

識経験者、産業界代表、動物愛護団体代表によるStakeholder Meetingを開催している。

また、上記第七改正が成立したことを受け、ECVAMは、今後の重点課題を以下の11項目に整理した<sup>15)</sup>。1) 全身毒性(単回投与毒性、反復投与毒性、神経毒性、肺毒性、腎毒性、肝毒性、免疫毒性、血液毒性)、2) 局所毒性(光毒性、皮膚腐食性、皮膚刺激性、眼刺激性)、3) 感受性(皮膚感受性、吸入感受性)、4) 発ガン性、5) 生殖毒性、6) トキシコキネティクス、7) 環境毒性、8) 科学的な情報サービス、9) 定量的構造活性相関、10) 生物学材料(発熱性物質試験)、11) 戦略開発(in vitro 毒性試験やバリデーションにおけるGLP, Good Cell Culture Practice (GCCP)のガイドライン、トキシコゲノミクス)。これらの内、光毒性試験、皮膚腐食性試験、及び経皮吸収試験についてはOECDレベルでのガイドライン或いはその案が存在する。また、生殖毒性試験としては、胚性幹細胞試験、全胚培養試験、及びマイクロマス試験などを胎児毒性試験代替法として合意している。一方、単回投与毒性試験と皮膚刺激性試験、及び眼刺激性試験についてはバリデーション中、或いはその準備中である。また、in vitro 胎児毒性試験、皮膚刺激性試験、急性毒性試験、免疫毒性試験、トキシコゲノミクスについてECVAM主催のWorkshopを開催し、検討を進めている。

### 3-2) 米国の状況

ICCVAMは代替法を受け入れるための基準に基づいて、米国内外から提供されたバリデーションデータのPeer Reviewによる代替法評価を行っている。まず、1998年に皮膚腐食性試験法としてのCorrositex<sup>®</sup>について資料のPeer Reviewを行い、本方法が動物愛護の点で問題無いこと、また、全ての化学物質に有用とは言えないが、Department of Transport (DOT)で必要とされる状況においては有用であると評価した<sup>16)</sup>。なお、DOTが適当とする被験物質の範囲とICCVAMの専門家が適当と評価した物質の範囲との間で若干食い違いが有る。本試験で陰性の場合には皮膚刺激性試験により確認の必要があるが、false positiveを許容するならば陽性の場合の動物試験は不要とした。同様のpeer reviewにより、感受性試験法としてのLLNA法を完全な代替法ではないが、妥当な方法であるとして認知した<sup>17)</sup>。また、皮膚腐食性試験としてEpiDerm<sup>™</sup>、およびEPISKIN<sup>™</sup>法の評価が終了している。このように米国ではICCVAMの設立以来、着々と代替法の評価と行政への取り入れを進めている。

一方、ICCVAMはECVAMと密接な協力関係を結び、お互いが承認した試験法については、簡易の評価促進プロセスを採用している<sup>18)</sup>。また、急性全身毒性を評価するための細胞毒性試験についての共同バリデーションを実施中であり、Phase Ia試験およびPhase Ib試験結果を踏まえて再現性向上の観点からプロトコルを修正したPhase II試験が2003年11月に完了した。現在、最適化されたプロトコルを使用

した試験Phase IIIの段階にある。更に、4種の眼刺激性試験代替法(BCOP法、HET-CAM法、ICE (Isolated Chicken Eye Test)法及びIRE (Isolated Rabbit Eye Test)法)のプレバリデーションを計画しており<sup>19)</sup>、各国に情報提供を求めている。特に良質なin vivo試験結果を求めており、我々の行ったin vivo眼刺激性試験結果を提供した。なお、ICCVAMでの評価には日本の専門家も協力している。

### 3-3) 最近のOECDの状況

OECDでは安全性試験法ガイドラインの策定にあたって、動物福祉の問題を重視しており、安全性試験法専門家会議にInternational Council on Animal Protection in OECD Programmes (ICAPO)のような動物福祉団体代表の参加を許容している。

動物実験代替法関連のガイドラインとしては、これまでに急性経口投与毒性試験法として、動物使用数の少ない固定用量法(420: Fixed Dose Method, Updated 2001.12.20)、毒性クラス分け法(423: Acute Toxic Class Method, 2001.12.20)及びUp-and-Down法(425: Up-and-Down Procedure, 2001.12.20)を受け入れるとともに、多数の動物を用いる従来のガイドライン(401)を廃止した(2002.12.20)。また、旧ガイドラインに基づくデータの受け入れ拒否を求めた。また、安全性試験における倫理的な実験法についてのガイドライン案(1998)を作成した。皮膚感受性試験ガイドライン429 (LLNA法)が正式にガイドライン化した(2003)。また、光毒性試験ガイドライン432 (In Vitro 3T3 NRU phototoxicity test)、皮膚腐食性試験ガイドライン430 (In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER))、皮膚腐食性試験ガイドライン431 (In Vitro Skin Corrosion: Human Skin Model Test, EPISKIN<sup>™</sup>, EpiDerm<sup>™</sup>法)、経皮吸収試験ガイドライン428 (Skin Absorption: in vitro Method)が2002年5月のNational Coordinator会議で合意された。しかし、ガイドラインとしての正式な通知はまだなされていない。

### 2-4) 日本における代替法の受入状況

日本にはECVAMやICCVAMのような安全性試験評価のための常設組織は存在せず、事例ごとに研究班が組織され、検討されてきた。in vitroの安全性試験法としては、従来より実施されている遺伝毒性試験以外のin vitro試験法として医療用プラスチック容器抽出物の安全性評価のための細胞毒性試験法や注射用水の微生物性発熱物質評価のためのin vitroエンドトキシン試験法(リムラステスト)が承認され、日本薬局方に組み入れられている。また、OECD加盟国としてOECD承認試験法で得られた結果の受け入れ義務がある。一方、ICHでは表4に示したような安全性試験法ガイドラインの変更がなされ、我が国のガイドラインにも導入されたが、それらも前記の3Rの原則に合致するものである。

表4：代替法に関連したICH（医薬品の承認申請に必要な試験についての国際的なハーモナイゼーションのための会議）での検討

- 1) 単回投与毒性試験において統計学的に厳密なLD50値を要求しない。非齧歯類では必ずしも死亡するまで用量を上げなくとも良い。  
⇒使用動物数の削減、動物の苦痛の削減
- 2) 反復投与毒性試験において12ヶ月試験を要求しない。  
⇒毒性試験期間の短縮、動物使用数の削減
- 3) 雄性生殖臓器毒性検出系としての詳細な病理観察を供う2週間反復投与毒性試験結果を受け入れる。  
⇒早期の臨床試験 無駄な動物実験の削減
- 4) 発癌性試験における動物種数を1種に削減し、代替法で補足する。  
⇒使用動物数の削減
- 5) 臨床試験との関係における非臨床試験実施タイミングについて合意  
⇒科学的・倫理的に妥当な医薬品開発の促進  
⇒無駄に終わる動物試験実施の減少

ウサギの眼を用いる化粧品の眼刺激性試験については、適切な方法があればとの留保つきではあるが、代替法を受入れることが厚生省により表明されている<sup>20)</sup>。しかし、どの試験法が適切な方法であるのか不明であった。そこで、我々は日本化粧品工業連合会と協力し、眼刺激性試験代替法のバリデーションを実行し、血清を添加した培養液を用いる細胞毒性試験法など、いくつかの方法が従来のウサギを用いるドレイズ試験結果との対応が良いことを示した<sup>21)</sup>。

また、代替法を用いて化粧品原料の眼刺激性を評価するためのガイドライン案を作成した。<sup>22)</sup>

一方、代替法を国際的な基準に基づいたバリデーションによって評価するには、準備と実施および結果の解析、さらにはガイドラインの作成等に多額の費用と時間、及び労力を要する。そこで我々は「動物実験代替法の使用を促進するための研究」のもとで、日本動物実験代替法学会と協力し、文献やバリデーション結果を客観的・科学的に評価し、試験法の長所や短所、限界などを明らかにし、その妥当性検討プロジェクトを平成13年度より開始した。本研究班は、代替法やバリデーションの専門家から構成され、①代替法に関する情報を収集して一次的な解析・評価を行う評価委員会、②その報告をもとに、より広い視野で評価するための（臨床医師、毒性学者、行政担当者等からなる）評価会議、の二段階で評価するスキームを構築した。今までに、光毒性試験代替法について広く調査し、施設間比較の出来るバリデーション結果の存在する3T3細胞を用いてneutral red (NU)取り込みを指標とする方法(3T3-NRU光毒性試験法)を評価した<sup>23)</sup>。また、難水溶性物質に使用できる可能性の高い光毒性試験法と

して、酵母光生育阻害試験と赤血球光溶血試験法を組み合わせた試験法バッテリーについて文献的評価を基に多施設バリデーションを実施している。また、放射性同位元素を用いない皮膚感作性試験(LLNA試験法の改良法)の評価を計画している。

