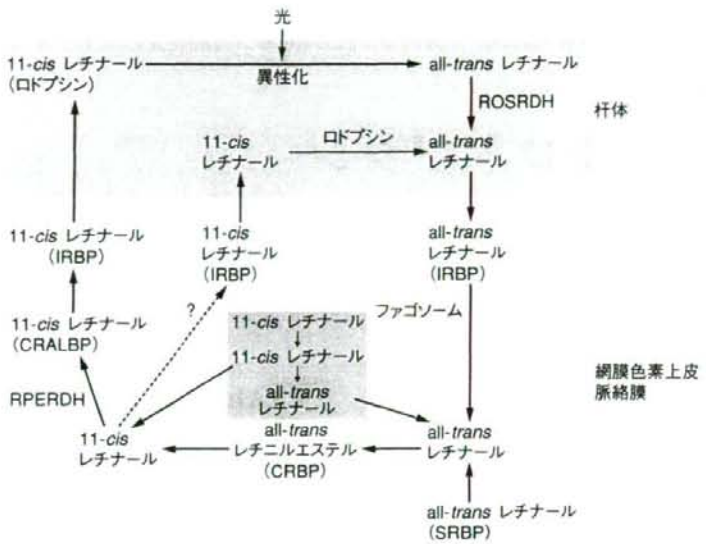

図1　視サイクル

光照射により 11-*cis* レチナールから all-*trans* レチナールへ構造が変わる。レチナールの再生過程には，視細胞，網膜色素上皮細胞に存在するさまざまな酵素が関与する。

のの，これらの感光色素分子を利用した光受容様式は同一である。

一方，古細菌型ロドプシンとして，バクテリアにおいてロドプシン類似蛋白質が発見されている。高度好塩菌から見いだされた光受容蛋白質は，網膜のロドプシンと同様に発色団としてレチナールを有する7回膜貫通型の蛋白質である[4]。バクテリアが産生する視物質という点からバクテリオロドプシンと名づけられている。バクテリオロドプシンは光受容に際し（光エネルギーを利用して），プロトンを細胞膜の内から外へ輸送するプロトンポンプとして機能する。そして，光センサーとして機能しているとの説もあるが，その主な目的は，細胞内外のプロトンの濃度勾配を利用したエネルギー生産のための機能であり，利用面から脊椎動物のロドプシンと大きな違いがある。

本項のテーマであるチャネルロドプシンは，古細菌型ロドプシンとして，クラミドモナスから見いだされた。チャネルロドプシンは，その名前から推測できるように，光受容能とイオンチャネルの機能を併せもつ蛋白質である。チャネルロドプシン1はバクテリオロドプシンと同様にプロトンチャネルとして働くが，その反応は，バクテリオロドプシンがプロトンを能動的に輸送するのに対し，受動的である[5]。チャネルロドプシン2は，光受容能と陽イオン選択的チャネルとして働く[3]。チャネルロドプシン2のこの特徴的な機能が神経科学への応用に最も重要な点である。

これまで古細菌型ロドプシンで，チャネルロドプシン2のように，光受容＋陽イオンチャネル選択的チャネルを併せもつ蛋白質は見いだされていない。この特徴的な機能から，チャネルロドプシン2を神経細胞に発現させた場合，光刺激で細胞を興奮させることができると考えられた（図2a）。実際，Nagel ら[3]は培養細胞に導入し光刺激によって陽イオンの流入が生じることを確認した。また，その反応速度はミリ秒単位であることを報告した。

先に古細菌型ロドプシンも同様に発色団としてレチナールを有すると述べたが，詳細にはロドプシンと異なり，古細菌型ロドプシンでは，光照射によって all-*tran* 型から 13-*cis* 型へ異性化すると

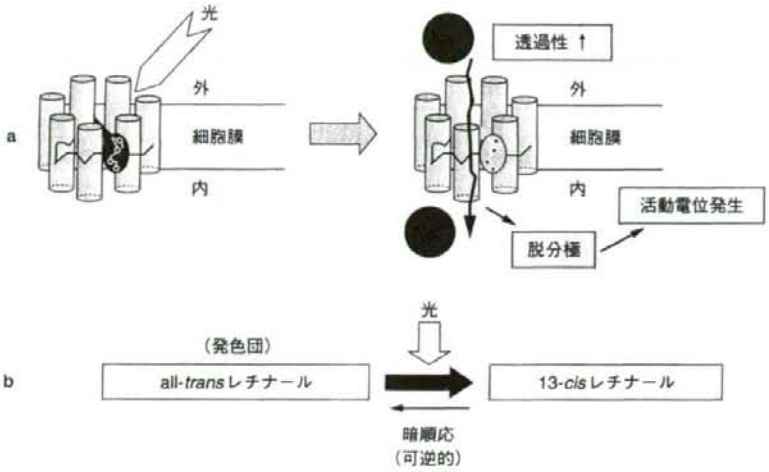

図2　チャネルロドプシン2の特徴
7回膜貫通型の膜蛋白質で，光照射によるレチナールの異性化に伴い細胞内に陽イオンを透過させる。神経細胞では脱分極を引き起こし興奮する。

