many kinds of chemicals showing mutagenic/carcinogenic potency are present in aquatic environment. Ohe et al. (2004) reviewed that 7–15% of river water around

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the world showed genotoxicity toward *Salmonella typhimurium* TA98 and TA100 strain which detect frameshift point mutations and base substitution, respectively. Genotoxicity studies of river water have employed the *Salmonella*/genotoxicity test mainly, but it is important to evaluate the river water using aquatic animals. Micronucleus (MN) test has been used in genotoxicological study with fish (Al-Sabti and Metcalfe, 1995; Hayashi et al., 1998), and it detects MN resulting from either chromosomal breakage during cell division or chromosome loss events in anaphase damages (Kirsch-Volders et al., 2003). MN test is often applied with comet assay. Comet assay detects DNA damage including single-strand breakage or other lesions such as alkali-labile sites and DNA cross-link by measuring the migration of DNA from immobilized nuclear DNA (Singh et al., 1988). Comet assay has been widely used for studies on genotoxicity of environmental chemicals with fish (Lee and Steinert, 2003). Induction of hepatic cytochrome P450 enzymes in fish is also known to be sensitive biomarkers for monitoring polychlorinated biphenyl (PCB) or poly aromatic hydrocarbons (PAHs) (Ahokas et al., 1976). The determination of ethoxy resorufin-*O*-dealkylase (EROD) activity in fish has been used as an indicator of PAHs (Wassenberg et al., 2005). Methoxy resorufin-*O*-dealkylase (MROD), penthoxy resorufin-*O*-dealkylase (PROD), and bezyloxy resorufin-*O*-dealkylase (BROD) activities are known, and Russell et al. (2004) evaluated PCB using these enzyme activities.

It is well known that not only mutagens/carcinogens but also endocrine disrupting chemicals (EDCs) are present in aquatic environment (Whaley et al., 2001). Vitellogenin (VTG) is known to be an effective biomarker for monitoring EDCs (Li et al., 2005). VTG is a protein synthesized in female liver and delivered into blood, then taken up by oocytes to be processed into egg yolk. However, VTG production can be induced in males by administration of EDCs. So VTG has been used as an indicator of EDCs. They are known to be effective and sensitive biomarkers, but there is little study to determine genotoxicity and estrogenic activity simultaneously using fish. Among all kinds of aquatic organisms, goldfish (*Carassius auratus*) is suitable in size to collect any organs. We thought that goldfish is a good model for evaluation of aquatic environment. In this study, we checked the validity of multiple-biomarkers in goldfish using standard chemicals and applied the river water. Recently, Watanabe et al. (2006) reported that Ho River, which flows through the textile dyeing factory in Shizuoka Prefecture, showed genotoxicity toward TA98 and YG1024 (*O*-acetyltransferase overexpressing strain) with metabolic activation (+S9 mix). So we evaluate the toxicity of Ho River not only genotoxicity but also estrogenic activity and P450 enzyme activity using goldfish.

MATERIALS AND METHODS

Chemicals

β-naphthoflavone (β-NF), low-melting point agarose, *O*-phenylenediamine, 7-ethoxyresorufin, 7-methoxyresorufin, 7-penthoxyresorufin, 7-benzyloxyresorufin, and resorufin were purchased from SIGMA (St. Louis, MO). HRP-conjugated-anti-rabbit IgG goat antibody was purchased from Seikagaku Corporation (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Fish

We obtained goldfish weighing 20–25 g from a local dealer in Hamamatsu City, Japan. Before the experiments, goldfish were acclimatized for 2 weeks in a well-aerated aquarium maintaining at 20°C ± 2°C under 12 h light/12 h dark photoperiod. For estrogenic activity assay, a low estrogenic diet which does not contain any soybean meal was developed with Kyorin Corporation (Himeji, Japan).

