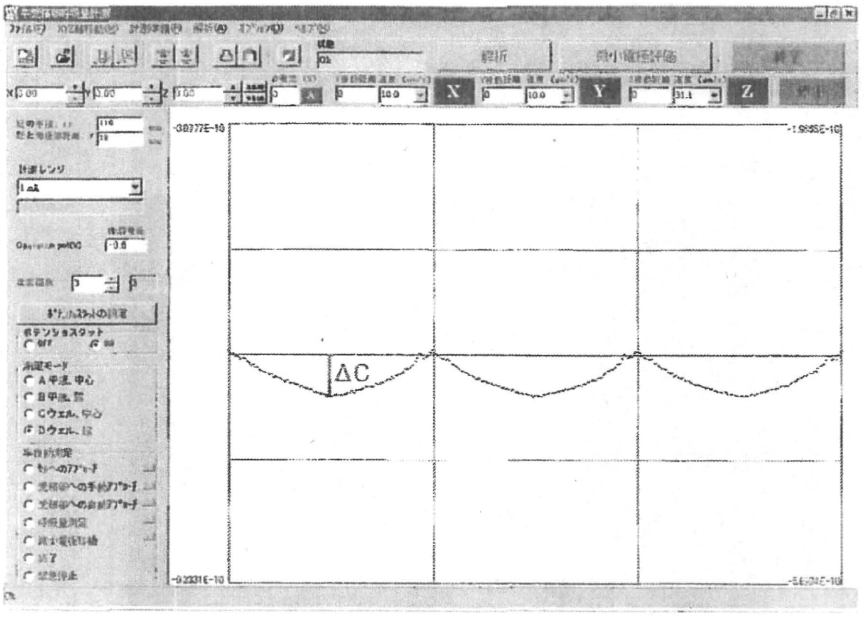

図3　呼吸解析ソフトの画面
マイクロ電極の走査の始点と終点の電流値の差（ΔC）から胚の酸素消費量（呼吸）を算出する．

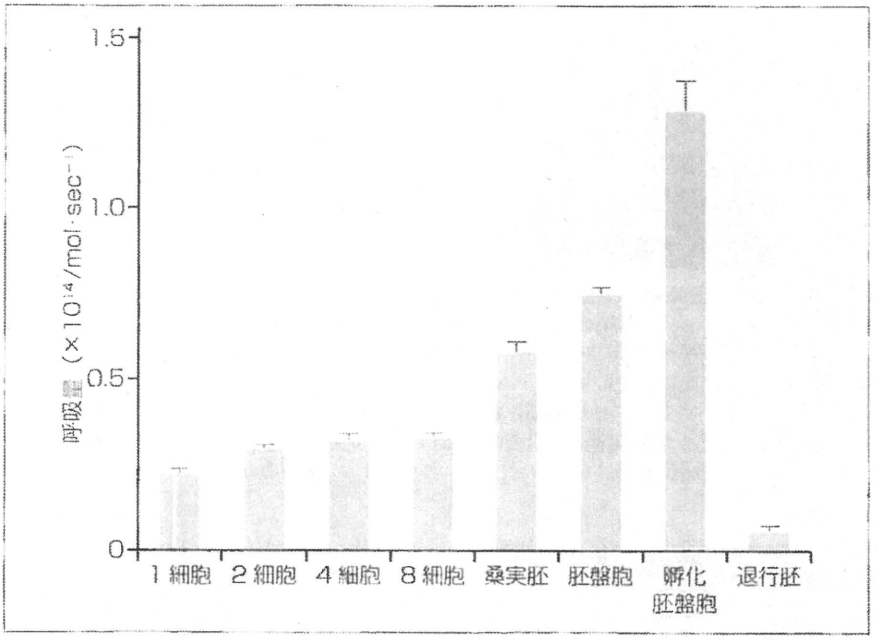

図4　マウス胚の発生過程における呼吸量変化
桑実胚から胚盤胞期にかけて呼吸量が増加する．

あり，発生が進んだ胚の呼吸活性は高い傾向にある．電子顕微鏡を用いたミトコンドリアの微細構造解析により，day 5胚では発達したミトコンドリアが多く観察される．このようにヒト胚においても，受精卵呼吸測定装置を用いたミトコンドリア呼吸機能解析研究が進んでいる．

今後，IVF-ETを中心とする不妊治療成績の向上には，移植の対象となる胚のクオリティ評価がこれまで以上に重要になってくる．精度の高い胚クオリティ評価法確立のためには，現行の形態的評価法と比べて客観性の高いクオリティ評価法の開発が不可欠である．

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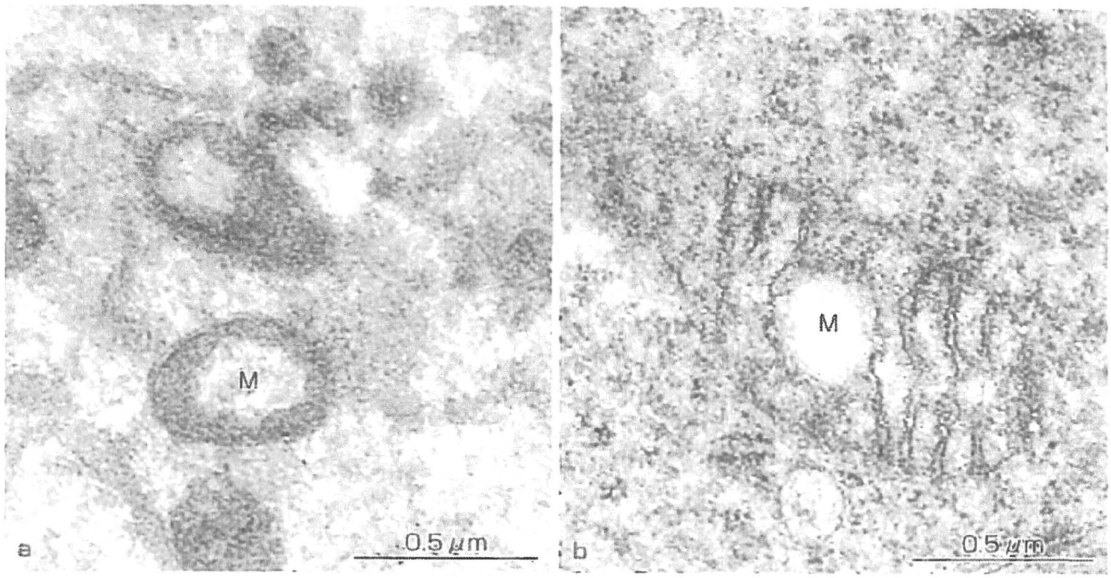

図5　マウス胚のミトコンドリアの微細形態
a：2細胞期胚，b：胚盤胞．M：ミトコンドリア．

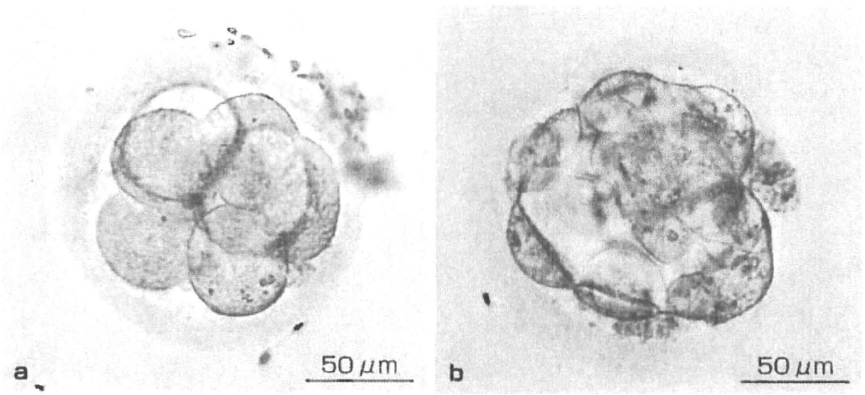

図6　ヒト体外受精胚の形態と酸素消費量
a：day 3 胚 (0.45×10^{-14} mol・sec^{-1})，b：胚盤胞 (1.15×10^{-14} mol・sec^{-1})．

本稿で述べた受精卵呼吸測定装置は，高精度・非侵襲的に胚の呼吸代謝機能を解析できることから，新しい胚のクオリティ評価の有効な基盤技術として期待できる．

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電気化学計測技術を応用したシングルセル呼吸機能解析と応用

阿部宏之*

＊　山形大学　大学院理工学研究科　物質化学工学分野　教授

シングルセル解析の最前線」
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5　電気化学計測技術を応用したシングルセル呼吸機能解析と応用