### 3-5) その他の国際的状況

第3回国際動物実験代替法会議(イタリア、ポロニア)において、動物実験と代替法についての「ポロニア宣言」が採択された。これはRusselとBurch(1959)が1954年に提案した3Rを再確認するとともに、それを更に促進するためのものである。それを以下に要約した。

- ① 全ての国が全ての研究・試験・教育に3Rの原則を積極的に組み入れるための法的な枠組みを作るべきである。
- ② いずれの動物実験においても、関係する科学者や行政官の全てに教育や訓練を行う公式あるいは非公式の機構が無くてはならない。
- ③ 全ての動物実験は事前に専門家により科学および倫理の両面について、独立した審査を受けなくてはならない。
- ④ 動物実験の結果得られる利益と想定される動物の苦痛の両方を評価し、計ることが審査委員会の重要な機能の一部である。
- ⑤ どのような状況においても許されるべきでない動物の苦痛のレベルについての国際的な合意があるべきである。
- ⑥ より厳しい実験動物に対する規制を避けるために動物実験を他の国に依頼することを受け入れるべきではない。

### 4. 我が国における代替法研究・評価センターの必要性

今まで述べたように、欧米では1990年代の半ばよりEUはECVAM、米国はICCVAMという新規安全性試験法の開発とバリデーション、或いは評価のための専門機関を設置し、検討を進めてきた。しかし、日本においてはECVAMやICCVAMに相当する組織はなく、試験法毎に当該専門家が対応しているのが現状である。また、我が国独自の試験法のバリデーションや評価が遅れている。そこで、in vitro新試験法の開発研究と、それによって得た専門的知見を基礎に新規代替法のバリデーションと評価、及び国際対応を行う日本における代替法研究の中心、JaCVAM (Japanese Center for Validation of Alternative Methods)を設立する必要がある。しかし、欧米並の予算と人員の確保は不可能であり、関連学会や業界との協力体制が望ましい(図4)。これにより、1) JaCVAM主催による国際レベルのバリデーションにより新しい試験法の利点と限界が明確化されることにより、医薬品や化粧品、農薬、その他の化学物質の安全性評価のレベルが向上する、2) 適切に評価された、経済的に有利な方法が利用できることにより、安全性評価がスピードアップされる、3) トキシコゲノミクスやプロテオミクスなどの新しい手法や考えを利用した安全性評価手法を適切に評価するこ

とにより、利用を図ることができる、4) ECVAM や ICCVAM と協力して代替試験法を開発することにより、新しい試験法開発についての国際貢献ができる、更に、5) 動物実験代替法開発に関する我が国の姿勢と貢献を印象づけることができる、と考える。

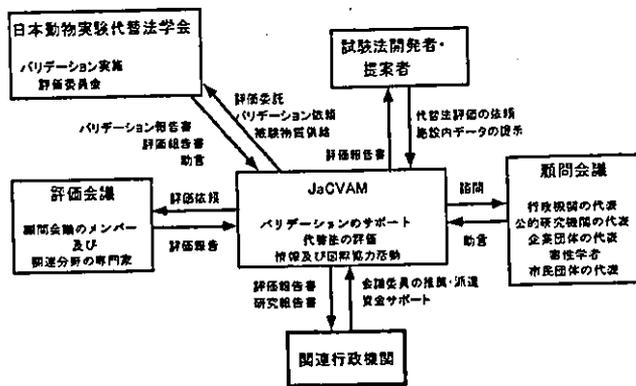


図 4 : JaCVAM 構想案

## 5. まとめ

安全性試験法の改良は科学の進歩とともに継続して進めなくてはならない。欧米では ECVAM や ICCVAM の様に代替法や新しい安全性試験法について専門的に取り扱う組織があり、代替法の研究やバリデーションおよびその支援、および科学的評価を行っている。日本においても同様な組織が作られることが望ましい。

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# 日本における動物実験代替法の開発と活動状況

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## 日本における動物実験代替法の開発と活動状況

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**Abstract** : For the promotion of 3R's principle of alternative methods, EU and US established ECVAM and ICCVAM for the evaluation and validation of alternative methods (AMs), respectively. This is because reliability and relevance of AMs should be determined by intra- and inter-laboratory validation for their regulatory acceptance. Appropriate procedures of the validation and regulatory acceptance of AMs were recommended by OECD in 1996. It is difficult to prepare data to satisfy the requirement by single laboratory or company. Therefore, it is recommended to establish similar institute in Japan like Japanese Center for the validation of AMs (JaCVAM). However, there is not enough resources in Japan. It is necessary to cooperate with Japanese Society of Alternatives to Animal Experiments (JSAAE) and Japanese industry group like Japanese Cosmetic Industry Association (JCIA). JSAAE is the only scientific community that is specified to research on alternatives. It has been contributing to the communication between scientists and animal protection groups during these 20 years. Research group supported by the Ministry of Health, Labor, and Welfare has been cooperating with JSAAE to develop and evaluate new alternative methods. Those were in vitro alternatives to Draize eye irritation tests, 3T3-NRU phototoxicity test, battery system of phototoxicity tests using yeast and red blood cells, and non-R1 method of LLNA. We are expecting that the establishment of JaCVAM will contribute to the valid alternative test methods originated in Japan.

**Key words** : Japanese Society of Alternatives to Animal Experiments (JSAAE), Japanese Center for the validation of alternative methods (JaCVAM), Japanese Cosmetic Industry Association (JCIA), alternative methods, validation

### 1. はじめに

多数の動物に苦痛を与え、生命を奪うことにつながる研究や教育への実験動物の使用についての

“Regulatory acceptance of alternative tests and the establishment of JaCVAM.”

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1976年東京大学薬学系大学院博士過程修了。現在、国立医薬品食品衛生研究所安全性生物試験研究センター薬理部長、日本薬理学会評議員、日本動物実験代替法学会評議員、HAB協議会評議員、日本薬物動態学会理事、薬学博士。

動物福祉と人道の立場からの批判や研究者の自覚から、動物に替わる試験法の検討が行われるようになり、1954年にRusselとBurchにより動物実験代替法（代替法）についての3Rの原則が提案された。

イギリスでは、代替法研究とその社会への受け入れを促進するために、医学分野における実験動物を他のものに置き換えるための基金 (FRAME) が1969年に設立された。米国では、ジョンズホプキンス大学に代替法センターが1981年に、日本では、京都大学の菅原 努先生を中心に日本動物実験代替法学会の前身となる研究会が1982年に設立された。これらの活動を通じて多くの安全性試験代替法が開発され、行政試験法としても可

能性のある試験法が現れて来た。

そこで、EUは代替法開発の拠点とし、代替法についてのデータベースを設置・維持するため、また、行政、産業、生物・医学分野の科学者、消費者、および動物愛護運動グループの対話を促進することを目的に1991年に代替法バリデーションセンター（European Center for the Validation of Alternative Methods: ECVAM）を設立した（1994年開所）。米国は毒性試験法の開発、バリデーション、受け入れ、および国内・

国際レベルでのハーモナイゼーションに関する問題を連邦政府内で調整するためにNICEATM（NTP Interagency Center for the Evaluation of Alternative Toxicological Methods）の下にNIEHS（National Institute of Environmental Health Sciences）を含む14の行政機関および研究機関によりICCVAM（Interagency Coordinating Committee on the Validation of Alternative Methods）を1993年に設置した。現在欧米の行政機関においては、新たに開発された

表1 動物実験代替法への日米欧の取り組み

1954	RusselとBurchが動物実験代替法についての3Rを提案
1969	医学分野における実験動物を代替法に置き換えるための基金（FRAME）設立（イギリス）
1979	動物実験の改善と実験動物数削減を求める法律の制定（スウェーデン）
1981	ジョンズ・ホプキンス大学、代替法センターの設立（米国）
1982	動物実験代替法研究会の設立（日本）
1986	動物実験反対シンポジウム（日本みどりの連合） 日本学術会議毒科学研連による代替法に関するシンポジウム
1989	日本毒科学会による代替法についてのシンポジウム
1990	政府機関代替法グループ（IRAG）設置（米国） 欧州議会、医薬分野以外の動物実験禁止を提案
1990	厚生省の眼刺激性試験代替法に関する研究班発足
1992	厚生省研究班、眼刺激性試験代替法のバリデーション開始
1993	EU議会が化粧品の安全性評価のための動物実験を1998年までに廃止するとのDirective 76/768修正案採用 厚生省、単回投与毒性試験に使用する動物数を削減
1993	米国Interagency Coordinating Committee on the Validation of Alternative Methods（ICCVAM）設立。
1994	EU代替法バリデーションセンター（European Center for the Validation of Alternative Methods: ECVAM）開所。
1996	OECDで代替法を行政的に受け入れるための基準を検討。
1997	EU議会がDirective 76/768修正案の施行を2000.6.30まで延期。
1998	ECVAMがEpiskin法およびラット皮膚電気抵抗法（TER法）を皮膚腐食性試験として、3T3-NR法を光毒性試験として承認。
1999	厚生省研究班、代替法を用いる眼刺激性試験評価ガイドライン案報告 ICCVAMがCorrositex法を皮膚腐食性試験代替法として承認。
2000	EU議会がDirective 76/768修正案の施行を2002.6.30まで延期 EU 3T3-NR光毒性試験法および皮膚腐食性試験を官報に掲載
2001	OECD急性経口毒性試験ガイドライン（401）を削除し、固定用量法（420）、急性等級法（423）、上げ下げ法（425）を採用。
2002.5	3T3-NR光毒性試験法（432）、皮膚腐食性試験ガイドライン（TER法430およびヒト皮膚モデル431）をOECD専門家会議で採択
2003.3	化粧品およびその原料の安全性評価に関する化粧品指令第7次改正公布 ①ECVAMやOECDで承認された代替法があるものはすべて即時禁止 ②2009年までに動物を用いる全ての安全性試験を全面的に禁止、および動物実験を行った化粧品の販売禁止 ③薬物動態試験や生殖発生毒性、反復投与毒性試験については2013年まで猶予
2004.2	第六回国際動物実験代替法会議（2007）を日本で開催することが決定