いうように（図2b），ロドプシンの異性化とは逆向きである（ロドプシンは11-*cis*型からall-*trans*型）。この点も視覚への応用を考慮した場合，重要な特徴である。ロドプシンでは一度光にさらされたレチナールは，視細胞および網膜色素上皮細胞に存在する酵素によって再生される。しかし，チャネルロドプシン2では，反応の向きが逆であるために可逆的な反応を示す。その理由は明らかではないが，1つには，all-*trans*型の励起状態が13-*cis*型よりも低いために，光照射によって13-*cis*型となったレチナールは暗状態になると自発的にall-*trans*型となるためと推測されている[6]。

視覚への応用

筆者らは2005年よりチャネルロドプシン2遺伝子の視覚への応用を目ざして研究を行ってきた[7]。その研究の概略は非常にシンプルなもので，遺伝盲ラットの網膜細胞にチャネルロドプシン2遺伝子を導入，発現させ，視機能が回復されるか，というものである（図3）。まず，問題になったのが，網膜細胞への遺伝子導入法である。効率よく特定の細胞に遺伝子導入できること，そして将来を見据え，臨床応用が可能な方法であることが必要であった。そこで，臨床応用実績のあるアデノ随伴ウイルス（rAAV）ベクター，そのなかでも網膜神経節細胞に親和性の高い2型を選択した。

実験には，RCS（Royal College of Surgeons）ラットを用いた。RCSラットはヒト色素変性症と同様の常染色体劣性網膜変性を起こすラットである。生後いったん網膜が正常に形成されるが，生後20日頃から視細胞の変性が始まり，3か月で視細胞が消失する。筆者らは，視細胞を完全に消失した生後10か月齢のRCSラットを用いて研究を行った。視細胞を消失していることを確認するため，暗順応後に網膜電図（ERG）および視覚誘発電位（visual evoked potential：VEP）を測定し，視細胞からの情報伝達がないことをあらかじめ確認し，実験に用いた。

rAAVウイルスベクターは，チャネルロドプシン2遺伝子とそれを可視化するために蛍光を融合させた遺伝子を組込み作製した。このベクターをRCSラットの硝子体内に投与し，投与後，経時的にVEPを測定した。この結果，投与後6週で光刺激に対する典型的なVEPが記録されるよ

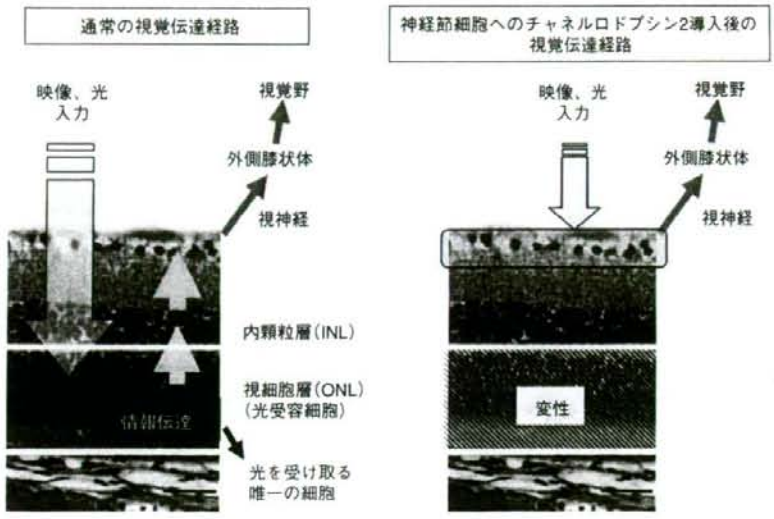

図3　チャネルロドプシン2を用いた視覚再生
神経節細胞へチャネルロドプシン2を導入した場合，入射した光情報は神経節細胞から直接脳に伝えられる。

うになった（図4）。この反応については，生後1年半が経過した現在についても続いている。網膜での発現部位，遺伝子の導入効率を調べたところ，発現部位は主として網膜神経節細胞で，全神経節細胞の約30%の導入が確認された（図5）。1回のウイルス投与で30万個の細胞が光受容細胞になったと予想できる。