阿部宏之*

5.1　はじめに

ミトコンドリアは生命活動に必要なエネルギーの生産やアポトーシスなど重要な生物現象に関与しており，その機能が障害されると代謝異常や種々の疾患の原因となる。また，ミトコンドリアの呼吸活性は細胞の代謝活動や機能評価の有効な指標となることから，シングルセルレベルでの高精度呼吸計測技術は，細胞のクオリティー評価や疾患の診断などに極めて有効な手段となる。本節では，局所領域における生体反応をリアルタイムで追跡できる電気化学計測技術を応用した高精度・非侵襲細胞呼吸計測システムを解説するとともに，この技術を応用したシングルセルレベルでの呼吸機能解析や細胞間ネットワーク解析，受精卵クオリティー評価への応用研究を紹介する。

5.2　マイクロ電極を用いた細胞呼吸測定装置

電気化学計測法はプローブ電極による酸化還元反応を利用し，局所領域における生物反応を電気化学的に検出する技術であり[1,2]，この技術の有効な装置としてマイクロ電極を探針とする走査型電気化学顕微鏡（Scanning electrochemical microscopy：SECM）が注目されている。SECMの空間分解能は探針であるマイクロ電極径に依存するため原子や分子レベルの解析は困難であるが，局所空間での化学反応の評価やイメージング，生体材料を用いたリアルタイム解析や化学反応誘起が可能であることから，局所領域の電気化学センシングなど種々の系で用いられている[3-6]。例えば，酸素の還元電位を検出できるマイクロ電極を用いることで細胞の酸素消費量（呼吸）を高感度・非侵襲的にリアルタイムで測定することができる（図1）。

従来，SECMは微量な酸素消費を検出できることから，金属錆の検出装置として用いられてきた。筆者らは，従来型SECMを生物試料，特に受精卵の呼吸計測に応用するために，呼吸測定に関連した要素技術の開発を行ってきた。その結果，SECMをベースに受精卵や微小組織などの球状試料の酸素消費量を非侵襲的に測定できる「受精卵呼吸測定装置」の開発に成功した[7]。この呼吸測定システムは，倒立型顕微鏡，マイクロ電極の電位を一定に保持するポテンショスタット，マイクロ電極の移動を制御するコントローラー，酸素消費量算出のための専用解析ソフトを内蔵したノート型コンピューターにより構成されている（図2）。倒立型顕微鏡のステージ上には，マイクロ電極の3次元走査を可能とするXYZステージが設置されており，生物試料の呼吸計測のために気相条件制御が可能な測定用チャンバーや保温プレートが設置できる。また，計測

* Hiroyuki Abe　山形大学　大学院理工学研究科　物質化学工学分野　教授

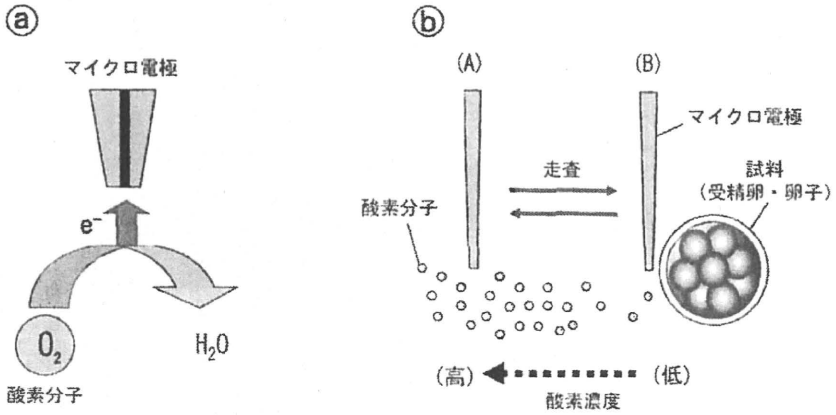

図1　マイクロ電極を用いた受精卵呼吸測定法
ⓐマイクロ電極は酸素の還元電位を検出する。
ⓑ走査型電気化学顕微鏡による呼吸測定。呼吸により胚近傍の溶存酸素が減少するため，沖合との間に溶存酸素の濃度勾配が生じる。その酸素濃度差（電流値の差：ΔC）から球面拡散理論式[8]を用いて試料の酸素消費量を算出する。

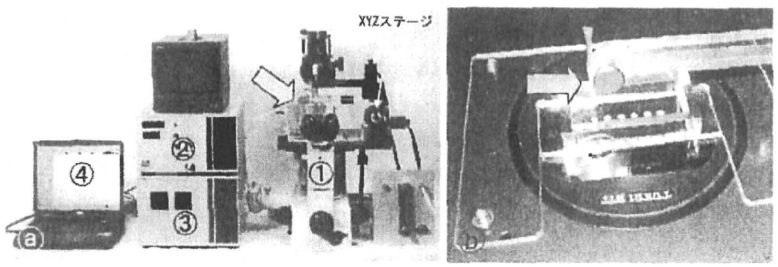

図2　走査型電気化学顕微鏡を改良した「受精卵呼吸測定装置」（北斗電工㈱製）
①倒立型顕微鏡，②ポテンショスタット，③コントローラー，④ノートパソコン（呼吸解析ソフトを内蔵）。矢印は，専用の測定チャンバーを示す。
ⓑ測定ステージ部。矢印は，ホルダーに設置したマイクロ電極を示す。

精度と操作性の向上を目的に，マイクロ電極の改良と専用の多検体測定プレートおよび測定液の開発を行っている。従来型SECMは，金属などの腐食部位を検出する装置として先端径10～20 μmのマイクロ電極を探針として用いているが，酸素消費量が非常に小さい細胞や受精卵の呼吸測定には電極の感度が不十分であるため，より高感度のマイクロ電極が必要である。マイクロ電極の計測感度は先端径が小さいほど高いため，受精卵や細胞の呼吸測定では先端径2～4 μmにエッチング加工した白金電極をガラスキャピラリー先端部に封止したディスク型マイクロ電極（図3ⓐ，ⓑ）を使用している。

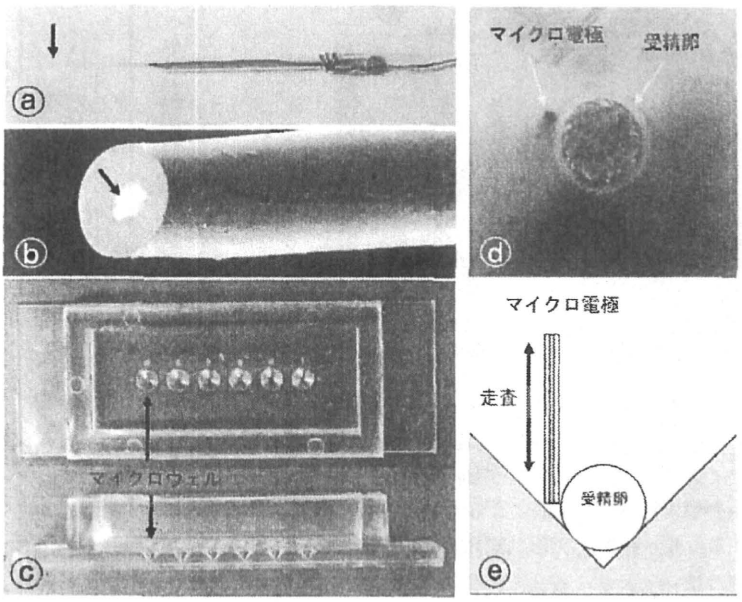

図３　受精卵呼吸測定用のマイクロ電極ⓐと多検体測定プレートⓒ
ⓐディスク型白金マイクロ電極。
ⓑマイクロ電極の走査電子顕微鏡像。先端部（矢印）が直径２～４μmにエッチング加工された白金電極がガラスキャピラリーに封止されている。
ⓒ多検体プレート底面には円錐形のマイクロウェルが６穴施されている。
ⓓマイクロウェル底部に静置したウシ胚。
ⓔマイクロ電極を胚に対して鉛直方向に走査し酸素消費量を測定する。