代替法を科学的に評価し、可能なものについては取り入れていこうという作業が行われている。

なお、ECVAMやICCVAMでは従来の安全性試験の代替法のみならず、定量的構造活性相関や内分泌かく乱化学物質検索のような新たな毒性評価の要請やトキシコゲノミクスのような新しい技術を行政的試験として、取り入れるための検討も視野に入れている。また、*in vitro*の安全性試験法の結果の信頼性を保証するためのGLP基準も検討され、OECDレベルでのposition paperも作成された。また、EU政府と議会はついに2009年までに化粧品の安全性評価のための動物実験を原則として禁止する化粧品指令第7次改正を2003年3月に公布した。

日本動物実験代替法学会では設立以来、動物実験代替法バリデーションや評価を行うとともに、補助金を通じた研究支援、大会やシンポジウムを通じた研究発表と代替法や倫理的な動物実験に関する教育、市民講座を開催し、研究者間の情報交換や市民との交流を続けてきた。また、2007年に第6回国際動物実験代替法会議(6th World Congress on Alternatives and Animal Use in Life Sciences)を主催、東京都江戸川区のタワーホール船堀で開催する予定である。本大会は2009年のEUの化粧品の安全性評価のための動物実験の原則廃止に向けて、それまでの研究成果をまとめ、必要な研究計画を立てる重要な時期にあたる。一方、本大会の内容は広く、代替法学会員のみでは十分対応できない。そこで、日本トキシ

コロジ学会、日本実験動物学会、日本実験動物環境研究会、日本環境変異原学会、国際トキシコロジ学会の協賛も得て、それぞれの学会代表者にも大会準備委員会に入っただき、準備を進めている。

## 2. 日本における代替法の受入状況

日本にはECVAMやICCVAMのような安全性試験法に関する新試験法を評価する常設組織は存在せず、事例ごとに研究班が組織され、検討されてきた。*in vitro*の安全性試験法としては、従来より実施されている遺伝毒性試験以外にも医療用プラスチック容器抽出物の安全性評価のための細胞毒性試験法や注射用水の微生物性発熱物質評価のための*in vitro*エンドトキシン試験法(リムラステスト)が承認され、日本薬局方に組み入れられている。一方、OECD加盟国としてOECD承認試験法で得られた結果の受け入れ義務がある。なお、ICHでは三極の統一ガイドラインが作られ、不必要な動物実験の繰り返しは少なくなったが、表2に示したような安全性試験法ガイドラインの変更も行われ、我が国のガイドラインにも導入された。それらも代替法に関する3Rの原則に合致するものである。

ウサギの眼を用いる化粧品の眼刺激性試験については、適切な方法があればとの留保つきではあるが、代替法を受入れることが厚生省により表明されている<sup>2)</sup>。しかし、どの試験法が適切な方法であるのか不明であった。そこで、我々は日本化

表2 代替法に関連したICH(医薬品の承認申請に必要な試験についての国際的なハーモナイゼーションのための会議)での検討

- 1) 単回投与毒性試験において統計学的に厳密なLD50値を要求しない。  
非齧歯類では必ずしも死亡するまで用量を上げなくとも良い。  
→ 使用動物数の削減、動物の苦痛の削減
- 2) 反復投与毒性試験において12カ月試験を要求しない。  
→ 毒性試験期間の短縮、動物使用数の削減
- 3) 雄性生殖臓器毒性検出系としての2週間反復投与毒性試験を承認  
→ 早期の臨床試験 → 無駄な動物実験の削減
- 4) 発癌性試験における動物種数を1種に削減し、代替法で補足する。  
→ 使用動物数の削減
- 5) 臨床試験との関係における非臨床試験実施タイミングについて合意  
→ 科学的・倫理的に妥当な医薬品開発の促進  
→ 無駄に終わる動物試験実施の減少

粧品工業連合会と協力し、眼刺激性試験代替法のバリデーションを実行し、血清を添加した培養液を用いる細胞毒性試験法など、いくつかの方法が従来のウサギを用いるドレイズ試験結果との対応が良いことを示した<sup>3)</sup>。

一方、代替法を国際的な基準に基づいたバリデーションによって評価するには、準備と実施および結果の解析、さらにはガイドライン案の作成などに多額の費用と時間、および労力を要する。そこで我々は厚生労働科学研究「動物実験代替法の使用を促進するための研究」のもとで、日本動物実験代替法学会と協力し、文献やバリデーション結果を客観的・科学的に評価し、試験法の長所や短所、限界などなどを明らかにし、その妥当性を明らかにするための検討を平成13年度より開始した。本研究班は、代替法やバリデーションの専門家から構成され、①代替法に関する情報を収集して一次的な解析・評価を行う評価委員会、②その報告をもとに、より広い視野で評価するための（臨床医師、毒性学者、統計学者、行政担当者等からなる）評価会議、の二段階で評価するスキームを構築した。今までに、光毒性試験代替法について広く調査し、多施設バリデーション結果の存在する3T3細胞を用いてneutral red (NU) 取り込みを指標とする方法（3T3-NRU光毒性試験法）を評価した<sup>4)</sup>。また、難水溶性物質に使用できる可能性の高い光毒性試験法として、酵母光生育阻害試験と赤血球光溶血試験を組み合わせた試験法バッテリーについて一次評価を行い、その結果を基に多施設バリデーションを実施した。平成16年度より開始した厚生労働科学研究「安全性評価のための動物実験代替法の開発および評価体制の確立に関する研究」においても継続して、光毒性試験法バッテリーの評価を行い、評価委員会では多施設バリデーションの結果も合わせ、さらに評価を行っている。また、放射性同位元素を用いない皮膚感作性試験（Local Lymph Node Assay法の改良法）の評価を行った。評価委員会での一次評価結果では本改良法が適切かつ有用な方法であると評価されたが、提案者作成によるデータのみしかなかったことから、さらに多施設バリデーションを行い、その上で最終評価を行う予定である。

なお、本研究班では代謝活性化能を含む細胞の開発（小澤正吾博士）や代謝活性化能を添加した試験系を用いた急性毒性予測のための細胞毒性試験法の開発（田中憲徳博士）、感作性試験代替法の開発および代替法に関する国際情勢の調査（豊田英一博士）、光感作性試験代替法（戸倉新樹博士）、代替法開発のための統計解析手法の研究（吉村功博士）、およびバリデーションデータの統計解析（大森 崇博士）を行っている。

### 3. 我が国における代替法研究・評価センターの必要性

先に述べたように、欧米では1990年代の半ばよりEUはECVAM、米国はICCVAMという新規安全性試験法の開発とバリデーション、あるいは評価のための専門機関を設置し、検討を進めてきた。また、ECVAMとICCVAMは協力関係を構築し、共同バリデーションの実施や評価結果の相互承認を行っている。また、我が国への協力要請も行われている。しかし、日本においてはECVAMやICCVAMに相当する組織はなく、我が国独自の試験法のバリデーションや評価が遅れており、国際的な認知を得ることも困難である。

*in vitro*新試験法の開発研究と、それによって得た専門的知見を基礎に新規代替法のバリデーションと評価、および国際対応を行うためには、日本においても代替法研究の中心として、JaCVAM (Japanese Center for Validation of Alternative Methods) を設立する必要がある。しかし、欧米並の予算と人員の確保は不可能であり、関連学会や業界との協力体制が望ましい（図1）。新規代替法の評価は前項で示したような主に当該代替法の専門家からなる評価委員会での評価と臨床医師やトキシコロジーの専門家、行政担当者等からなる評価会議での評価による2段階評価を考えている。また、十分なバリデーションデータが無いが、価値の高いと評価された試験法については、関連学会や業界の協力を得て、国際的レベルに則ったバリデーション実施を考えている。