次にチャネルロドプシン2を用いた視覚情報がどのようなものであるかを調べるため，RCSラットの行動学試験を行った。この方法は，ラットを中央に置き，その周りに縦縞のドラムを回転させるというもので，小動物の視覚行動学試験で広く用いられている（図6）。縦縞の大きさを変更すること，またはドラムの回転スピードを変更させることにより，視力を予測できる[8,9]。

この方法により，視覚について調べてみると，遺伝子導入前，RCSラットは回転する縞模様に対し反応を示さなかったが，同一個体の遺伝子導入後では回転する縞模様を追いかけるような首振り行動が観察された。また，縞模様の回転スピードを上げると，それに伴い首振り回数も増加した。この回転数に比例した行動は，導入前のRCSラットではみられなかった。しかし，網膜変性を起こさない同種のラットで比較してみると，遺伝子導入後のRCSラットの運動数は低く，正常では8 rpmの回転スピードまで追順した行動を起こすのに対し，遺伝子導入ラットでは追従できなかった（図6）。さらに，正常に比較し遺伝子導入ラット

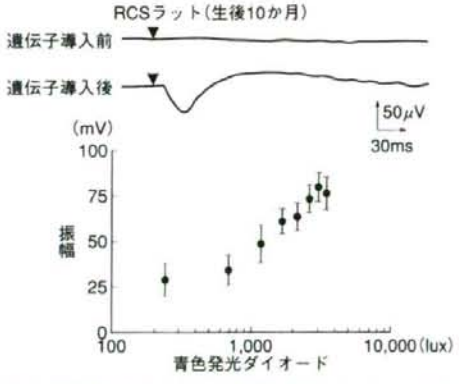

図4　遺伝盲（RCS）ラット網膜へのチャネルロドプシン2遺伝子の導入
遺伝子導入後，視覚誘発電位が現れる。しかし，網膜電図は依然として記録されない。（文献[7]より改変して引用）

図5　チャネルロドプシン2の発現部位と導入効率
チャネルロドプシン2の発現は，主に神経節細胞層と内網状層にみられる（蛍光緑，a）。蛍光青色は逆行性標識された神経節細胞を示す。チャネルロドプシン2は細胞膜に限局していることがわかる（b）。GCL：神経節細胞層，IPL：内網状層，INL：内顆粒層，RPE：網膜色素上皮。

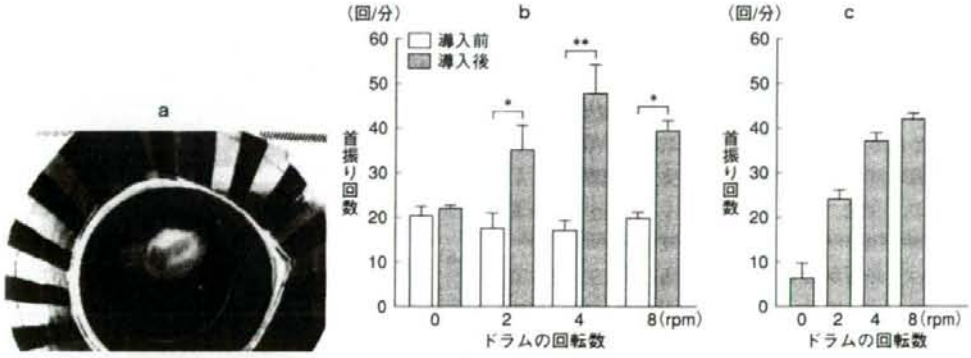

図6　回転台を用いた視機能解析
チャネルロドプシン2の波長特性を考慮し，青色（ピーク波長460 nm）と黒の縞模様を用いた（a）。遺伝子導入前は，回転台（ドラム）の回転数にかかわらず首を振る回数に変化はみられなかった。遺伝子導入後，回転数の増加に伴い首振り回数の増加が観察された（b）。c：網膜変性のない同種ラット。

では，高い照度下でなければ行動を起こさないことが明らかとなった。以上のことからチャネルロドプシン2遺伝子の導入により，RCSラットが縞模様を認識できるようになったことがわかったが，その感度は，正常と比較し劣るものであった。

チャネルロドプシン2には感受波長特性があることが知られている。そこで，遺伝子導入したRCSラットで詳細に感受波長域を調べた結果，感受波長域は450 nmを中心に550 nm以下に限局していることが明らかとなった。