5.3　単一受精卵の呼吸量測定

「受精卵呼吸測定装置」を用いた呼吸測定には，専用の多検体測定プレートと測定液を用いる。多検体測定プレートは測定操作の簡易化を目的に開発され，プレートの底面には逆円錐形のマイクロウェルが施されている（図３ⓒ）。マイクロ電極が検出する微弱な電流は溶液の成分によって影響を受けるため，呼吸量測定にはマイクロ電極の測定感度に影響を与えず，胚に対して無侵襲な専用の測定液を用いる。測定液を満たしたマイクロウェル内に試料（胚）を導入した後，マイクロウェルの底部中心に静置させる（図３ⓓ）。胚のサイズ（半径値）を解析ソフトに入力した後，マイクロ電極を胚の透明帯直近に手動で移動する。マイクロ電極は酸素が還元可能な−0.6 V *vs.* Ag/AgClに電位を保持した後，移動速度20～30 μm/sec，走査距離150～300 μmの条件に設定し，コンピューター制御により透明帯近傍を鉛直方向に自動的に走査する（図３ⓔ）。通常，１回の呼吸測定ではマイクロ電極を２～３回走査した後，胚の酸素消費量は球面拡散理論式[8,9]を基本に開発した解析ソフトを用いて算出する。

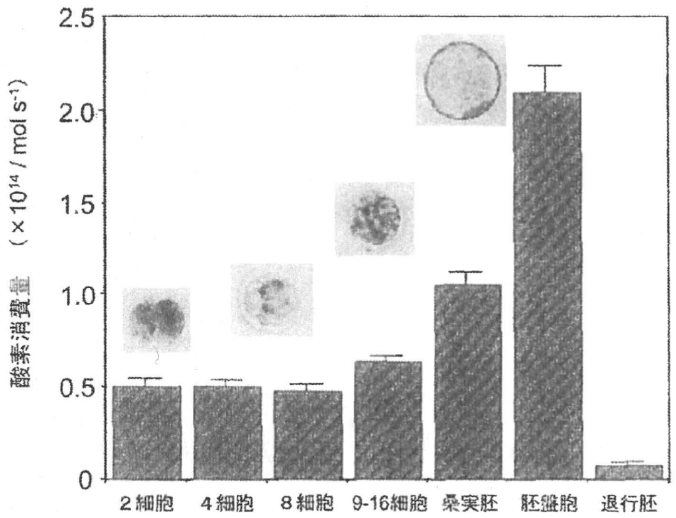

図4　ウシ体外受精胚の発生過程における呼吸量変化
桑実胚から胚盤胞期にかけて呼吸量が増加する。退行胚ではほとんど呼吸量は検出されない。

これまでに「受精卵呼吸測定装置」を用いて，ウシ，ブタ，マウスの単一胚の呼吸量測定を行っている。ほとんどの動物胚において，８細胞期までの発生初期では酸素消費量は少なく，桑実胚から胚盤胞にかけて顕著に呼吸量が増加する（図４）。呼吸測定の有用性を評価するために呼吸能とミトコンドリアの関係を調べた結果，呼吸活性の低い８細胞期まではほとんどのミトコンドリアは未成熟であるが，桑実胚から胚盤胞にかけてミトコンドリアの顕著な発達（クリステの拡張）が認められた[10]。このように，ミトコンドリアの発達と呼吸量の増加が一致して起こることから，「受精卵呼吸測定装置」はミトコンドリア呼吸を高精度でモニタできることがわかる。

5.4　呼吸測定による受精卵の品質評価

体外受精・胚移植技術は，不妊治療の最も有効な治療法となっている。不妊治療では，質的に良好な胚の選択は妊娠率向上，多胎妊娠回避，流産率低下のために不可欠である。現在，胚の品質は割球の数や形態を基準に評価されているが，このような形態的特徴は定量性に欠けるため判定基準が観察者の主観に左右される可能性が指摘されている。筆者らは，形態良好胚ではミトコンドリアは正常に発達しているが，形態不良胚や発生停止胚ではミトコンドリアの多くは未成熟あるいは退行していることを発見した[11-13]。そこで，ミトコンドリア呼吸活性を指標に受精卵の品質を評価するという独創的発想に至り，「受精卵呼吸測定装置」を用いた精度の高いシングルセルレベルでの細胞呼吸計測システム開発を進めてきた。これまでに，ウシを用いた動物実験によって，呼吸活性の高い胚は呼吸測定後に追加培養を行うと高い確率でクオリティー良好な胚

表１　ウシ胚の呼吸量と妊娠率の関係[9]

移植時の発生ステージ	酸素消費量（$F\times10^{14}$/mol s^{-1}）	受胎胚数/移植胚数（妊娠率%）
胚盤胞	$F\geqq1.0$	21/36 (58.3)
	$F<1.0$	0/6 (0)
初期胚盤胞	$F\geqq0.8$	16/25 (64.0)
	$F<0.8$	0/6 (0)
桑実胚	$F\geqq0.5$	17/28 (60.7)
	$F<0.5$	1/12 (8.3)

盤胞へと発生すること[11]，凍結時に呼吸量の大きい胚盤胞は融解した後の生存率も良好であることを明らかにしている[15]。さらに，呼吸測定後の胚を借腹牛に移植し胚の呼吸活性と受胎率の関係を調べた結果，移植前の呼吸量が基準値以上（胚盤胞で1.0×10^{14}/mol s^{-1}，初期胚盤胞で0.8×10^{14}/mol s^{-1}，桑実胚で0.5×10^{14}/mol s^{-1}）の胚を移植した場合，60%以上の高い確率で妊娠するが，基準値に満たない胚のほとんどは受胎しないことが確認されている（表１）。このように，「受精卵呼吸測定装置」を用いた呼吸測定は，妊娠が期待できる品質良好胚の有効な選別法になると考えられる。

5.5　呼吸測定システムの医療応用

「受精卵呼吸測定装置」は，短時間で非侵襲的に細胞の呼吸量を測定できることから，不妊治療や移植医療における受精卵や微小組織のクオリティー評価への応用が可能である。ウシやマウスを用いた動物実験により，呼吸測定した胚を移植し誕生した個体の染色体や行動などを調べているが，染色体異常や奇形，行動異常などの事例は確認されていない。電気化学呼吸計測技術は，医療応用へ向けて安全面での問題もクリアできると考えられる。現在，探索的臨床研究として「受精卵呼吸測定装置」を用いたヒト胚の呼吸量測定と品質評価システムの確立を試みている。これまでに，ヒト胚（余剰胚）ではミトコンドリアの発達に伴い呼吸量が増加すること（表２，図５），呼吸活性の高い胚は胚盤胞への発生率が高い傾向にあることが示されている[16]。これらの研究成果を踏まえ所定の倫理承認を得た後，不妊治療への臨床応用を目的に呼吸測定胚の移植試験を予定している。

表2　ヒト胚（余剰胚）の胚発生過程における酸素消費量変化

発生ステージ	胚　数	酸素消費量（$F\times10^{14}$/mol s^{-1}）
2-8細胞	18	0.51±0.05
桑実胚	5	0.61±0.11
初期胚盤胞	13	0.72±0.06
胚盤胞	4	1.01±0.02

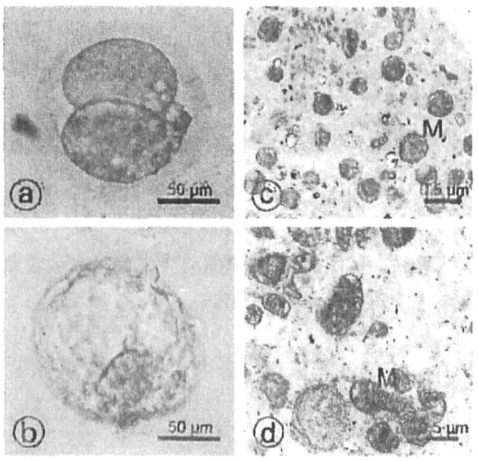

図5　ヒト胚におけるミトコンドリアの超微細形態変化
ⓐ，ⓒ2細胞期胚，ⓑ，ⓓ胚盤胞。
ⓐ，ⓑ光学顕微鏡像，ⓒ，ⓓ電子顕微鏡像。M：ミトコンドリア。

5.6　シングルセル呼吸測定と細胞間ネットワーク解析

本節の最後に，哺乳類卵子をモデル系とする細胞間ネットワーク解析研究を紹介する。卵巣から回収した直後の卵子は数層の卵丘細胞に被われており（卵丘細胞—卵子複合体：COC），卵丘細胞と卵子はギャップ結合を介した細胞間ネットワークを形成している（図6）。卵子—卵丘細胞間のギャップ結合は，卵丘細胞と卵子間の物質交換に関与することで，卵子の成長や機能成熟に極めて重要な役割を果たしている。例えば，未成熟卵子では減数分裂抑制作用を示す環状AMP（cyclic AMP）が卵丘細胞から卵子に供給されている[17]。一般に，卵巣から採取した卵子は未成熟であり，血清や成長因子を加えた培地で成熟培養を行う。そこで，「受精卵呼吸測定装置」を用いて単一のCOCおよび卵子の呼吸量を測定し，卵子成熟過程における呼吸活性と細胞間ギャップ結合の関連を調べた。その結果，卵子は成熟の進行に伴い呼吸活性が顕著に高くなるが，一方の卵丘細胞は成熟培養により卵丘細胞層が膨潤し，卵子—卵丘細胞間ギャップ結合は呼吸活性の著しい低下とともに消失することが示された（図7）。