これにより、3Rの目的に合致する新規試験法の受け入れが促進されるとともに、1) JaCVAM主催による国際レベルのバリデーションにより新

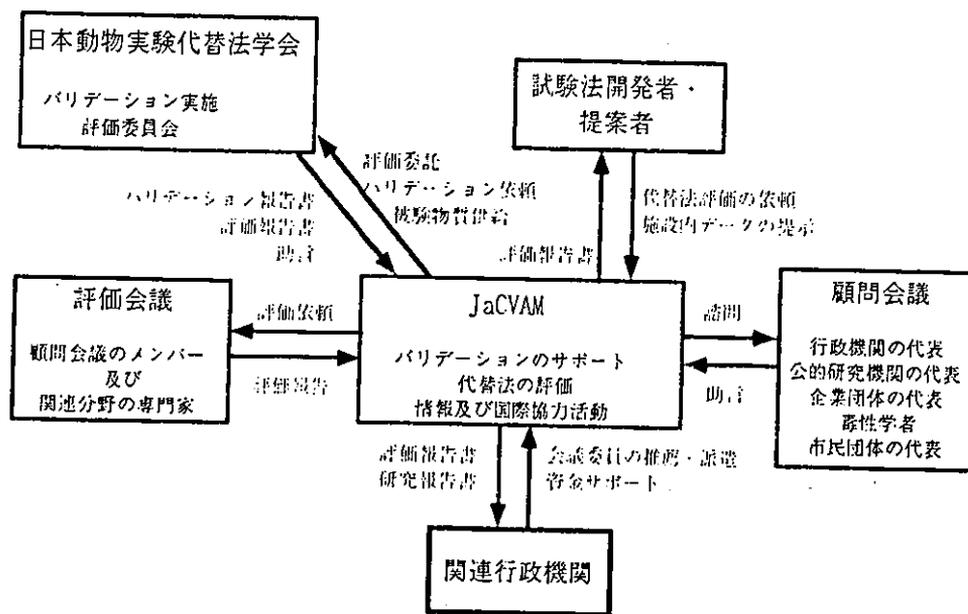


図1 JaCVAM構想

しい試験法の利点と限界が明確化されることにより、医薬品や化粧品、農薬、その他の化学物質の安全性評価のレベルが向上する、2) 適切に評価された、経済的に有利な方法が利用できることにより、安全性評価がスピードアップされる、3) トキシコゲノミクスやプロテオミクスなどの新しい手法や考えを利用した安全性評価手法を適切に評価することにより、利用を図ることができる、4) ECVAMやICCVAMと協力して代替試験法を開発することにより、新しい試験法開発についての国際貢献ができる、さらに、5) 動物実験代替法開発に関する我が国の姿勢と貢献を印象づけることができる、と期待している。

現在、本構想は、国会での承認を待っているところである。

#### 4. おわりに

1999年に開催された第3回国際動物実験代替法会議（イタリア、ポーニア）において、動物実験と代替法についての「ポーニア宣言」が採択された<sup>9)</sup>。これはRusselとBurch（1959）が1954年に提案した3Rを再確認するとともに、それをさらに促進するためにのものである。その法的、科学的、倫理的な部分<sup>10)</sup>を以下に要約する。

①全ての国が全ての研究・試験・教育に3Rの

原則を積極的に組み入れるための法的な枠組みを作るべきである。

②いずれの動物実験においても、関係する科学者や行政官の全てに教育や訓練を行う公式あるいは非公式の機構が無くてはならない。

③全ての動物実験は事前に専門家により科学および倫理の両面について、独立した審査を受けなくてはならない。

④動物実験の結果得られる利益と想定される動物の苦痛の両方を評価し、計ることが審査委員会の重要な機能の一部である。

⑤どのような状況においても許されるべきでない動物の苦痛のレベルについての国際的な合意があるべきである。

⑥より厳しい実験動物に対する規制を避けるために動物実験を他の国に依頼することを受け入れるべきではない。

これらが採択された時点では、実現困難に思われたところも多かったが、EUの化粧品指令第七改正によれば、2009年以後はEU域外においても動物を用いて安全性評価を行った化粧品の輸入が禁止される予定であるし、動物実験に関する人道的なエンドポイントについては、2000年にOECDからガイドライン<sup>11)</sup>が通知された。3Rの原則や動物実験委員会による動物実験の審査につ

いては現在改訂作業が進んでいる「動物の愛護および管理に関する法律」にも盛りられることも予想される。

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## Three-Dimensional High-Density Culture of HepG2 Cells in a 5-ml Radial-Flow Bioreactor for Construction of Artificial Liver

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A three-dimensional high-density cell culture is essential for the construction of an artificial tissue. Many researchers have reported that three-dimensional cell culture enhances cell function. The use of a radial-flow bioreactor (RFB) has enabled the cultivation of cells at high density for constructing a three-dimensional tissue. In this study, we have developed a novel, small RFB, which has a bed volume of 5 ml and is equipped with a porous support as an immobilized scaffold; its performance was tested using the hepatoblastoma cell line, HepG2. Among the other supports tested here, hydroxyl apatite was selected from the viewpoint of its ability to support good cell growth at high density with uniform distribution in a bioreactor. The HepG2 cells grew well in the scaffold under a sufficient supply of nutrients by radial flow and were used to construct a three-dimensional tissue in the scaffold. The concentration of the cells cultivated in this 5-ml RFB reached  $10^8$  cells/ml and the glucose consumption rate was almost similar to that obtained when using a 30-ml RFB, which has already been reported previously. This high glucose consumption continued over 7 d after the growth phase. Furthermore, albumin production was maintained in the stable phase. Gene expression profiles of cells obtained from long-term cultures in the 5-ml RFB were analyzed. It was found that the expressions of genes encoding the cell cycle-related proteins, cyclins, and cell cycle division 2 (*cdc2*) were suppressed in the stable phase. In addition, the number of cells incorporating 5'-bromo-2'-deoxyuridine (BrdU) in the stable phase markedly decreased compared with that in the growth phase. These results indicated that the majority of cells in the stable phase remain in the G0/G1 phase. Furthermore, this implies that the three-dimensional tissue constructed in the 5-ml RFB showed the high function similar to a normal liver in the human body. Therefore, the 5-ml RFB was considered as a useful tool and a substitute method for animal experiments.

[Key words: three-dimensional high-density cell culture, radial-flow bioreactor, DNA microarray]

In order to maintain cellular functions, it is essential to cultivate cells and construct high-density three-dimensional tissue structures. For this purpose, several researchers have developed various types of bioreactors. The functional features of the constructed high-density three-dimensional proliferated cell culture have been investigated and compared with those of a dish culture. One of the aims of high-density three-dimensional cell culture is the fabrication of a functional bioartificial organ and its transplantation. In particu-

lar, artificial liver is an important target since there is a high demand for its use as bridging treatments for patients awaiting hepatocyte replacement therapy or those suffering from fulminant hepatic failure (1). However, the artificial liver is expected to perform multiple liver-specific functions such as detoxification, lipid metabolism, serum protein production and bilirubin production, among others. At present, the most frequently studied bioreactors for this purpose are capillary hollow fiber systems. Sussaman *et al.* (2) developed a device for incorporating a highly differentiated human liver cell that was cultured in a hollow fiber cartridge; this device was capable of supporting dogs suffering from fulminant

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hepatic failure for a period long enough for their own livers to have resumed normal functioning. Furthermore, this device has been utilized in human clinical trials, and in general, the patient's condition is reported to have improved (3). However, the standard hollow fiber reactor showed some limitations in terms of mass-transfer capacity and transfiber fluid convection in the case of axial flow geometry. Morsiani *et al.* (4) developed a new type of hollow fiber bioreactor, which consisted of a woven/nonwoven polyester fabric sandwiched between layers of woven polyester screen fabric, and the medium flowed across the fabric component. They reported that albumin production and ammonia detoxification of porcine hepatocytes was performed for 2 weeks. Miyashita *et al.* (5) developed a new circulatory bioreactor and cultivated glutamine synthetase-transfected HepG2 (GS-HepG2). The large-scale culture of GS-HepG2 in this reactor was applied to pigs suffering from ischemic liver failure, and the increase in blood ammonia was suppressed. Yamashita *et al.* (6) evaluated the efficacy in terms of the serum parameters of a hybrid artificial liver support system (HALSS) containing porcine hepatocytes. Pigs suffering from warm ischemic liver failure were subjected to HALSS, and the increase in blood ammonia was completely suppressed; the blood lactate level was also suppressed.

In hepatocyte cultures, it has become increasingly evident that spheroid culture enhances liver cell functions and maintains them for a long period compared with monolayer culture. In particular, the surface modification of cultivation equipment for a spheroid culture was examined in detail. Yamada *et al.* (7) reported that spheroids induced by a polymer containing lactone enhanced liver function. Kurosawa *et al.* (8) performed cultivation on a hydrophobic porous expanded poly-tetrafluoroethylene (ePTFE) membrane. The spheroids were created in the ePTFE membrane and their albumin production was maintained. A rotation cell culture system and a small number of scaffolds were used for the creation of spheroids (9, 10). Since the spheroids are constructed using three-dimensional multi-cellular aggregates, further progression of high-density three-dimensional culture in the bioreactor or spheroid culture is essential for developing a hyperactive artificial liver support system.