Genotoxicity Assay

Genotoxicity was assessed by comet assay and MN test according to Deguchi et al. (2007). In both assay, methyl methanesulfonate (MMS) and mitomycin C (MMC) were used as positive control. MMS and MMC were dissolved in dimethylsulfoxide (DMSO) and injected intraperitoneally (i.p.) once at the following doses: 10, 50 mg/kg body weight for MMS and 2, 4 mg/kg body weight for MMC. In the negative control group, DMSO was injected i.p. into fish. Five fish were used in each group, and kept in a 10-L glass tank. In comet assay, blood was collected 3, 6, and 24 h after injection. The comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with CCD camera. Two slides were prepared for each fish, and 50 cells per slide were examined. The tail moment of DNA was measured by using Komet 4.0 image analyzed program (Kinetic Imaging, Liverpool, UK) or Comet Analyzer (Youworks, Tokyo, Japan). In MN test, blood was collected 48, 96, and 144 h after injection. Two slides were prepared for each fish. At least 1000 erythrocytes per slide were observed by a fluorescence microscope (magnification 400×), and the number of micronucleated cells were recorded. MN frequency was calculated as the number of micronucleated cells per 1000 cells.

Estrogenic Activity Assay

Male goldfish were exposed to 10-L that contained 1, 10, 100 μg/L of 17β-estradiol (E2), bisphenol A (BPA), and 4-nonylphenol (NP) in glass tank in static condition. Negative control group was only exposed to solvent carrier (DMSO

100 μL/L). Five fish were used in each group, and test solutions were exchanged every day. Blood was collected after 9 days and centrifuged at 3000 rpm for 20 min then the plasma was divided into aliquots and stored at −20°C until use. The concentrations of VTG in plasma were determined by enzyme-linked immunosorbent assay (ELISA) using monoclonal mouse antibody against goldfish lipovitellin (Lv). The goldfish VTG and Lv were purified from sexually mature female goldfish plasma and oocyte, respectively, as described by Hara et al. (1993). Purified VTG was used as a standard, and diluted samples were used for measurement of VTG in duplicate. Visualization was accomplished using HRP-conjugated-anti-rabbit IgG goat antibody and *O*-phenylenediamine as a HRP substrate. After 10 min, the reaction was stopped by adding 2 M H_2SO_4 and absorbance was measured with a microplate reader (Thermo Fisher Scientific, MA) at 492 nm. These assays were performed at room temperature. The concentrations of VTG were calculated from the linear part of the log-transformed standard curve. The detection limit of VTG in present study was 0.02 μg/mL.

P450 Enzyme Activity Assay

P450 enzyme activity was measured using β-NF and phenobarbital (PB) as positive control. β-NF and PB were dissolved in DMSO and injected i.p. once at the following doses: 1, 10 mg/kg body weight for β-NF and 50, 100 mg/kg body weight for PB. In the negative control group, DMSO was injected i.p. into fish. Five fish were used in each group, and kept in a 10-L glass tank. Liver was collected 24, 48, and 96 h after injection and rinsed in 1.15% KCl then homogenized with three volumes of homogenization buffer (100 mM Tris-HCl containing 1 mM EDTA-2Na, 100 mM KCl, pH 7.4) in a Potter-Elvehjem homogenizer, then centrifuged at 9000 × *g* for 20 min at 4°C. The supernatant was ultracentrifuged at 105 000 × *g* for 60 min at 4°C. The microsome pellet was resuspended and homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA-2Na and 20% glycerol. Microsome was divided into aliquots and stored at −80°C until use. The protein content was determined according to BCA protein assay (PIERCE, USA) using bovine serum albumin as a standard. EROD, MROD, PROD, and BROD activities of liver microsome were measured using the method described by Burke et al. (1994) with some modifications. Microsome (50 μg) and 25 μL of NADPH generation system (100 mM $MgCl_2$, 10 mM NADP, 100 mM G-6-P, and 1000 U/mL G-6-P DH) were preincubated for 5 min at 30°C (total volume: 200 μL), and then the 50 μL of each substrates (25 μM of 7-ethoxyresorufin, 25 μM of 7-methoxyresorufin, 25 μM of 7-pethoxyresorufin, or 25 μM of 7-benzyloxyresorufin) were added to start the reaction. After 5 min, the reaction was stopped by adding 250 μL of ice-cold ethanol. The mixture was centrifuged at 15 000 rpm for 3 min at 4°C. The fluorescence of the supernatant was measured on a fluorescence microplate reader (Thermo Fisher Scientific, MA) with an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each enzyme activities were expressed as the rate of resorufin formation, and were calculated based on the fluorescence of a standard curve of resorufin standards.