チャネルロドプシンを用いた視覚の再生では，感度などに改善の余地があるものの，視細胞変性による失明に対し，新たな視覚再生方法となり得ると考えられた。チャネルロドプシン2を用いた視覚再生法の特徴は，従来ある神経細胞に光受容能を与えることから，元来，網膜がもつ神経ネットワークを利用できるという点にある。筆者らの研究では網膜神経節細胞を遺伝子導入の標的細胞

として選択したが，遺伝子導入のためのベクターを変更することで，その標的細胞はON型双極細胞への導入も可能となると考えられる。

今後の課題

世界で初めての遺伝子治療が実施されたのは1990年のことである。アデノシンデアミナーゼ（ADA）欠損症の4歳の少女に行われた遺伝子治療は成功をおさめ，遺伝子治療がその他の病気の救世主となると思われた。しかし，最初の遺伝子治療から約20年が経過した現在でも，確立した治療法になるに至っていない。問題点として，ベクターの安全性，遺伝子の挿入変異，生殖細胞への影響などが挙げられる。

チャネルロドプシン2は緑藻類クラミドモナス由来で，元来ヒトがもたない蛋白質であることから，このような問題に加えて，導入遺伝子が作り出す蛋白質そのものが生体に与える影響を調べる必要がある。筆者らは，1年6か月の観察期間を通してチャネルロドプシン2遺伝子導入よる視機能の回復効果が持続していることを確認しているが，今後，ラットだけでなく，サルなどの高等動物で視力評価や免疫学的な副作用検討を実施していく必要があると考えている。

おわりに

チャネルロドプシン2を神経細胞に発現させることで「光で神経細胞を活性化できる」という技術を利用して，現在，神経回路網の解析のためのツールとして研究分野で用いられている。チャネルロドプシン2の生体に対する安全性が明らかとなれば，研究ツールとしてだけでなく，さまざまな疾患の治療に利用できると考えられ，安全性の確立が待ち望まれる。

文　献

1) Zemelman BV, Lee GA, Ng M et al：Selective photostimulation of genetically chARGed neurons. Neuron **33**：15-22, 2002
2) Banghart M, Borges K, Isacoff E et al：Light-activated ion channels for remote control of neuronal firing. Nat Neurosci **7**：1381-1386, 2004
3) Nagel G, Szellas T, Huhn W et al：Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci USA **100**：13940-13945, 2003
4) Henderson R, Unwin PN：Three-dimensional model of purple membrane obtained by electron microscopy. Nature **257**：28-32, 1975
5) Nagel G, Ollig D, Fuhrmann M et al：Channelrhodopsin-1：a light-gated proton channel in green algae. Science **296**：2395-2398, 2002
6) Hubbard R：The stereoisomerization of 11-cis-retinal. J Biol Chem **241**：1814-1818, 1966
7) Tomita H, Sugano E, Yawo H et al：Restoration of visual response in aged dystrophic RCS rats using AAV-mediated channelopsin-2 gene transfer. Invest Ophthalmol Vis Sci **48**：3821-3826, 2007
8) Haruta M, Sasai Y, Kawasaki H et al：In vitro and in vivo characterization of pigment epithelial cells differentiated from primate embryonic stem cells. Invest Ophthalmol Vis Sci **45**：1020-1025, 2004
9) Lund RD, Adamson P, Sauvé Y et al：Subretinal transplantation of genetically modified human cell lines attenuates loss of visual function in dystrophic rats. Proc Natl Acad Sci USA **98**：9942-9947, 2001

ELSEVIER

ISLETS
Human

A Novel Predictive Method for Assessing the Quality of Isolated Pancreatic Islets Using Scanning Electrochemical Microscopy

M. Goto, H. Abe, T. Ito-Sasaki, M. Goto, A. Inagaki, N. Ogawa, K. Fujimori, Y. Kurokawa, T. Matsue, and S. Satomi

ABSTRACT

Introduction. The current methods for evaluating islet potency are not useful in clinical transplantation. Therefore, we need reliable, rapid methods enabling accurate prediction of islet quality.

Materials and Methods. We evaluated respiratory activity using scanning electrochemical microscopy (SECM), glucose-stimulated respiratory activity, glucose-stimulated insulin release, ADP/ATP assays, insulin/DNA levels, and Trypan blue exclusion tests as predictive methods for the ability of isolated rat islets to cure syngeneic diabetic rats.

Results. Although glucose-stimulated respiratory activity, basal respiratory activity, ADP/ATP ratio, and glucose-stimulated insulin release were significantly correlated with the outcome of transplantation into diabetic rats, there was no correlation between outcomes, insulin/DNA ratios, and Trypan blue exclusion tests. The glucose-stimulated respiratory activity in islet preparations that could cure diabetic rats was significantly greater than those unable to cure diabetes. Rat islets with >1.5-fold glucose-stimulated respiratory activity consistently cured diabetic rats, whereas those with a value <1.5 hardly cured any rats.