Hepatocyte culture systems in which the liver function can be maintained are useful for *in vitro* drug metabolism experiments (11, 12). Laugsch and Bader developed a cell-based flat membrane bioreactor, and porcine liver cells were maintained in a 3-D co-culture between two gel layers in the bioreactor with a sandwich configuration (13). The results showed that porcine liver cells preserved their phase I and phase II drug metabolism activities and responded to the drugs for over 3 weeks.

The radial-flow bioreactor (RFB) is a packed-bed-type bioreactor; the bed is filled with scaffolds such as porous glass beads, cellulose beads, and so on. The medium is made to flow from the periphery to the center of the bed under low shear stress. Yoshida *et al.* (14) utilized an RFB with a bed volume of 400 ml for the production of monoclonal antibodies for 2 weeks, and 10 g of the monoclonal antibody was obtained during the culturing process. The cell density of the RFB reached  $1.3 \times 10^8$  cells/ml-scaffold. Kawada *et al.* (15) reported the culturing of the human liver

cell line, FLC-7, in an RFB at high density ( $1.1 \times 10^8$  cells/ml-scaffold); the albumin production level was 2.34-fold greater than that obtained from the monolayer culture using a culture dish. Iwahori *et al.* (16) indicated that the CYP3A4 mRNA expression level was approximately 100-fold greater than that obtained from the monolayer culture, 48 h after rifampicin treatment in the 30-ml RFB. Therefore, the expression of CYP3A4 was strongly correlated with drug metabolism. Aizaki *et al.* (17) showed that hepatocytes cultured in an RFB were transfected with full-length HCV RNA, and *in vitro* propagation of HCV was clearly observed. Therefore, we assume the RFB to be the best bioreactor to cultivate cells having a high-density three-dimensional structure.

The 400-ml and 30-ml RFBs are too large to construct small organs with high biological function. The 400-ml RFB needs 13 l/d of culture medium in order to maintain over  $1 \times 10^8$  cells/ml-scaffold. However, the status of cells having a three-dimensional structure in a high-density culture is still unclear. In the present study, we cultivated the hepatoblastoma cell line, HepG2, in our newly developed 5-ml RFB. In comparison with the 400-ml and 30-ml RFBs, the 5-ml RFB does not require much space for the installation of the RFB culture system; therefore, multiple bioreactors can be operated concomitantly. In the present study, we indicate that when the cells remain in the G0/G1 phase under high-density culture conditions, the cells cultivated in three-dimensional carriers in the 5-ml RFB can maintain liver tissue function. Therefore, the 5-ml RFB may possibly be used as an alternative to the animal liver, especially, as an alternative method to drug metabolism and/or toxicity tests using animals.

## MATERIALS AND METHODS

**Culture of HepG2** HepG2, human hepatocellular carcinoma cells, were obtained from the American Type Culture Collection (Rockville, MD, USA). HepG2 ( $3 \times 10^6$  cells) were cultured on tissue culture dishes (59 cm<sup>2</sup>; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in 10 ml of Dulbecco's modified Eagle medium (DMEM; Gibco/BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS, BioSource International, Camarillo, CA, USA), 100 units/ml of penicillin, and 10 µg/ml of streptomycin (Gibco/BRL) at 37°C in an incubator containing 5% CO<sub>2</sub>. Cell passaging was performed every 4 d with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA·4Na; Gibco/BRL), which was utilized to detach the cells.

**Culture in RFB** RFBs (ABLE Corporation, Tokyo) of 30 ml and 5 ml capacity as well as the RFB culture system were used in this study. SIRAN™, a block of SIRAN™ (QVF GmbH, Mainz, Germany), hydroxyl apatite (Pentax Corporation, Tokyo), porous PVA resin (Muromachi Kagaku, Tokyo), polyurethane foam (Inoac Corporation, Tokyo), and calcined cattle bone calcium powder (Excelsa, Tachikawa) were used as scaffolds in the RFBs (Table I). The inner space of scaffolds in the 5-ml RFB was  $\phi 20 \times 15$  mm in height with a central spiral coil of 3 mm $\phi$  being set for medium circulation. The inner space was completely filled with SIRAN™, hydroxyl apatite, calcined cattle bone calcium powder, a shaped block of SIRAN™, polyurethane foam, or porous PVA resin.

Isolated cells were injected into the medium chamber of the RFB culture system, which was filled with DMEM supplemented with 10% FBS (pH 7.6). The medium was circulated between the

TABLE 1. Various scaffolds used in the present study

Scaffold	Shape	Support size (mm)	Porosity (%)
SIRAN™	Particle	0.6	55–60
Hydroxyl apatite	Particle	0.6–1.0	80–90
Calcined cattle bone calcium powder	Particle	0.6	70
Porous PVA resin	Block	0.13	90
Block of SIRAN™	Block of particles	0.2–0.3	55–60
Polyurethane foam	Block	0.2–0.4	94

RFB and the medium chamber using the circulation pump. The cells remained attached to and grew on the scaffold during the circulation. Sampling was carried out every 24 h to analyze the concentration of glucose, lactic acid, glutamine, and ammonia using the biosensor model BF-4 (Oji Scientific Instruments, Amagasaki). During the experiment, dissolved oxygen (DO) present in the medium, the effluent from the RFB, pH, and temperature were all monitored and controlled. Some of the spent medium was removed from the medium chamber and fresh medium was added to supplement the consumed glucose. The albumin concentration in the medium was measured using the Human Serum Albumin ELISA Kit (Cygnus Technology, Wrentham, MA, USA).

**Thin section observation** After cultivation in the 5-ml RFB, each scaffold with the cells was removed from the reactor and fixed with 10% formalin neutral buffer solution (pH 7.4; Wako Chemicals, Osaka) at 4°C for 1 h, washed twice with PBS, and then dehydrated using ethanol solutions, the concentrations of which increased in series. The dehydrated scaffolds were embedded in resin using the Histo-resin Plus Embedding Kit (Laica, Heidelberg, Germany). Sections 5 µm in thickness were prepared and each section was stained by Toluidine blue according to a general method and was observed under a microscope (BF-50; Olympus, Tokyo).

**mRNA isolation and DNA microarray analysis** After culturing the cells for 7 and 17 d in the 5-ml RFB, hydroxyl apatite beads with HepG2 cells were removed from the reactor and washed twice in PBS. Three independent samples were collected from each of the 5-ml RFB cultures. Subsequently, the cells were lysed and the mRNAs were extracted using the RNeasy Mini Total RNA Extraction Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Conversion of the total RNA (10 µg) to the target for Affymetrix GeneChip DNA microarray hybridization was performed according to the manufacturer's instructions. The targets were left for 16 h at 45°C to be hybridized to human U133 GeneChip DNA microarrays (Affymetrix, Santa Clara, CA, USA). After hybridization, the DNA microarrays were washed and stained on a Fluidics Station (Affymetrix) according to the manufacturer's protocol. The DNA microarrays were scanned and the obtained images were analyzed by the GeneChip Expression Analysis Software (ver. 5.0) (Affymetrix). DNA microarray analysis was performed in duplicate for each sample.

**Immunohistochemistry** Cells in the 5-ml RFB were treated with 0.01 mM 5-bromo-2-deoxyuridine (BrdU; Wako Chemicals) for 24 h. The scaffolds with the cells were fixed and embedded as described above. Immunohistochemistry was performed using the ZYMED BrdU Staining Kit (Zymed Laboratories, South San Francisco, CA, USA), and the scaffolds were observed microscopically.

## RESULTS

### Growth comparison between the 30-ml RFB and 5-ml RFB

In order to confirm the performance of the novel

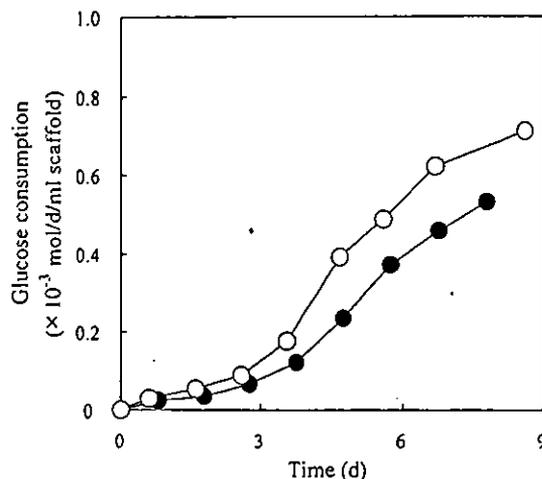


FIG. 1. Comparison of glucose consumption between 30-ml and 5-ml RFBs. Symbols: open circles, 5-ml RFB; closed circles, 30-ml RFB. Inoculated cell number was  $1.2 \times 10^8$  cells in the 30-ml reactor ( $4 \times 10^6$  cells/ml-scaffold) and  $3 \times 10^7$  cells in the 5-ml reactor ( $6 \times 10^6$  cells/ml-scaffold).