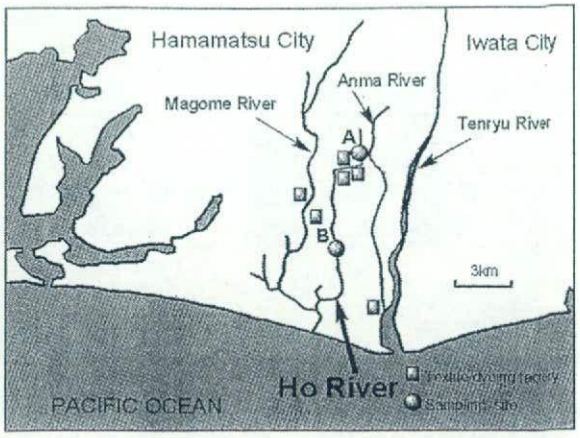

Fig. 1. Geographic locations of the sampling points, the textile dyeing factories, and the Ho River in Shizuoka Prefecture.

Exposure to River Water

We collected river water from upstream (point A) and downstream (point B). The sampling points were shown in Figure 1. At first, temperature, pH, and COD_{Mn} of Ho River were measured. Male goldfish were exposed to 10-L river water in glass tank in static condition. Negative control group was tap water. Five fish were used in each group, and a half volume of the river water was exchanged with same river water every day. After exposure for 1 and 2 weeks, the goldfish were removed from each tank and we examined genotoxicity, estrogenic activity, and P450 enzyme activity.

Statistical Analysis

Dunnett's test after one-way ANOVA was used to evaluate the significance of the differences in each test between the control group and the treated group; *p* values lower than 0.05 were considered to indicate statistical significance.

RESULTS

Genotoxicity

DNA damage and MN induction were evaluated using MMS and MMC. Figure 2 showed the mean values of

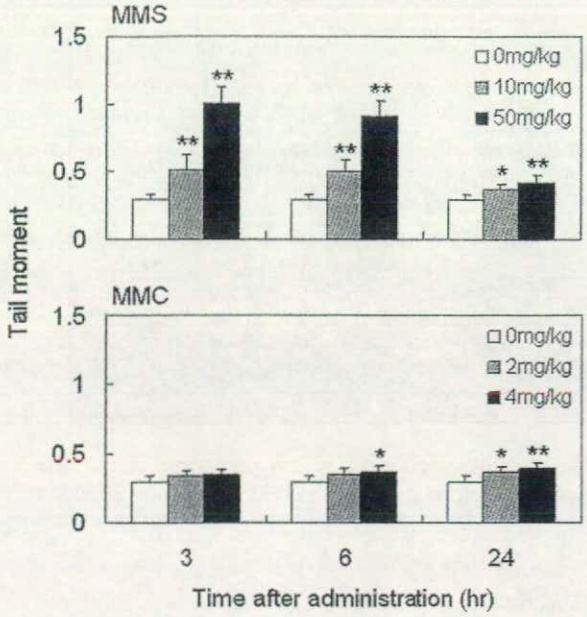

Fig. 2. DNA damage in peripheral blood cells of goldfish administered MMS (upper graph) and MMC (lower graph). One hundred cells were counted per fish. The mean values were obtained from 500 cells. The bars represented the SEM values. Tail moment = DNA migration × Tail intensity (using Komet 4.0 image analyzed program). Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 mg/kg).

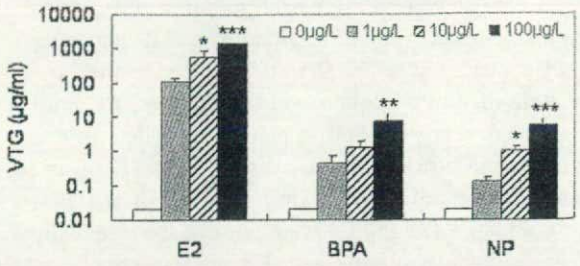

Fig. 4. Induction of VTG in plasma of male goldfish exposed to E2, BPA, and NP for 9 days. The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 g/L).

DNA tail moment in blood. MMS caused stronger DNA damage compared to MMC. In MMS, DNA damage was increased until 3 or 6 h after injection and then decreased. In MMC, DNA damage was not observed at 3 h after injection, but then showed weak DNA damage after 24 h. Figure 3 showed the frequencies of MN in blood. MMC caused stronger chromosomal aberration compared to MMS, and the frequencies of MN significantly increased until 96 h after injection and then decreased. The average of frequencies for 48, 96, and 144 h (MMC 4 mg/kg) were 1.18‰ (=12/10 204, $p < 0.05$), 2.79‰ (=28/10 028, $p < 0.01$), and 1.18‰ (=12/10 186, $p < 0.05$), respectively.