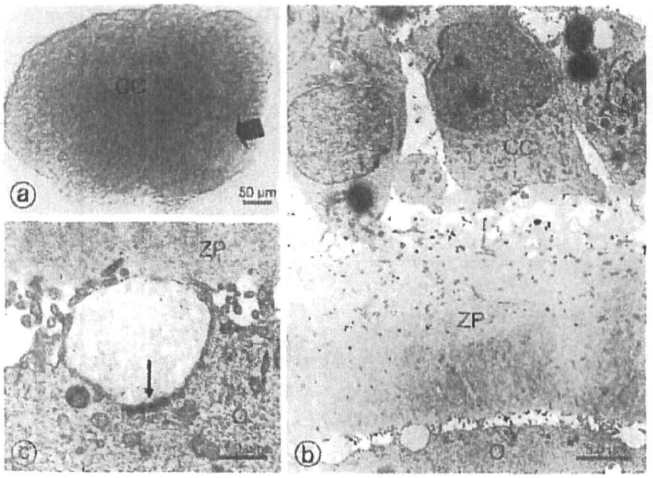

図６　ヒト卵丘細胞―卵子複合体（COC）の形態
ⓐ光学顕微鏡像。矢印は，卵子を示す。
ⓑ，ⓒ電子顕微鏡像。矢印は，卵丘細胞と卵子間のギャップ結合を示す。
CC：卵丘細胞，O：卵子，ZP：透明帯。

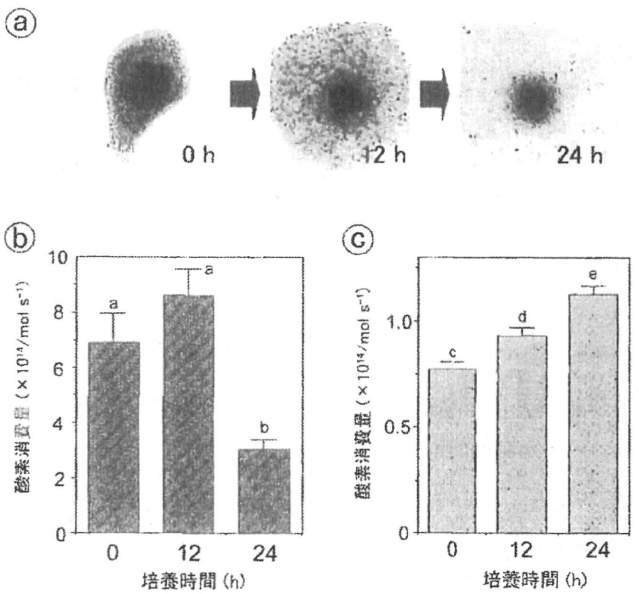

図７　ウシ卵子成熟過程における呼吸活性変化
ⓐ卵子成熟過程における卵丘細胞層の膨潤。
ⓑ卵丘細胞―卵子複合体（COC）の酸素消費量。
ⓒ卵子の酸素消費量。

卵子は単一の細胞であるため，受精卵のように割球の数や形態を基準に品質を評価することは困難である。このため，卵細胞質の形態（透明度や顆粒の分布状態など）や卵丘細胞の付着状態を基準にクオリティーが評価されている[17~19]。一般に，卵丘細胞が密にほぼ均一に付着し卵丘細胞間とのギャップ結合が正常に発達している卵子は，成熟率が高くクオリティー良好胚へと発生する割合も大きい[20,21]。このことは，卵子と卵丘細胞の細胞間ネットワークが正常に機能していることが卵子の機能維持に極めて重要であることを示している。「受精卵呼吸測定装置」による呼吸測定は，卵子―卵丘細胞間のネットワークをリアルタイムで解析することができ，これまで困難であった卵子品質評価の有効な方法になるものと期待される。

5.7 おわりに

本節で解説した電気化学計測技術は，細胞呼吸を無侵襲的に測定できることから，細胞の代謝機能解析，受精卵や卵子の品質診断システムへの応用が期待できる。さらに，この技術は超高感度計測であることからシングルセルレベルでの呼吸能解析やミトコンドリア機能解析が可能であり，卵子においては卵子―卵丘細胞間ネットワーク解析に有用な方法であることが示された。細胞呼吸は動物・植物問わずほとんどの細胞に共通する生物現象であることから，電気化学計測技術および装置の応用範囲は極めて広い。今後，筆者らが開発した「受精卵呼吸測定装置」がシングルセル機能解析や不妊治療などの医療において活用されることを期待している。

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Attenuation of Cross-Talk Between the Complement and Coagulation Cascades by C5a Blockade Improves Early Outcomes After Intraportal Islet Transplantation

Kazuaki Tokodai,[1] Masafumi Goto,[1,2,6] Akiko Inagaki,[2] Wataru Nakanishi,[1] Norihiko Ogawa,[1] Kazushige Satoh,[1] Naoki Kawagishi,[1] Satoshi Sekiguchi,[1] Bo Nilsson,[3] Noriko Okada,[4] Hidechika Okada,[5] and Susumu Satomi[1]

Background. Complement 5a factor (C5a) elicits a broad range of proinflammatory effects, including chemotaxis of inflammatory cells and cytokine release. C5a is also linked to the coagulant activity in autoimmune diseases. Therefore, C5a most likely plays a crucial role in the instant blood-mediated inflammatory reaction.
Methods. Intraportal transplantation of 2.5 islet equivalents/g of syngeneic rat islet grafts was performed in two groups of streptozotocin-induced diabetic rats: controls and C5a inhibitory peptide (C5aIP)-treated group.
Results. The thrombin-antithrombin complex was significantly suppressed in the C5aIP group ($P=0.003$), and both the curative rate and the glucose tolerance were significantly improved in the C5aIP group ($P<0.05$ and $P<0.005$, respectively). Expression of tissue factor on granulocytes in recipient livers was up-regulated 1 h after islet infusion ($P<0.0001$), which was significantly suppressed by C5aIP ($P<0.005$). However, C5aIP was unable to regulate tissue factor expression on isolated islets. Furthermore, no differences were detected between the groups, regarding infiltration of CD11b-positive cells and deposition of C5b-9 on the islet grafts.
Conclusions. These data suggest that C5aIP attenuates cross-talk between the complement and coagulation cascades through suppressing up-regulation of tissue factor expression on leukocytes in recipient livers but not on islet grafts, a process leading to improvement in islet engraftment. Therefore, C5aIP in combination with conventional anticoagulants could be a strong candidate strategy to control the instant blood-mediated inflammatory reaction induced in clinical islet transplantation.

Keywords: Islets, Transplantation, Complement, C5a, Tissue factor.

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Islet transplantation is a promising clinical strategy for type 1 diabetic patients. Although the protocol, introduced by Shapiro et al. (*1*), has greatly improved the clinical outcomes using this strategy, islets derived from, on average, two to four donor pancreata are still needed to cure a diabetic patient. The instant blood-mediated inflammatory reaction (IBMIR), characterized by activation of both the coagulation and complement cascades, is one of the major obstacles to successful islet engraftment (*2–4*). We have shown that a low-molecular weight dextran sulfate efficiently attenuates xenogeneic IBMIR triggered by porcine islets (*3, 5*). However, it was also found that low-molecular weight dextran sulfate alone was less effective in inhibiting complement activity but effective as an anticoagulant (*5, 6*).