5-ml RFB during cell culturing, the cell growth was investigated using the HepG2 cell line. As an index of cell proliferation, the glucose consumption rate was calculated based on its concentration in the medium chamber and the volume of the feeding medium. Figure 1 shows the time course of the glucose consumption rate in the 5-ml RFB culture. This consumption rate was very similar to that obtained in the 30-ml RFB culture. After the culture, a part of the scaffold was removed from the 5-ml RFB and the cell number in the scaffold was counted. The cell density was  $1 \times 10^8$  cells/ml-scaffold, and this was very similar to that obtained in the 400-ml or 30-ml RFBs (14, 15). Therefore, it was assumed that the 5-ml RFB could provide similar culture conditions as those provided by the previous RFBs.

**Five-ml RFB culture using various scaffolds** Various scaffolds for bioreactors involving hepatocyte cell cultures have been previously studied (18–21). In the present study, we performed a cell culture in the 5-ml RFB using various scaffolds. The time courses of the glucose consumption rates in the RFB cultures using each of the scaffolds listed in Table 1 are shown in Fig. 2. This figure indicates that the glucose consumption rates of all the scaffolds except for the polyurethane foam were almost the same. The consumption rate in the culture using the polyurethane foam did not exceed  $2.0 \times 10^{-3}$  mol/day. After cultivation, thin sections of scaffolds were prepared and observed microscopically. Photographs of the observed sections are shown in Fig. 3. Figure 3D is a photograph of the polyurethane foam; it shows the three-dimensionally growing cells attached to the polyurethane fibers, and that these cells did not exist in the porous spaces of the polyurethane. On the other hand, three-dimensionally growing cells were present in the porous spaces of hydroxyl apatite (Fig. 3A), calcined cattle bone calcium powder (Fig. 3B), and PVA resin (Fig. 3C). The hydroxyl apatite beads have high porosity and a wide surface area for attachment. In addition, hydroxyl apatite has been used as a scaffold to achieve the three-dimensional growth of hepatocytes. Higashiyama *et al.* (22) experimented with

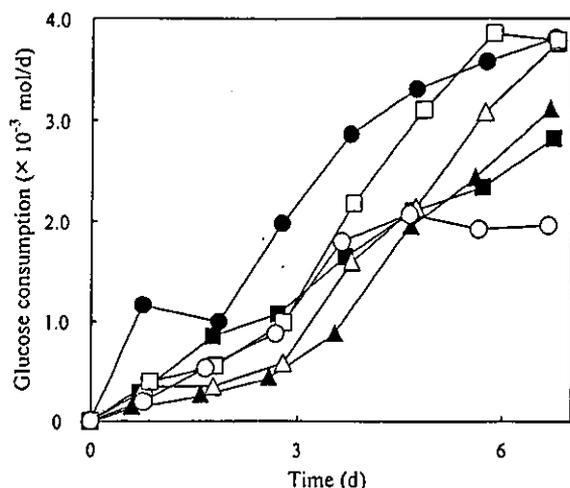


FIG. 2. Comparison of glucose consumption in culture with various scaffolds. Symbols: closed triangles, SIRAN™; closed squares, hydroxyl apatite; closed circles, calcined cattle bone calcium powder; open triangles, porous PVA resin; open squares, block of SIRAN™; open circles, polyurethane foam. Inoculated cell number was  $6 \times 10^6$  cells/ml-scaffold.

rat hepatocytes that were inoculated on a hydroxyl apatite disk; subsequently, the transplanted hepatocyte-packed disk was transferred to the rats. The hepatocyte packed on the hydroxyl apatite disk could survive and maintain their functionality by receiving oxygen and nutrients from the blood stream. Therefore, we used hydroxyl apatite in subsequent cultures. Observation of the condition of cells in thin sections obtained from the 5-ml RFB, indicated that the cell growth and density were very similar between the upper and lower sides of this reactor (data not shown). Therefore, the 5-ml RFB could maintain a uniform medium flow and showed no influence of downsizing.

**Long-term culture in the 5-ml RFB** A 17-d culture was performed in order to confirm whether the 5-ml RFB could be utilized in long-term cultures. During the culture, the concentrations of glucose, lactic acid, glutamine, and ammonia present in the medium were measured. The time course of the consumption of glucose (Fig. 4A) and glutamine (Fig. 4C) as well as that of the production of lactic acid (Fig. 4B) and ammonia (Fig. 4D) in a culture are shown in Fig. 4. The glucose consumption was found to be almost stable after day 10. The curves corresponding to lactic acid production, glutamine consumption, and ammonia production were the same as that of glucose; therefore, we assumed that constant cell conditions were maintained

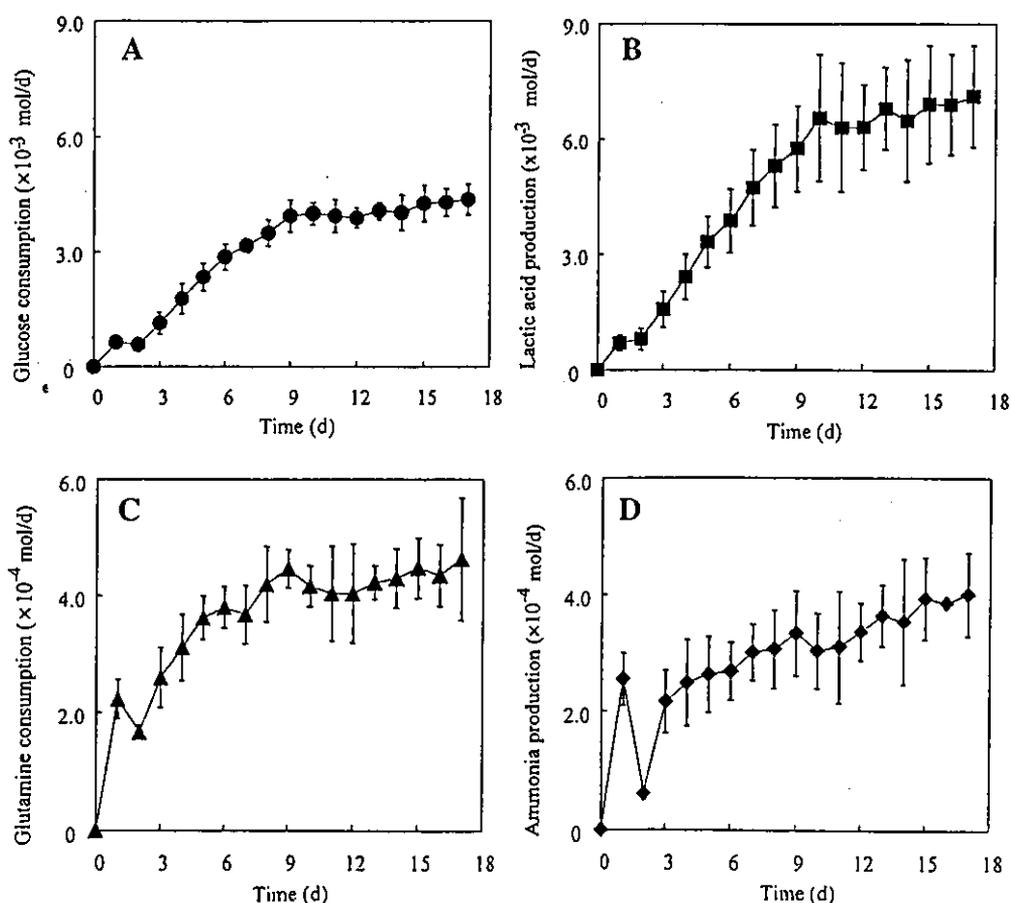


FIG. 4. Time course of the consumption or production rate in long-term cultures in the 5-ml RFB. Values are represented as average  $\pm$  SD of four independent cultures. The graphs are as follows: (A) glucose consumption, (B) lactic acid production, (C) glutamine consumption, and (D) ammonia production.

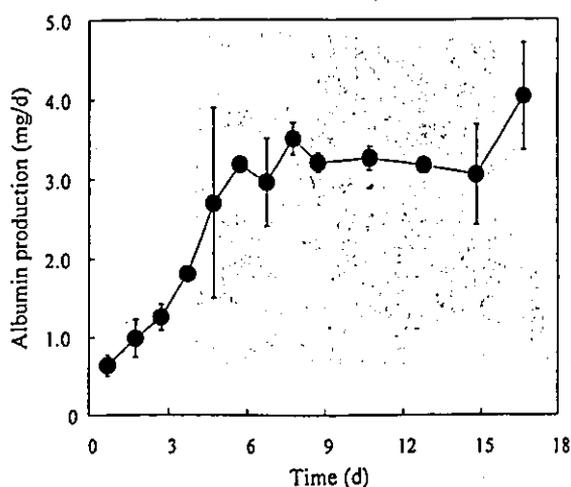


FIG. 5. Time course of albumin production rate in long-term cultures in the 5-ml RFB. Values are represented as average  $\pm$  SD for four independent cultures.

between days 10 and 17. Albumin is one of the functional marker proteins in the liver. Figure 5 shows the production rate of albumin that was stable from day 10 to day 17. This result indicated that the hepatocyte specific function could be maintained in the HepG2 cells, cultivated in the 5-ml RFB, during a long-term culture.