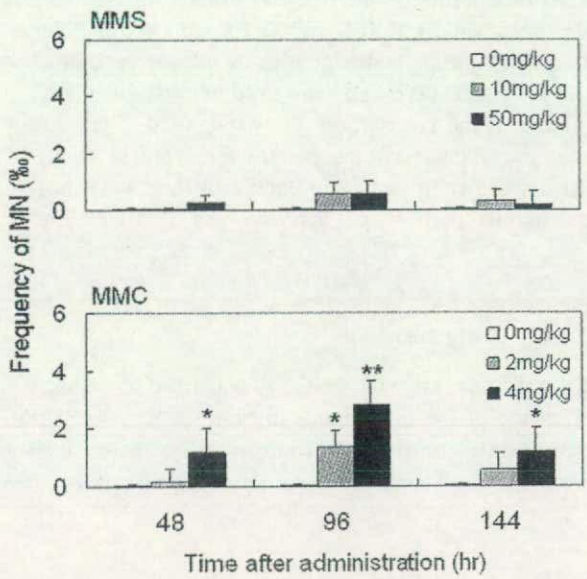

Fig. 3. The frequency of MN in peripheral blood cells of goldfish administered MMS (upper graph) and MMC (lower graph). The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 mg/kg).

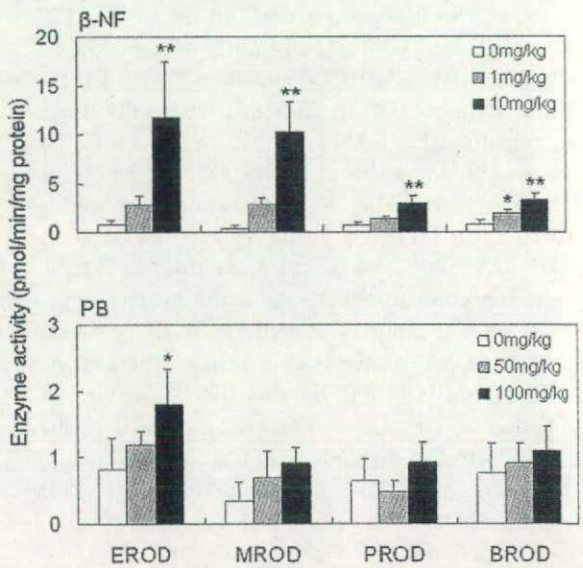

Fig. 5. Activity of four liver enzymes (EROD, MROD, PROD, and BROD) of goldfish administered β-NF (upper graph: after 48 h) and PB (lower graph: after 24 h). The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 mg/kg).

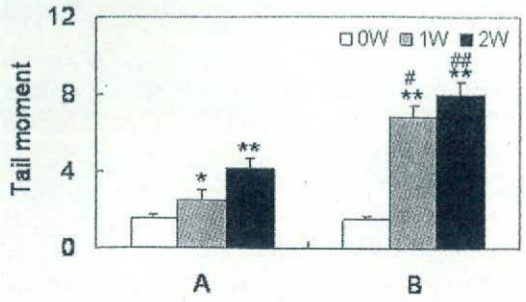

Fig. 6. DNA damage in peripheral blood cells of goldfish exposed to Ho River for 2 weeks. One hundred cells were counted per fish. The mean values were obtained from 500 cells. The bars represented the SEM values. Tail moment = Tail distance × Ratio (using Comet Analyzer). Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 W), $\#p < 0.05$ (vs. point A; 1 W), $\#\#p < 0.05$ (vs. point A; 2 W).

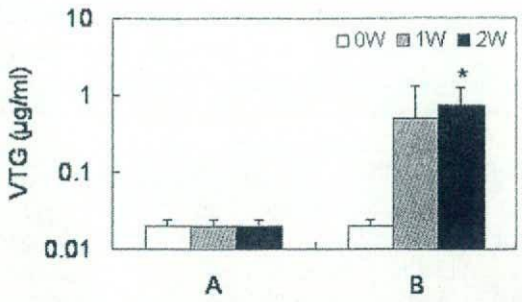

Fig. 7. Induction of VTG in plasma of male goldfish exposed to Ho River for 2 weeks. The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{*}p < 0.05$ (vs. control; 0 W).