In the complement system, complement 5a factor (C5a) is well known to elicit a broad range of proinflammatory reactions including chemotaxis, cell activation, and cytokine release (*7–10*). However, few studies have been performed on how C5a influences the IBMIR. Recent studies have demonstrated that there is an interaction between the coagulation and complement systems in several situations (*11, 12*). Of special interest is that C5a, through its receptor

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[1] Division of Advanced Surgical Science and Technology, Tohoku University, Sendai, Japan.
[2] Tohoku University International Advanced Research and Education Organization, Tohoku University, Sendai, Japan.
[3] Division of Clinical Immunology, the Rudbeck Laboratory, University Hospital, Uppsala, Sweden.
[4] Department of Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan.
[5] Choju Medical Institute, Fukushimura Hospital, Toyohashi, Japan.
[6] Address correspondence to: Masafumi Goto, M.D., Ph.D., Tohoku University International Advanced Research and Education Organization, Tohoku University 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-0872, Japan.
E-mail: gotokichi@aol.com

M.G. participated in research design and the writing of the manuscript; A.I., W.N., N.O., K.S., N.K., and S.S. participated in the performance of the research; B.N., N.O., and H.O. contributed new reagents or analytic tools; and S.S. participated in the writing of the manuscript.

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C5aR, has been shown to mediate the exposure of tissue factor (TF) on neutrophils, and thereby significantly enhance the procoagulant activity in them (*13*). Therefore, in islet transplantation, the regulation of C5a might contribute to the suppression of the IBMIR through TF inhibition in the host neutrophils or by preventing proinflammatory reactions.

It is well known that there are two types of receptors, C5aR and C5L2, for C5a (*14*). C5a is known to mediate its proinflammatory effects through interaction with C5aR, which was reported to be expressed by various cells (*15*). C5L2 has been regarded as a default receptor for C5a (*14, 16, 17*). However, recent studies have suggested that C5L2 is capable of mediating the biologic activities of the complement anaphylatoxins C5a and C3a through mitogen-activated protein kinase activation (*18, 19*). Rittirsch et al. (*20*) reported that the role of C5L2 is specifically linked to the release of high-mobility group box 1 (HMGB1), which has been identified recently as a potent lethal and procoagulant mediator (*21–23*). Furthermore, it was reported that pancreatic islets contain abundant HMGB1, which can be released into the circulation soon after islet transplantation into the liver (*24*).

C5a inhibitory peptide (C5aIP), a complementary peptide (ASGAPAPGPAGPLRPMF: m.w. 1594 Da) against an active region of C5a (RAARISLGPRCIKAFTE), has been shown to inhibit the intracellular Ca^{2+} mobilization in neutrophils and is effective to inhibit rapid lethal endotoxin shock in two rat models (*7, 25*). C5aIP, unlike the antibody to C5a, seems to be free from detrimental side effects because of its extremely low molecular mass and short half-life. C5aIP is currently in a phase II clinical trial for the treatment of sepsis.

In this study, we sought to evaluate the effect of C5aIP on islet engraftment. To elucidate the potential direct effect of C5aIP on islet grafts, we also analyzed the expression of complement receptors on isolated islets. We found C5a-derived TF, induced on the granulocytes of the recipients, as a novel target to inhibit the IBMIR. This shows that attenuation of cross-talk between the complement and coagulation cascades improves early outcomes after intraportal islet transplantation.

RESULTS

Inhibition of the IBMIR by C5aIP

The coagulation products, thrombin-antithrombin complex (TAT), regarded as the key marker of the IBMIR, increased immediately, peaked at 1 h after the islet infusion, and thereafter gradually decreased to the basal levels in the absence of C5aIP (Fig. 1A). Both the peak value and the area under the curve (AUC) of TAT were significantly suppressed in the presence of C5aIP (peak: 9.9±3.4 vs. 19.2±9.0 ng/mL, $P=0.01$; AUC: 1664±526 vs. 3487±1516 min ng/mL, $P=0.003$; Fig. 1A, B). At any time points examined during 24 h after islet transplantation, significant differences were not observed between the two groups for any of the cytokines investigated.

Effect of C5aIP on the Islet Engraftment

All diabetic rats used in this study were severely hyperglycemic (>400 mg/dL) before transplantation. Although none of the animals transplanted with 2.5 islet equivalents (IEQs)/g of recipients became normoglycemic, 4 of 6 rats (66.7%) transplanted with the same number of islets in the C5aIP group became normoglycemic during the study period ($P<0.05$; Fig. 2A). The glucose tolerance response was significantly ameliorated in the C5aIP group than that in control group (AUC: 20,396±2683 vs. 25,940±2021 min mg/dL, $P<0.005$; Fig. 2B). Furthermore, the insulin amount in the liver of the recipients was considerably higher in the C5aIP group (17.7±12.1 vs. 5.6±4.1 ng/IEQ, $P=0.06$; Fig. 2C). Notably, the body weight of the recipients was not affected by the treatment.

C5aIP Suppresses TF Expression on Granulocytes in the Recipient Livers After Intraportal Islet Transplantation

To elucidate the mechanism by which C5aIP effectively suppressed the coagulant activity, TF expression on granulocytes in the livers of the recipients was examined using fluorescence-activated cell sorting (FACS) analysis (Fig. 3A–D). Granulocytes were separated from the hepatic mononuclear cells by both forward scatter and side scatter in FACS analyses. The expression of TF on granulocytes was up-regulated within 1 h after islet infusion ($P<0.0001$), and the up-regulation was significantly suppressed by C5aIP ($P<0.005$).

Expression of Complement Receptors on Pancreatic Islets

Although C5a receptor (C5aR) was not detected in the pancreatic tissues before isolation procedures, both C5aR and

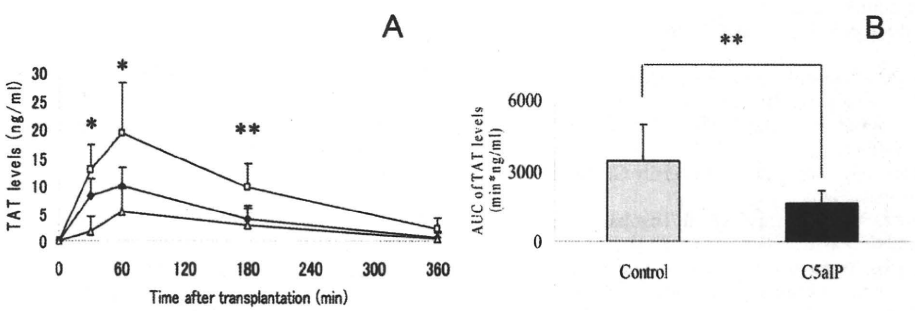


FIGURE 1. Inhibition of the instant blood-mediated inflammatory reaction by C5a inhibitory peptide (C5aIP) after intraportal islet transplantation. The ethylenediaminetetraacetic acid (EDTA)-treated blood was drawn from a jugular vein catheter of the transplanted rats treated with saline (square) or C5aIP (diamond) at varying time points after syngeneic islet transplantation. In addition, EDTA blood drawn from the rats to which the transplantation was not done served as a negative control (triangle). Thrombin-antithrombin complex (TAT) levels were assessed (A). The pretransplant values were subtracted from all the values. The area under the curve (AUC) of TAT levels was calculated (B). *P less than 0.05; **P less than 0.005.

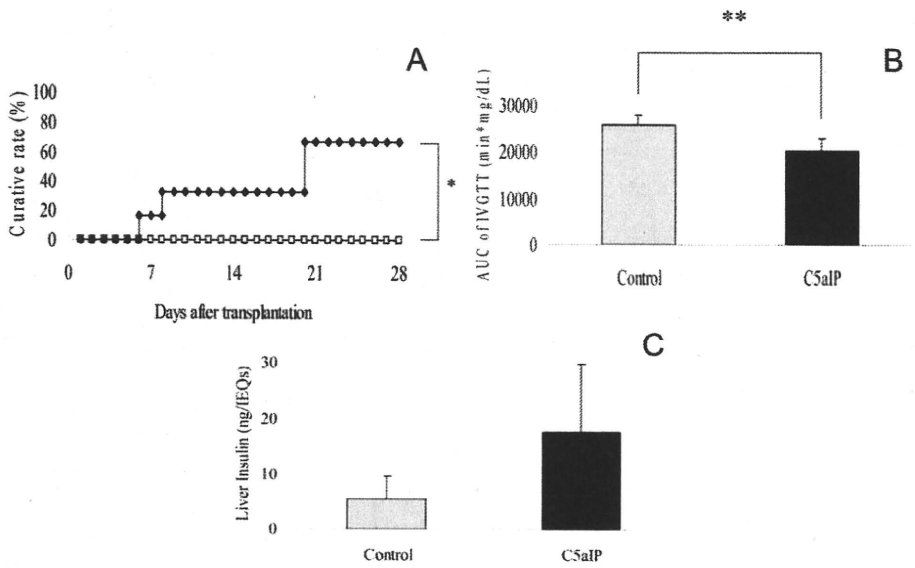


FIGURE 2. Effect of C5a inhibitory peptide (C5aIP) on the islet engraftment. Pancreatic islets from Lewis rats were transplanted into male Lewis rats that were rendered diabetic by streptozotocin (65 mg/kg intravenously [IV]). (A) Diabetic animals received 2.5 islet equivalents (IEQs)/g of islets with saline (*square*: control group) or 2.5 IEQs/g of islets with C5aIP (*diamond*: C5aIP group). Serial blood glucose levels were measured. Recipients whose nonfasting blood glucose levels at less than 200 mg/dL on two consecutive measurements were considered to be cured (*P=0.038). D-glucose (1.0 g/kg body weight) was infused IV at 4 weeks after islet infusion, and the blood glucose concentrations were measured before and at 5, 10, 20, 30, 60, 90, and 120 min after the glucose injection. The area under the curve (AUC) was calculated (B). After the intravenous glucose tolerance test (IVGTT), the recipient livers were retrieved, and hepatic insulin content per transplanted IEQs was evaluated (C). *P less than 0.05; **P less than 0.005.