**Effect of long-term culture in the 5-ml RFB on gene expression** We assumed that a change in the gene expression level occurred from the growth phase to the stable phase. Therefore, to confirm the differences in the gene expression level for each gene between the growth and stable phases, mRNA was isolated on day 7 and day 17. Genes fulfilling the following criteria were considered as "suppressed or induced genes from day 7 to day 17" for a given DNA microarray analysis: (i) genes whose expression levels on day 17 when divided by the data on day 7 yielded less than a value of 0.33 were considered to be suppressed, or larger than a value of 3 were considered to be induced, (ii) genes whose expression levels of which from a duplicate analysis were within 1.5-fold, (iii) genes whose p-value was less than 0.01 when the *t*-test was performed on the microarray data from day 7 to day 17. As a result, 13 and 19 were selected as suppressed and induced genes, respectively. Genes of the organic anion transporters and the glucose transporter, which related to the liver function, were included as induced genes. The suppressed genes are listed in Table 2. In Table 2, collagen type II, alpha 1 is listed as a suppressed gene. At present, there is no evidence that the collagen type II gene is expressed in liver tissue, but it is known that collagen genes are overexpressed in liver fibrosis. Our result showed that the collagen type II gene was suppressed in the stable phase. Therefore, we assumed that the condition of cells in the stable phase in the 5-ml RFB was similar to that of cells under normal liver conditions. On the other hand, at least five types of cell cycle-related proteins, such as cyclin A2, cell division cycle 20, replication protein A3, cell division cycle 2 and cyclin B2, are included in Table 2. In particular, *cdc2* plays an important role in cell division and it is required for both G1/S and G2/M transitions (23, 24). Cyclin B associates with *cdc2* to constitute the M-phase-

TABLE 2. Ratio of gene expression level of 17-d culture to 7-d culture in 5-ml RFB

Gene	Accession no.	Fold (-)
Collagen, type II, alpha 1	X06268	0.18
Ribonucleotide reductase M2 polypeptide	BC001886	0.24
Baculoviral IAP repeat-containing 5	NM_001168	0.29
Cyclin A2	NM_001237	0.29
H2A histone family, member X	NM_002105	0.30
Cell division cycle 20	NM_001255	0.31
Forkhead box M1	NM_021953	0.31
Centromere protein A	NM_001809	0.32
Polo-like kinase	NM_005030	0.32
Replication protein A3	BC005264	0.32
Cell division cycle 2	D88357	0.33
Maternal embryonic leucine zipper kinase	NM_014791	0.33
Cyclin B2	NM_004701	0.33

promoting factor (MPF) (25). In addition, Wang *et al.* (26) showed that forkhead box M1B protein enhanced the transcriptional networks of cell cycle progression genes that included cyclin A2 and cyclin B2 and the expression of forkhead box protein was restricted to the proliferation of cells. Table 2 indicates that gene expression of not only forkhead box M1 but also cyclin A2 and cyclin B2 were suppressed in the stable phase. Many researchers reported that the expression and activation of cell cycle-related genes were affected by some additives and drugs, resulting in cell cycle arrest and growth inhibition of HepG2 (27–31). Therefore, we assumed that the cell cycle was arrested in the stable phase.

**BrdU incorporation** To confirm our hypothesis, we added BrdU to the medium and fixed the cells 24 h after the incorporation of BrdU. If the cells were able to proliferate, then BrdU would be incorporated into the cells. Thin sections were prepared and immunostained using the anti-BrdU antibody. Photographs of immunostained sections are shown in Fig. 6. It was found that the ratio of stained cells markedly decreased on day 17. The gene expression of *cdc2* is well known to be required for both G1/S (start-point) and G2/M transitions. Since the gene expression of *cdc2* was suppressed on day 17 (Table 2), we assumed that cells in the stable phase remained in the G0/G1 phase. In addition, Loyer *et al.* (32) reported that in a normal adult liver, hepatocytes were arrested in the G0 phase, and *cdc2*, cyclin A, and cyclin B were not produced in adult hepatocytes. Our findings are in agreement with their results.

## DISCUSSION

Various types of bioreactors have been developed to support the growth of high-density three-dimensional cell cultures. In particular, cultures using microcarriers and hollow-fiber modules are well known and many types of investigations using these have been reported. Werner *et al.* (33) reported that the hepatocyte-derived cell line, HepZ, was grown on a microcarrier in a 1-l scale bioreactor at  $7.1 \times 10^6$  cells/ml, and this cell line formed a multilayer on the microcarrier surface. Jasmund *et al.* (34) indicated that the new concept of the hollow-fiber reactor supported a high-density culture ( $2.5 \times 10^7$  cells/ml) for 25 d. However, the working

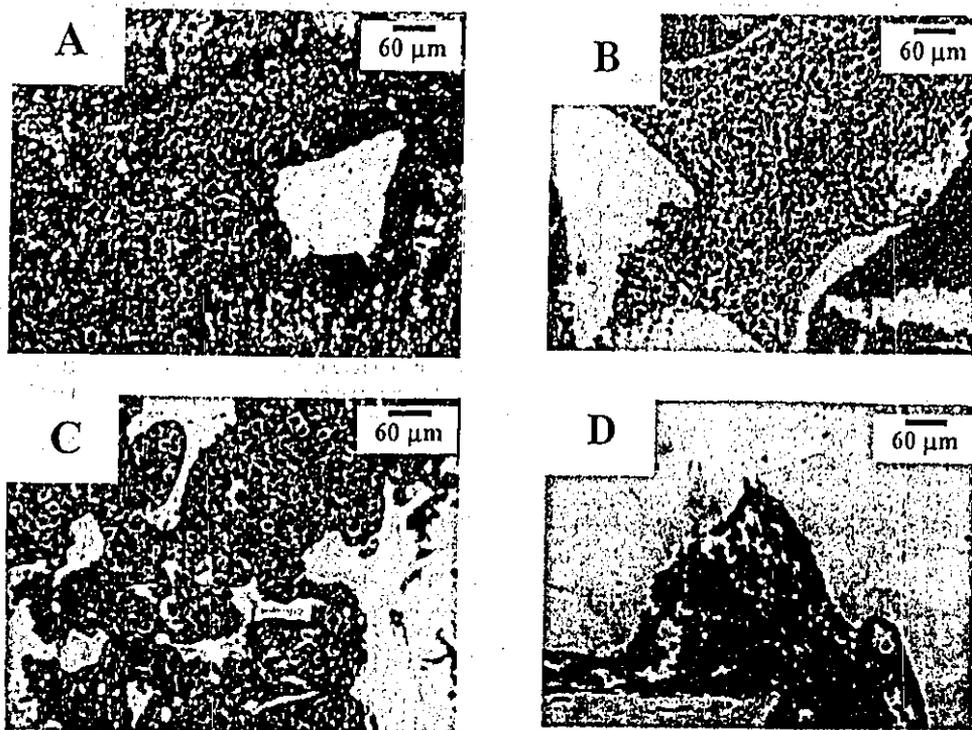


FIG. 3. Thin-section observation of each scaffold after 7 d cultivation in 5-ml RFB. The sections were stained with Toluidine blue. The photographs show hydroxyl apatite (A), calcined cattle bone calcium powder (B), porous PVA resin (C), and polyurethane foam (D). Bars indicate 60  $\mu\text{m}$ .