Estrogenic Activity

VTG synthesis was evaluated using E2, BPA, and NP. Figure 4 showed the concentrations of plasma VTG in male goldfish exposed to E2, BPA, and NP for 9 days. VTG were significantly increased by treating with 10 μg/L of E2 and NP ($p < 0.05$), 100 μg/L of BPA ($p < 0.01$), and 100 μg/L of E2 and NP ($p < 0.001$).

P450 Enzyme Activity

EROD, MROD, PROD, and BROD activities in liver were measured at 24, 48, and 96 h after injection of β-NF and PB. Figure 5 showed the enzyme activities at 48 h after injection of β-NF and at 24 h after injection of PB. In β-NF, all activities were gradually increased until 48 h after injection, and then decrease at 96 h (data not shown). All activities were significantly increased by β-NF at 48 h after administration. In PB, only EROD activity was significantly increased at 24 h after injection, and then decrease. Other activities were not increased until 96 h (data not shown).

Evaluation of Ho River

The temperature of point A and point B were 22.2 and 23.5°C, respectively. The pH of point A and point B were 7.42 and 7.39, respectively. The COD_{Mn} of point A and point B were 4.95 and 13.86, respectively. Ten male goldfish were exposed to Ho River for 1 or 2 weeks. The survival ratio of goldfish was 100% in both the points. In comet assay, Ho River showed DNA damage in both the points, however DNA damage was stronger in point B than that of point A (Fig. 6). On the other hand, MN was not significantly increased in both the points (data not shown). The plasma VTG synthesis was not observed in point A, however the plasma VTG levels was significantly increased in point B (Fig. 7). Although all hepatic P450 activities were not significantly increased in point A, significant induction of MROD activity was found in point B (Fig. 8).

DISCUSSION

A lot of bioassay techniques using aquatic animals have been developed so far. We evaluated genotoxicity, estrogenic activity, and P450 enzyme activity using goldfish. In

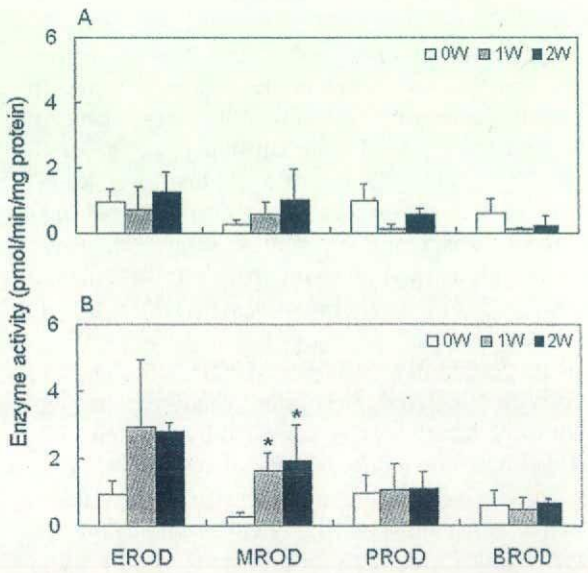

Fig. 8. Activity of four liver enzymes (EROD, MROD, PROD, and BROD) of goldfish exposed to point A (upper graph) and point B (lower graph) for 2 weeks. The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{*}p < 0.05$ (vs. control; 0 W).

comet assay and MN test, we used MMS (alkylating agent) and MMC (DNA crosslinker). In comet assay, MMS showed stronger DNA damage than that of MMC, but in MN test, MMC showed higher frequency of MN than that of MMS. The numerous biomarkers such as DNA strand breaks, DNA adducts, chromosomal aberrations, sister chromatid exchange, and MN were used to monitor mutagens/carcinogens in aquatic environment. Mitchelmore and Chipman (1998) commented that DNA strand breaks, particularly as measured by the comet assay, act as a biomarker of genotoxicity in fish and other aquatic species. They also emphasized that this approach should be combined with the use of other biomarkers. Thus, a combination of the comet assay and the MN test is a useful tool for monitoring mutagens/carcinogens.