C5L2 were expressed on the isolated islets (C5aR: 7.91%±2.83%; C5L2: 2.45%±1.34%). The expression of both C5a receptors on the isolated islets was markedly up-regulated by proinflammatory cytokines (C5aR: 90% up, C5L2: 75% up, respectively). In addition, most of the C5aR-positive cells in isolated islets also expressed CD11b (78.2%±5.4%). By contrast, C3a receptor was expressed minimally on isolated islets (0.44%±0.38%).

C5aIP is Unable to Regulate TF Expression on the Isolated Islets

Because both C5a receptors are expressed on the isolated islets, the relevance between C5a and TF expression in the islet grafts was examined using FACS analysis. The expression of TF on the isolated islets was up-regulated by 1 h incubation with syngeneic serum (P=0.01), whereas no difference was observed by culturing with recombinant C5a. Furthermore, the up-regulation of TF induced by serum was not inhibited by C5aIP (Fig. 3E–I).

Infiltration of CD11b-Positive Cells and Deposition of C5b-9 on the Islet Grafts

Immunohistochemical staining of islet grafts was scored semiquantitatively according to representative examples shown in Figure 4A. As summarized in Figure 4(B and C), no difference was detected between the C5aIP group and the control group regarding the infiltration of CD11b-positive cells (1.08±1.01 vs. 0.92±0.99; Fig. 4B) and the deposition of C5b-9 (0.99±0.79 vs. 0.80±0.89; Fig. 4C) on the islet grafts.

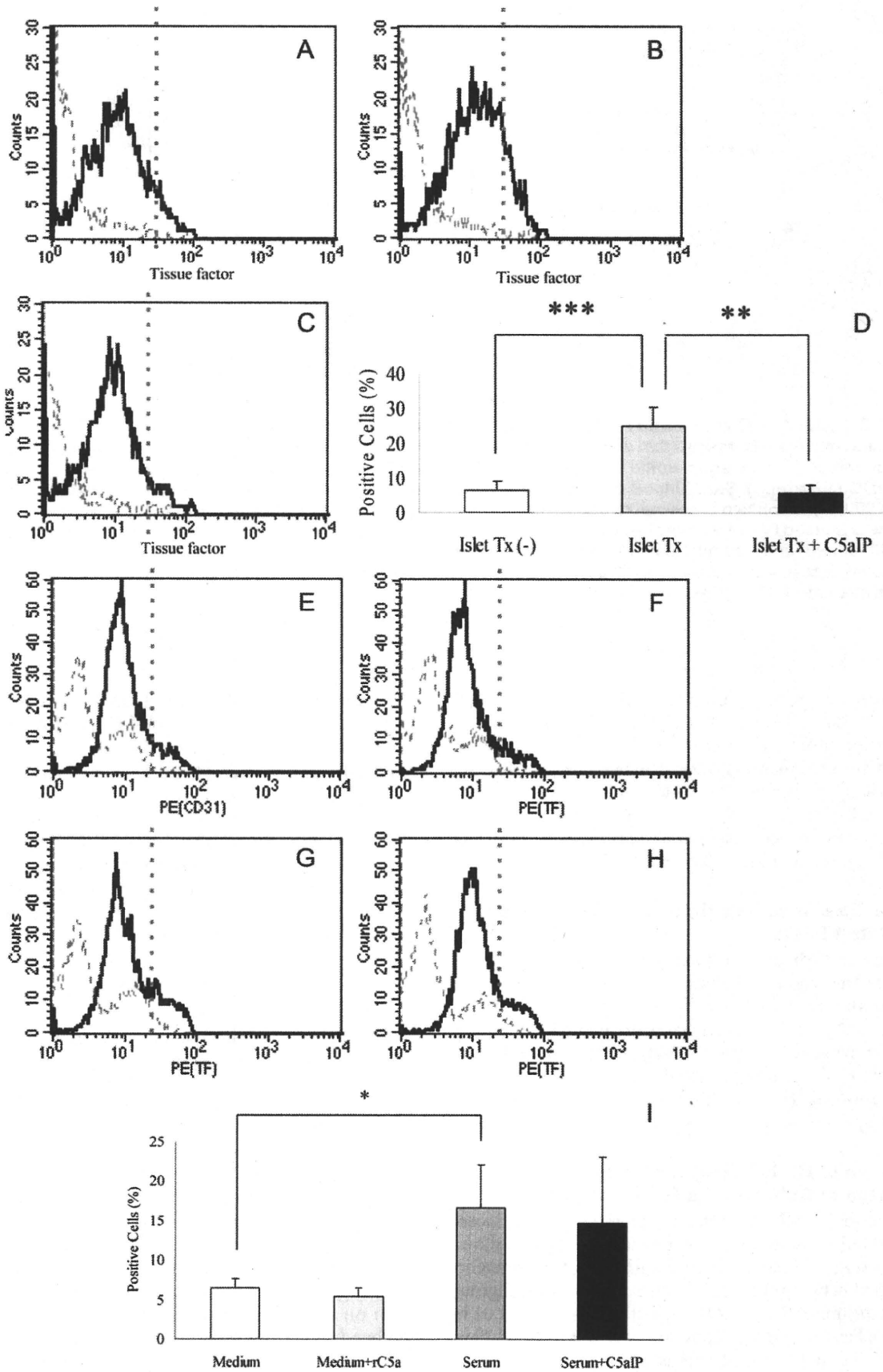
The Synergistic Effect of C5aIP Combined With Gabexate Mesilate on Islet Engraftment

C5aIP is unable to regulate the expression of TF on isolated islets. Therefore, gabexate mesilate was combined with C5aIP to suppress effectively the coagulant activation derived from TF expression on the islet grafts. As shown in Fig. 5, all rats transplanted with 2.5 IEQs/g of islets in the combination group became normoglycemic, whereas only 2 of 6 rats (33.3%) transplanted with the same number of islets in the gabexate group became normoglycemic during the study period (P<0.05).

DISCUSSION

C5aIP has been shown to improve early outcomes after intraportal islet transplantation by attenuating cross-talk between C5a and TF on granulocytes in the host livers. The beneficial effects of C5aIP were further enhanced by combining it with conventional anticoagulants such as gabexate mesilate.

The TAT levels, regarded as the key marker of IBMIR, were significantly suppressed, and islet engraftment was significantly improved by using C5aIP alone. Johansson et al. (*26*) reported that low TAT levels were associated with a high C-peptide production in clinical islet transplantation. Thus, the results of this study were in accord with clinical data. We thus far reported that complement activation occurs secondarily to coagulant activation most likely based on TF expression on islet grafts in the IBMIR (*3*, *27*). Recent report by Huber-Lang et al. (*28*) also supports our finding. Unexpectedly, C5aIP alone had significant effects on the inhibition of



coagulant activity in this study. In recent years, many researchers have reported a potential interplay between complement and coagulation cascades (*29–31*). Ritis et al. (*13*) found C5aR-TF cross-talk on neutrophils in an autoimmune model. In our study, TF expression on granulocytes in the recipient livers was up-regulated within 1 h of the islet infusion. The finding that TF is expressed not only on the islet grafts (*4*) but also on the activated leukocytes in the hosts after islet transplantation indicates to us the possibility that TF, induced on the recipient granulocytes, could be a novel target to inhibit the IBMIR. Notably, the up-regulated TF was effectively suppressed by C5aIP alone. In contrast, C5aIP had only a marginal effect on cytokine inhibition, on suppressing the infiltration of CD11b-positive cells, and on the deposition of C5b-9 to the islet grafts. These results suggest that C5aIP improved islet engraftment through an interruption of cross-talk between the complement and coagulation cascades and not through suppressing proinflammatory mediators or preventing islet damage by leukocyte infiltration or C5b-9 deposition.