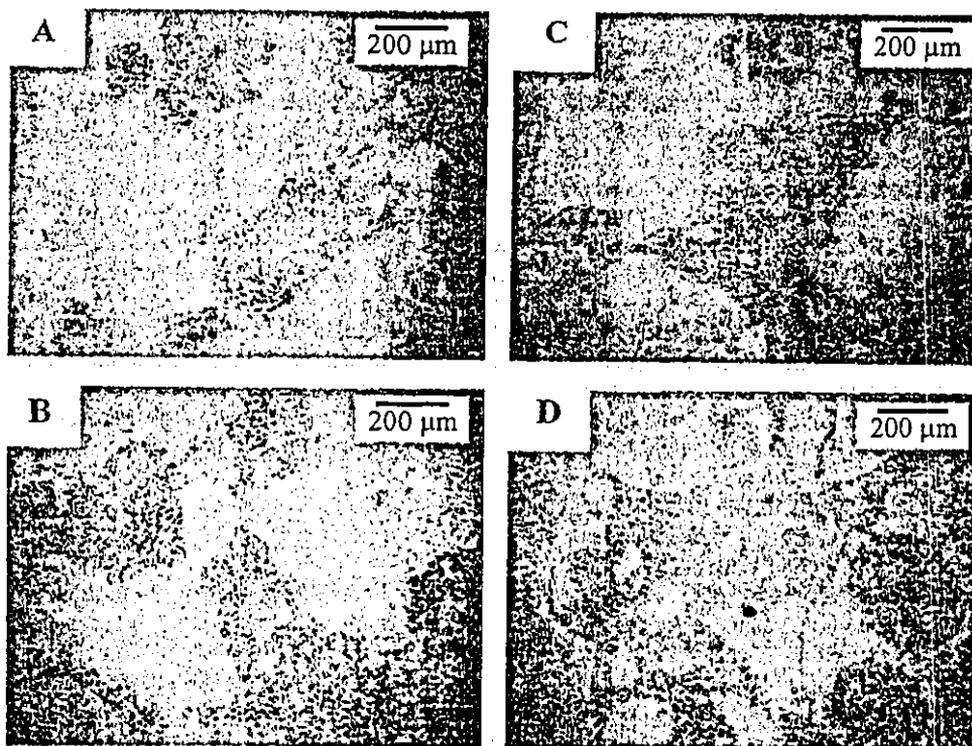


FIG. 6. Immunohistochemical staining using anti-BrdU antibody. Cells were cultured in the 5-ml RFB for 7 d in panels A and B, and for 17 d in panels C and D. BrdU was added to the medium 24 h before harvesting. Representative photographs show the incorporation of BrdU into freshly synthesized DNA in 5-ml RFB. Bars indicate 200  $\mu\text{m}$ .

volume of this reactor was over 100 ml and the reactor operators needed to prepare high numbers of cells for inoculation. Furthermore, a large amount of medium is required to maintain high-density culture. The RFB is one of the bioreactor types used for high-density three-dimensional cell culture, and the use of RFBs can enhance the cellular functions of the cultured cells (14–17). The cell density in the RFB was 4- to 15-fold higher than that in the microcarrier and hollow-fiber cultures. In addition, RFBs could achieve a uniform distribution of cultivated cells in the reactor. However, a detailed molecular biological analysis of the cellular status of the high-density three-dimensional cell culture in RFBs has not yet been sufficiently performed. The previous RFBs required substantial space for their installation, and a large amount of culture medium and cells for inoculation similar to the microcarrier or hollow-fiber cultures. In the present study, we developed a new RFB with a 5-ml capacity and evaluated the cellular status using the hepatoblastoma cell line, HepG2. We confirmed that the 5-ml RFB could be used to cultivate HepG2 at high density and predicted that a cell density of over  $1 \times 10^8$  cell/ml-scaffold could be achieved; furthermore, this density was almost similar to that predicted for the previous types of RFB (Fig. 1). The 5-ml RFB does not require much space for its installation, and it is easy to handle compared with the previous types of RFBs. Therefore, we were able to perform multiple experiments concurrently. Among the various types of scaffolds, the use of hydroxyl apatite and PVA resin helped achieve high density (Figs. 2 and 3). Therefore, we concluded that the 5-ml RFB could cultivate adhesive cells at a high density when a suitable scaffold was selected.

For long-term cultures in the 5-ml RFB, glucose consumption and albumin production were maintained for over 7 d (Figs. 4 and 5). Morsiani *et al.* (4) reported that the hollow-fiber reactor could be used to cultivate hepatocytes for 14 d, but the cellular functions were not stable. Fukuda *et al.* (35) developed the polyurethane foam packed-bed (PUF) module and cultivated HepG2 at a high density ( $4.8 \times 10^7$  cells/cm<sup>3</sup>-module) with a three-dimensional structure. The albumin production rate of HepG2 in a 7-d culture using the PAF module was 184  $\mu$ g/cm<sup>3</sup>-module/d. However, this production rate was higher than that obtained in long-term cultures cultivated in the 5-ml RFB (Fig. 5). Miyoshi *et al.* (36) reported that the fetal liver cells could be cultivated in polyvinyl foam (PVF) resin at a high density and confirmed this albumin production rate. They were able to successfully maintain the high-density cell culture ( $7 \times 10^7$  cells/cm<sup>3</sup>-PVF resin) for over 30 d; however, after the first two weeks, a decrease was observed in the albumin production rates. Figure 5 shows a good correlation between glucose consumption and albumin production. Thus, we assumed that the albumin production rate per cell was maintained during cell culturing in the 5-ml RFB. The 5-ml RFB proved to be advantageous in comparison with the previous types of RFBs — a long-term culture with higher cellular function could be performed. A comparison of DNA microarray analysis between the growth phase and the stable phase indicated that the expression of cell cycle-related genes had decreased in the stable phase (Table 2). In addition, an examination of BrdU incorporation showed that the ratio of cells which had incorporated

BrdU in the stable phase had markedly decreased when compared with that in the growth phase (Fig. 6). Some additives, as well as the depletion of nutrients, are known to trigger cell cycle arrest (27–31). Sugiyama *et al.* (37) demonstrated that glycerol had a potent inhibitory effect on hepatocyte proliferation. Yamashita *et al.* (38) indicated that trichostatin A inhibited cell proliferation and induced arrest at the G0/G1 phase in HepG2. In our study, we did not incorporate any of these additives into the culture media. Therefore, we can assume that our results were due to other mechanisms. From the results of the immunostaining (Fig. 6), a larger number of cells in the stable phase were found to remain in the G0/G1 phase, similar to the mature liver (32). Although we should investigate the induction and expression level of drug metabolism-related proteins in the stable phase, it was concluded from our results that HepG2 cells in the stable phase in long-term cultures are attractive as an alternative tool to the liver for the long-term testing of drug toxicity and drug metabolism.

In conclusion, we developed a 5-ml RFB and our results are the first demonstration of cellular status in a high-density three-dimensional cell culture. The 5-ml RFB may contribute to the evolution of an alternative methodology for drug testing and regenerative medicine.

#### ACKNOWLEDGMENTS

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## Improvement in Reliability of Probabilistic Test of Significant Differences in GeneChip Experiments

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A probabilistic test (FUMI theory) for GeneChip experiments has been proposed for selecting the genes which show significant differences in the gene expression levels between a single pair of treatment and control. This paper describes that the reliability of the judgment by the FUMI theory can be enhanced, when the selected genes are referred to biomolecular-functional networks of a commercial database. The genes judged as being differently expressed are grouped into a cluster in the biomolecular networks. It is also demonstrated that false positive genes have a trend in the networks to be isolated from each other, and also away from the clustered genes, since the false positive genes are randomly selected.

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### Introduction

The GeneChip technology has recently made rapid progress, but some important problems still remain open. For example: 1) The GeneChips are expensive, and the replication of experiments is not easy; 2) The GeneChips generate tens of thousands of data for every experiment, and a new method for handling such voluminous data efficiently is desired.

In a previous paper,<sup>1</sup> we put forward a method for detecting significant changes between two different conditions from a single pair of experiments, that is, treatment and control. The method is called FUMI theory (FUncion of Mutual Information).<sup>2,3</sup> Under the condition of the paper, out of 12559 genes on the chip, 200 - 310 genes were selected as differently expressed with 1% risk. Due to the risk of 1%, however, 126 genes must be falsely selected. This number of false positive genes is critical compared to the totally selected genes (200 - 310).

The simplest solution to the problems will be to refer to the results of repeated experiments under the same conditions.<sup>4</sup> It is quite probable that the genes of true differences in the expression will be selected once and again by the replicates. On the other hand, the false positive genes will be selected at random, and not many times.

This paper proposes a method to make the judgment based on the FUMI theory more reliable, *i.e.*, a method to distinguish between the true positive and false positive, even from a single pair of treatment and control experiments. For this purpose, a commercially available database which provides a biomolecular-functional network is integrated.

### Experimental

The details of experiments for the microarray analysis were previously described.<sup>1</sup> Human promyelocytic leukemia cell line (HL60) cells were exposed to 20 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) for 9 h and biotin-labeled cRNA was prepared and stored as a stock solution for later hybridization. A total of four GeneChip arrays (Human Genome U95A set, Affymetrix, Inc) were used for hybridization (two with the TPA-exposed stock solution and two with the control stock solution). One of four combinations of exposure and control experiments was taken as an example in the text. The others were used for reference.

The network of proteins (Fig. 1) was drawn with a commercial database (KeyMolnet, Institute of Medicinal Molecular Design Inc., Tokyo).

### Theory

In the FUMI theory, the *a priori* SD,  $\sigma$ , of microarray fluorescence measurements is described as a function of the averaged measurements,  $X$ :

$$\sigma = \sqrt{0.009636X^2 + 91897.8} \quad (1)$$

This relationship was obtained in our previous study from six replicate samples which were different from the target samples. Let  $X_E$  be the expression level (measurement) of a gene for the exposed sample and  $X_C$  be the measurement of the gene for the control sample.  $\sigma$  is given by Eq. (1) as  $X = (X_E + X_C)/2$ . The judgment of the significant differences is performed based on the inequality:

$$\frac{|X_E - X_C|}{\sqrt{2}\sigma} > 2.58 \quad (2)$$

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