Next, we measured VTG as a biomarker for estrogenic activity. Although VTG is regarded as female specific protein, male fish can also produce VTG in the liver if exposed to EDCs. Therefore the measurement of plasma VTG in male fish has been used as a sensitive biomarker of EDCs in aquatic environment. It is known that normal fish diets contain the phytoestrogens such as genistein, daidzein, equol, and coumestrol. Ishibashi et al. (2002) reported that VTG was detected by feeding normal fish diets, but VTG was not detected by feeding casein-based formulated fish diet which does not contain soybean and fish meal. So we developed a low estrogenic fish diet which does not contain soybean. The concentrations of VTG of male goldfish were decreased by feeding this fish diet (data not shown). When male goldfish were exposed to E2, BPA, and NP for 9 days, the concentrations of VTG were significantly increased by treating with 10 μg/L of E2 and NP ($p < 0.05$), 100 μg/L of BPA ($p < 0.01$), and 100 μg/L of E2 and NP ($p < 0.001$). The concentration of 1 μg/L was much higher than those detected in river water in Japan. In this study, VTG were detected by treating with 1 μg/L of each substance, so the measurement of plasma VTG in male goldfish was useful to monitor EDCs. Jobling et al. (1996) reported that VTG synthesis was found in rainbow trout (*Oncorhynchus mykiss*) after treating with 10 μg/L of NP. So goldfish seemed to have similar sensitivity to NP as rainbow trout.

We also determined EROD, MROD, PROD, and BROD activities in liver microsome. Many carcinogens are known to be metabolically activated by drug-metabolizing enzymes including CYP enzymes. CYP1A enzymes catalyzed PAHs and heterocyclic amine (HCA). CYP2B enzymes catalyzed the dichloro diphenyl trichloroethane (DDT) and PB. In this study, CYP1A and CYP2B activities were determined from EROD and MROD activities, PROD and BROD activities, respectively. EROD and MROD activities of goldfish were rose by β-NF (as a CYP1A inducer). In rats, EROD and MROD activities are related to CYP1A1 and CYP1A2, respectively. In fish, EROD activity is related to CYP1A1 (Stegeman et al., 1997), and Smeets et al. (2002) found a high correlation between EROD and MROD activities in rainbow trout (*Oncorhynchus mykiss*), dab (*Limanda limanda*), European flounder (*Platychthis flesus*), and lemon sole (*Microstomus kitt*). Therefore, the basic metabolic machinery in goldfish is similar to that of rats. PB was known to be a CYP2B inducer in rats, but neither PROD nor BROD activities rose when we administrated PB to goldfish. Sadar et al. (1996) reported that PROD activity did not rise by PB, but EROD activity rose in primary culture of rainbow trout hepatocytes. In this study, PROD activity did not rise, but EROD activity rose in goldfish. So it was strongly suggested that PB is metabolized by CYP1A in goldfish. On the other hand, Ruus et al. (2002) found high correlations between EROD and PROD activities in cod (*Gadus morhua*) and bullrout (*Myoxocephalus scorpius*). Like this, PROD and BROD activities in fish were unclear, so further investigations are required to elucidate these activities.

Through these studies, we could construct several biomarkers, such as genotoxicity, estrogenic activity, and P450 enzyme activity. So next, we applied these biomarkers to evaluate the river water. Recently, Watanabe et al. (2006) reported that Ho River, which flows through the textile dyeing factory in Shizuoka Prefecture, Japan, showed genotoxicity toward TA98 and YG1024 with S9 mix. We collected river water from upstream (point A) and downstream (point B) of textile dyeing factory, and we compared each point. It was assumed that mutagens/carcinogens, EDCs, and PAHs were existed in point B. Watanabe et al. (2006) also collected from same point and non-Cl phenylbenzotriazole (PBTA)-2, -3, and -7 were detected from this point. Several PBTAs were detected from river water in Japan (Morisawa et al., 2003; Ohe et al., 2006), and PBTAs were reported to show strong genotoxicity toward *Salmonella typhimurium* YG1024 in the presence of S9 mix (Ohe et al., 1999; Shiozawa et al., 2000; Nukaya et al., 2001; Watanabe et al., 2001, 2002). Masuda et al. (2004) reported that PBTA-6 showed genotoxicity toward goldfish. Although other PBTAs including non-Cl PBTAs were not tested *in vivo*, it was suggested that the non-Cl PBTAs might be contributed to their genotoxicity. Ho River also showed estrogenic activity and CYP1A induction. There is no data about PBTAs on VTG synthesis or P450 enzyme activity. So we must examine the effects of PBTAs on VTG synthesis and P450 enzyme activity in the future. Point A also showed DNA damage to goldfish. So we need to monitor Ho River including upstream in different season. Through these studies, we could evaluate both genotoxicity and estrogenic activity simultaneously, so goldfish might be a good experimental model for estimation of chemical contamination levels in aquatic environment.