Another important issue to clarify was whether C5aIP has any beneficial effects directly on the islet grafts, because TF is expressed on the surface of isolated islets (*4*). To examine the potential effect of C5a on islet grafts, we first analyzed the expression of complement receptors on pancreatic tissues and isolated islets. Both C5aR and C5L2, unlike C3aR, were expressed on isolated islets, whereas C5aR was not detected on pancreatic tissues before isolation procedures. These findings suggest that C5aR on the isolated islets could be induced during preservation or isolation procedures or both. Corroborating our findings, Lewis et al. (*32*) also reported that C5aR expression in cadaveric kidneys correlated positively with cold ischemia time. Based on the above findings, the potential cross-talk between C5a and TF on isolated islets was investigated. TF expression on isolated islets was significantly up-regulated by incubating with syngeneic serum but not by recombinant C5a. Notably, C5aIP was unable to suppress the up-regulated TF, despite its strong effect on TF inhibition in the recipient granulocytes. We speculate that TF expression on islet grafts could be increased immediately after having contact with the recipient's blood. Therefore, it is more likely that another anticoagulant treatment, one not focused on C5a, is required to regulate coagulant activation derived from TF expressed on the grafts. In this study, curative rate of diabetic rats was increased by combining C5aIP with gabexate mesilate, a clinically available anticoagulant.

Apart from the cross-talk between complement and coagulation cascades, C5a also plays a variety of crucial roles such as chemotaxis of leukocytes and cytokine induction. In particular, Rittirsch et al. (*20*) have recently reported that C5a interaction with C5L2, a secondary receptor for C5a, triggers HMGB1 release, which was identified as a lethal mediator of sepsis (*21–23*). Few differences were observed in the plasma HMGB1 levels after the islet infusion in the presence or absence of C5aIP. One possible explanation is the structural specificity of C5aIP. It is likely that C5aIP can specifically block the reaction of C5a with C5aR, but not with C5L2, because C5aIP was designated as the complementary peptide against an active region of C5a that is expected to play a crucial role when C5a binds to C5aR (*7*). The function of C5L2 per se is still controversial, and the role of C5L2 in islet transplantation also remains uncertain. Therefore, these issues are topics of interests for future study.

It has recently been reported that complement activation might be an essential entity of the damage induced by the IBMIR in an in vitro model (*33*). However, in this study, islet engraftment was markedly improved by introducing C5aIP despite that complement terminal products were not regulated. This finding suggests that the regulation of C5a-induced inflammatory responses could be more important to suppress the IBMIR. Therefore, the direct cytolytic role by complement terminal products on islet engraftment, at least in the present model, may not be crucial, although further in vivo investigations are required.

In summary, C5aIP attenuates cross-talk between the complement and coagulation cascades through suppressing the up-regulation of TF expression on leukocytes in the recipient livers, findings correlated with an improvement in islet engraftment. Therefore, C5aIP in combination with conventional anticoagulants, such as gabexate mesilate, are a good strategy to control the IBMIR induced in clinical islet transplantation, although the ideal regimen still needs to be determined in a clinical setting.

MATERIALS AND METHODS

Animals

All the animals in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (*34*). Male Lewis rats (Japan SLC Inc., Shizuoka, Japan) were used as islet donors and recipients.

Islet Isolation and Transplantation

Islet isolation and culture were performed as previously reported (*35*). Diabetic Lewis rats underwent intraportal islet transplantation after receiving isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) for anesthesia. Rat islets were infused in a total volume of 1 mL into the recipient liver through the portal vein using a 25-gauge insulin syringe.

Experimental Groups

Syngeneic rat islet grafts were transplanted intraportally into two groups of streptozotocin-induced diabetic rats: controls and C5aIP (Biologica

FIGURE 3. Continued Flow cytometry evaluation of tissue factor (TF) on the granulocytes in the liver of recipient rats, and on the isolated islets. (A–C) Cells prepared from the livers of diabetic rats. The expression of TF (*black histograms*) on liver granulocytes from diabetic rats that received no islets and were treated with saline (A, Islet Tx[−]) and diabetic rats that received 10 islet equivalents/g of islets and were treated with saline (B, Islet Tx) or treated with C5aIP (C, Islet Tx+C5aIP). A mouse isotype control was used as a negative control (*gray histograms*). Mean percentages of positive cells±SD were compared among the three groups (D). **P* less than 0.05; ***P* less than 0.005; ****P* less than 0.0001. (E–H) Cells prepared from isolated islets. Expression of TF (*black histograms*) on isolated islets that were cultured for 1 h with medium alone (E, medium), with medium supplemented with recombinant C5a (F, medium+rC5a), with syngeneic serum (G, Serum), or with syngeneic serum supplemented with C5aIP (H, serum+C5aIP). Mean percentages of positive cells±SD were compared among the four groups (I).

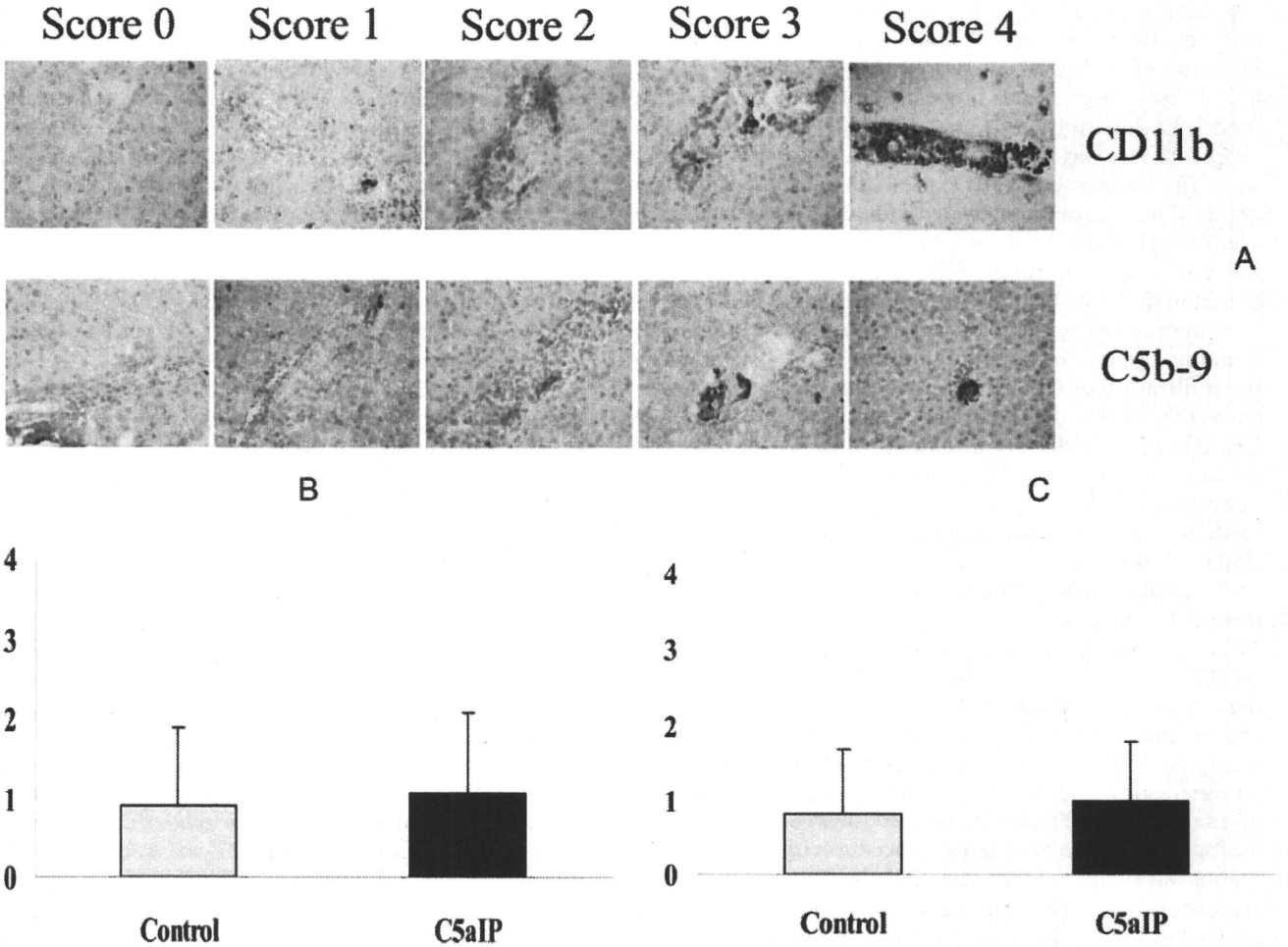


FIGURE 4. Immunohistochemical analyses. Islet cells were retrieved from the liver treated with saline (control group, n=3) or with C5a inhibitory peptide (C5aIP) (C5aIP group, n=3) at 3 h after islet transplantation (10 islet equivalents/g). In the each experimental group, more than 70 sections were stained with anti-CD11b or anti-C5b-9 antibody and were scored by blind evaluation with regard to the antibody used using a semiquantitative scoring scheme. Representative photomicrographs are shown (A). The results of scoring are compared between the two groups (B and C).