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Research Article

Functional analyses of neutrophil-like differentiated cell lines under a hyperglycemic condition

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Diabetic patients are prone to severe bacterial infections. The functional alterations of neutrophils by hyperglycemia are thought to be partially responsible for such infections. In this study, we investigated the functional changes of neutrophil-like differentiated cell lines (dHL-60, dTHP-1, and dNB-4) by treatment with 5.5 mM, 11 mM, or 35 mM of glucose. In dHL-60 cells, the incubation with high glucose (35 mM) resulted in the enhancement of cell aggregation, the suppression of cellular fragility, the induction of reactive-oxygen species (ROS) production by phorbol myristate acetate (PMA) stimulation, and the impairment of phagocytosis. In dTHP-1 cells, the treatment with higher glucose generated the suppression of cellular fragility and extremely impaired phagocytosis (by 35 mM), and induced ROS production due to PMA stimulation (by 11 mM). Furthermore, the higher glucose exposure to dNB-4 cells enlarged intracellular vacuoles (by 35 mM) and induced ROS production due to PMA stimulation (by 11 mM). Since the ROS generation of those cells was enhanced only after PMA stimulation under the higher glucose conditions, glucose may have a priming effect rather than a triggering effect. These extraordinary sensitivities caused by the higher glucose treatments may reflect the dysfunction or overactivation of neutrophils.

Keywords: Diabetes / High glucose condition / Neutrophil-like differentiated human myeloid leukemia cell lines / Phagocytosis / Reactive oxygen species production

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1 Introduction

Severe bacterial infections are common in diabetic patients with hyperglycemia [1]. Neutrophils serve as a first defense line against pathogenic bacteria. The bactericidal activity of neutrophil is primarily due to oxidative pathway. By the assembly of enzymatic components (known as NADPH oxidase) segregated into the cytoplasm and plasma membrane of resting polymorphonuclear leukocyte (PMN) [2, 3], the bactericidal oxidants such as superoxide anion are produced from the activated neutrophils. Previous studies have shown that PMN in diabetic patients have impaired chemotaxis, phagocytosis, and oxidative and bactericidal activities [4–9]. A possible explanation for the impairment of PMN phagocytosis activities in diabetics is the influence due to the elevation of serum glucose concentration [10] and advanced glycation end-products (AGEs) [11, 12]. The short-term treatment (30 min) of PMN from healthy donors with high glucose concentration reduced the cellular respiratory burst [13]. Moreover, neutrophils from poorly controlled diabetics have impaired the ability of superoxide generation in the response to formyl-Met-Leu-Phe (fMLP) but not phorbol myristate acetate (PMA), and phospholipase D activity is decreased in the response to fMLP [10]. However, the detailed mechanisms of functional abnormalities of neutrophils under the hyperglycemic condition remain elusive. In our preliminary *ex vivo* experiment for 8 h or more, it was hard to examine the neutrophil function, because most neutrophils prepared from healthy humans rapidly died due to apoptosis.

Human promyelocytic leukemia cell lines, HL-60, THP-1, and NB-4, have been extensively used for several

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Abbreviations: **AGEs**, advanced glycation end-products; **ATRA**, All-*trans* retinoic acid; **dHL-60**, differentiated HL-60; **dNB-4**, differentiated NB-4; **dTHP-1**, differentiated THP-1; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **NBT**, nitroblue tetrazolium; **PKC**, protein kinase C; **PMA**, phorbol myristate acetate; **PMN**, polymorphonuclear neutrophil; **ROS**, reactive-oxygen species

researches, *e. g.* the cellular differentiation and proliferation [14, 15], in worldwide laboratories. All-*trans* retinoic acid (ATRA) is well known as an inducer for cell differentiation, in particular it promotes the granulocytic maturation of human promyelocytic leukemia HL-60 cells [16–18]. The ATRA-differentiated HL-60 cells acquire the ability of reduction of nitroblue tetrazolium (NBT) [16–21], increase the tissue transglutaminase expression [19], modulate the proto-oncogene expression [20], induce the cell-surface expression of the Mac-i antigen [22], and up-regulate the protein kinase activity [21, 23]. NB-4 is a cell line derived from an acute promyelocytic leukemia patient and its differentiation can be induced by ATRA as well. Fleck *et al.* [24] have assessed the morphological changes such as cell spreading and flattening during the differentiation of HL-60 and NB-4 with ATRA toward a neutrophil-like phenotype by scanning electron microscopy. The cell-cycle progression of human monocytic cell line THP-1 is regulated by ATRA through the G1/S phase [25]. The mRNA expression of retinoic acid receptor (RAR) alpha of THP-1 cells is induced by granulocyte macrophage colony-stimulating factor (GM-CSF), and the NBT reducing activity of THP-1 cells is increased by the synergistic effect of ATRA and GM-CSF [26]. The expression of CD11b antigen (a cell surface marker of differentiation) on HL-60, NB-4 and THP-1 cells is increased by ATRA, as is phagocytosis activity [24, 25]. Thus, the HL-60 and NB-4 cells differentiated with ATRA, and the THP-1 cells differentiated with ATRA and GM-CSF are probably available for analysis of neutrophil-like function by treatment with high glucose for 24 h or more as hyperglycemic models. The aim of this study is to characterize the functional changes of those three differentiated cell lines under hyperglycemic conditions.

2 Materials and methods

2.1 Cell cultures

Three human leukemia cell lines (HL-60, NB-4 and THP-1) were maintained in RPMI 1640 medium including 11 mM glucose (a cell-maintaining medium, Iwaki, Japan) supplemented with 10% fetal calf bovine serum (FCS) at 37°C in 5% CO_2 atmosphere.

2.2 Cell differentiation

Cells were harvested during exponential growth and seeded at a density of 2×10^5 cells/ mL. The HL-60 and NB-4 cells were differentiated with 1 μM ATRA for 2 days in the cell-maintaining medium including 11 mM glucose (dHL-60 and dNB-4). For the differentiation of THP-1, the cells were cultured with 1 μM ATRA and 1 ng/mL GM-CSF for 2 days in the cell-maintaining medium (dTHP-1). The differentiation of those cells was monitored by NBT reducing assay as described later.

2.3 Treatment of cells with the different concentration of glucose

The differentiated or non-differentiated cells cultured in the cell-maintaining medium including 11 mM glucose were washed with PBS before experiments. For the mimicry of physiological conditions, the medium with addition of 5.5 mM glucose in glucose-free RPMI 1640 (Invitrogen, California, USA) was used. The cells (3×10^5 cells/mL of each) were incubated with a 5.5 mM glucose-containing medium (physiologic concentration), a 11 mM glucose-containing medium (*in vitro* cell-maintaining concentration), a 35 mM glucose-containing medium (addition of 24 mM glucose in cell-maintaining medium, high concentration), or a 24 mM mannitol-11 mM glucose-containing medium (addition of 24 mM mannitol in the cell-maintaining medium to rule out osmotic stress) for up to 4 days.

2.4 Cellular morphology, viability, vacuolization, and fragility

To characterize cellular morphology, the cells were prepared on glass slides by centrifugation using a Cytospin at $50 \times g$ for 2 min, and the slides were air-dried, fixed in methanol and stained with Diff-Quik (Dade Behring, Illinois, USA) and were observed at a magnification of 1000 × under a light microscope. To examine cell viability, the number of living and dead cells in the cultures was determined by trypan blue dye exclusion under a phase contrast microscope. The ratio of living/dead cells was estimated as cell viability. For the observation of cellular vacuolization, the cells were stimulated with 10 ng/mL of PMA for 10 min, cytospined, fixed, and stained with Diff-Quik. The vacuolated cells were observed under a light microscope. For analysis of cellular fragility, the cells were stimulated with 10 ng/mL of PMA for 10 min, cytospined, fixed, and stained with Diff-Quik, and the number of cells was counted under a light microscope. The cells on the slide lack fragile cells, which were destroyed physically by cytospining. The percentage of cellular fragility was determined by the following equation: % of cellular fragility = [destroyed-Diff-Quik-stained cells/total-Diff-Quik-stained cells] × 100. Each individual experiment was repeated at least three times.

2.5 Cell aggregation assay

The number of aggregated cells and total cells was counted under a phase contrast microscope. The percentage of cells in aggregates was determined by the following equation: %