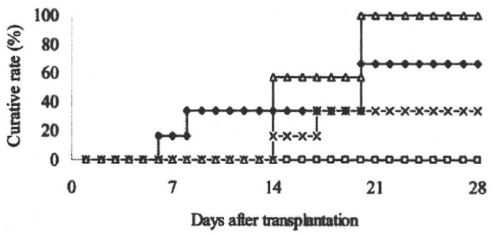


FIGURE 5. Synergistic effect of C5a inhibitory peptide (C5aIP) combined with gabexate mesilate on the islet engraftment. A total of 2.5 islet equivalents/g of islets from Lewis rats were transplanted into male Lewis rats that were rendered diabetic by streptozotocin (65 mg/kg intravenously). Diabetic animals were treated with saline (*square*), C5aIP (*diamond*), gabexate mesilate (*cross*), or C5aIP and gabexate mesilate (*triangle*).

Co.,Ltd., Nagoya, Japan)-treated group (n=11, respectively). The C5aIP group was treated with a bolus dose of C5aIP (4 mg/kg) followed by a continuous infusion (0.33 mg/kg/h) for 1 day. The recipients that were injected with equivalent amounts of saline served as controls.

Blood Analyses

After islet infusion, blood was collected through a tube inserted into the jugular vein at 0, 0.5, 1, 3, 6, and 24 h. Anticoagulated blood with 10 mM ethylenediaminetetraacetic acid was centrifuged immediately for 10 min at 8000*g*. The plasma was frozen at −80°C. Plasma levels of TAT, HMGB1, interleukin (IL)-6, and monocyte chemoattractant protein-1 were determined using Enzygnost TAT micro (Dade Behring, Tokyo, Japan), HMGB1 Kit II (Shino-Test Corporation, Kanagawa, Japan), Endogen Rat IL-6 Kit (Pierce Biotechnology, Inc., Rockford, IL), and Endogen Rat MCP-1 Kit (Pierce Biotechnology), respectively.

Induction and Diagnosis of Diabetes in the Recipients

Diabetes was induced by intravenous injection of streptozotocin (65 mg/kg) 7 days before surgery. Rats whose nonfasting blood glucose levels were at more than or equal to 400 mg/dL on two consecutive measurements were considered diabetic (*36*). Serial blood glucose levels were determined and recipients whose nonfasting blood glucose was less than 200 mg/dL on two consecutive measurements were considered to be cured.

Intravenous Glucose Tolerance Test

Intravenous glucose tolerance test was performed 4 weeks after islet infusion. D-glucose (1.0 g/kg) was infused intravenously, and the blood glucose

concentrations were determined before and at 5, 10, 20, 30, 60, 90, and 120 min after the glucose injection.

Insulin Amount in the Liver of the Recipients

Recipient livers were retrieved and homogenized in 5 mL of deionized water at 4°C. After adding 25 mL of deionized water and 75 mL of 0.18 M HCl in 96% ethanol, the homogenate was stored at 4°C for 24 h and was then centrifuged at 2150 *g* for 10 min. The resulting supernatant was stored at −80°C. The insulin concentration in a supernatant was evaluated using a commercial ELISA kit (Mercodia, Uppsala, Sweden).

Single Cell Preparation of Pancreatic Islets

The isolated islets were dispersed into individual cells by treatment with Accutase (Innovative Cell Technologies, San Diego, CA) at 37°C for 4 min.

Preparations of Hepatic Leukocytes

Rat hepatic leukocytes were prepared by anesthetizing the animals with isoflurane. They were killed by total bleeding from the incised axillary artery and vein. The liver was excised, cut into small pieces, pressed through a cell strainer mesh with the diameter of 70 μm (Becton Dickinson, Mountain View, CA), and suspended in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA). Leukocytes were separated from parenchymal and nonparenchymal cells by 36% Percoll gradient centrifugation.

Flow Cytometry Analysis

Single cells (2×10^5) suspended in PBS with 0.5% BSA were incubated with antibody for 30 min at 4°C on ice in the dark and subsequently washed and suspended in PBS with 0.5% BSA. The expression of C3aR, C5aR, and C5L2 on the rat islet surfaces was detected by FACSCalibur flow cytometry analysis (Becton Dickinson) using a monoclonal antibody to rat C3a receptor (Hycult biotech, Uden, Netherlands) followed by a goat anti-mouse IgG1 (Beckman Coulter, Miami, FL), a complement component 5a Receptor 1/CD88 mouse monoclonal antibody (Lifespan Biosciences, Atlanta, GA) followed by a goat anti-mouse IgG1, and a polyclonal antibody to rat C5L2 (Hycult biotech) followed by a donkey anti-rabbit IgG (Beckman Coulter). As a negative control of C3aR and C5aR, a mouse IgG1 isotype control (Beckman Coulter) was used. As a negative control of C5L2, a rabbit IgG isotype control (Beckman Coulter) was used.

Freshly isolated islets were cultured with or without 250 pg/mL mTNF-α (Roche Diagnostics, Indianapolis, IN), 100 pg/mL hIL1-β (Roche Diagnostics), and 20 ng/mL mIFN-γ (Roche Diagnostics) for approximately 12 h at 37°C. Likewise, to investigate the effect of C5a, syngeneic serum, and C5aIP, the islets were incubated with the medium alone, with the medium containing 10 μg/mL recombinant C5a (Recombinant Mouse Complement Component C5a, R&D Systems, Inc., MN), with syngeneic serum, and with serum containing 700 nM C5aIP for 1 h at 37°C. Furthermore, the expression of CD11b and CD31 of C5aR-positive cells was examined using mouse anti-rat CD11b antibody (LifeSpan Biosciences) and mouse anti-rat CD31 antibody (LifeSpan Biosciences), respectively.

The expression of TF on leukocytes in recipient livers was detected by flow cytometry analysis using Rabbit Anti-Mouse TF IgG (American Diagnostica, Greenwich, CT), followed by secondary phycoerythrin-labeled Donkey Anti-Rabbit IgG (Beckman Coulter). The clinical relevant graft dose (10 IEQs/g) was applied because the rate of TF-positive cells in whole liver cells could be expected rather low.

Immunohistochemical Staining

Pieces of pancreas were collected and snap-frozen in liquid nitrogen. The samples were sectioned and stained with a monoclonal antibody to rat C5a receptor (Hycult biotech), followed by staining using the EnVision kit (Dako, Denmark).

The recipient livers with islet grafts (10 IEQs/g) were retrieved 3 h after the islet infusion. The clinical relevant graft dose was applied to raise the possibility of finding the grafts in the host livers. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. Immunohistochemical staining was performed using mouse anti-rat C5b-9 (Santa Cruz Biotechnology, Santa Cruz, CA) and EnVision kit (Dako) for C5b-9 and mouse anti-rat CD11b (Lifespan Biosciences) and Streptavidin-APC (eBioscience, San Diego, CA) for CD11b. The deposits of C5b-9 and the infiltration of CD11b-positive cells on the islet grafts were divided semiquantitatively into five categories (Fig. 4A). More than 70 sections from the each experimental group were evaluated and scored by double-blind evaluations.

Statistical Analysis

All data are expressed as the mean ± SD. Statistical significance was determined using Student's *t* test, Mann-Whitney test, and one-factor analysis of variance with Bonferroni post hoc test. *P* value less than 0.05 was considered significant. Analysis of euglycemic conversion was performed by Kaplan-Meier method with a log-rank test.

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