Conclusion. Measurement of the glucose-stimulated respiratory activity using SECM technique is a novel method that may be useful as a rapid, potent predictor of the outcome of clinical islet transplantation.

From the Tohoku University International Advanced Research and Education Organization (Ma.G., Me.G., A.I.), Tohoku University, Sendai; Division of Advanced Surgical Science and Technology (Ma.G., N.O., S.S.), Tohoku University, Sendai; Faculty of Engineering (H.A., T.I.-S.), Yamagata University, Yonezawa; Division of Surgical Oncology (K.F.), Tohoku University, Sendai; Tohoku University Innovation of New Biomedical Engineering Center (Y.K.), Tohoku University, Sendai; and Graduate School Environmental Studies (T.M.), Tohoku University, Sendai, Japan.

This study has been supported by grants from Innovation Plaza Miyagi of JST (Japan Science and Technology Agency) and the Japanese Grant-in-Aid for Scientific Research (A).

Address reprint requests to Masafumi Goto, MD, PhD, Tohoku University International Advanced Research and Education Organization, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan. E-mail: gotokichi@aol.com

0041-1345/09/$–see front matter
doi:10.1016/j.transproceed.2008.10.075

THE CURRENT standard methods to evaluate islet potency are not useful in clinical islet transplantation. Furthermore, most tests are relatively subjective and time-consuming.[1] We have thus far shown that the ADP/ATP ratio correlated with in vivo viability of isolated islets.[2] However, insulin release from isolated islets is not entirely related to the ADP/ATP ratio. Moreover, it is difficult to continuously measure the ADP/ATP ratio of the same islets. Therefore, we sought to establish a reliable, rapid method enabling accurate prediction of both islet viability and insulin release. Scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode monitors the local distribution of electro-active species near the sample surface. SECM has been used to investigate numerous biological molecules, including DNA,[3] enzymes,[4] and antigen-antibody interactions.[5] This technique noninvasively measures respiratory activity of isolated islets under physiological conditions. We have used SECM to examine islet viability and potency of insulin release.

MATERIALS AND METHODS

In the present study, we evaluated respiratory activity using SECM, glucose-stimulated respiratory activity, glucose-stimulated insulin release, ADP/ATP assays, insulin/DNA levels, and Trypan blue exclusion tests as predictive methods to evaluate the ability of isolated rat islets exposed to various degrees of heat shock stress (0, 40, 50, 60 or 80 seconds) to cure syngeneic Streptozotocin-induced diabetic rats (n = 7, 6, 6, 7, and 7, respectively). SECM was programmed to automatically measure the reduction current of far and near points of samples based on spherical diffusion theory.[6] The respiratory activity of 10 islets in each group was calculated by evaluating the difference of the reduction current around the samples using 2–4 μm platinum-coated microelectrode. The glucose-stimulated respiratory activity was indicated by the stimulation index of the respiratory activity, defined as the ratio of the respiratory activity in high-glucose concentration (16.7 mmol/L) against that in basal glucose concentration (1.67 mmol/L). The ADP/ATP assay, insulin/DNA levels, and Trypan blue exclusion tests were performed as previously described.[5,7] In islet transplantation, 6 islet equivalents/g of body weight were transplanted into recipient livers via the portal vein using a 24-gauge butterfly needle using the previously described method.[8] Heat shock stress was induced by placing the isolated islets at 60°C for 0, 40, 50, 60, or 80 seconds.

RESULTS

On the one hand, significant correlations with the outcome of transplantation into diabetic rats were observed for glucose-stimulated respiratory activity (heat shock stress; 0 seconds; 2.39 ± 0.08; 40 seconds, 1.85 ± 0.17; 50 seconds, 0.86 ± 0.08; 60 seconds, 0.49 ± 0.03; 80 seconds, 0.37 ± 0.07; cured group: 1.94 ± 0.18; noncured group: 0.57 ± 0.07, respectively), basal respiratory activity (heat shock stress: 0 seconds, 5.65 ± 0.15, 40 seconds, 5.31 ± 0.51, 50 seconds, 4.18 ± 0.58, 60 seconds, 1.83 ± 0.27, 80 seconds, 0.31 ± 0.05; cured group: 5.27 ± 0.26; noncured group: 1.98 ± 0.46, respectively), ADP/ATP ratio (heat shock stress; 0 seconds, 0.003 ± 0.003, 40 seconds, 0.05 ± 0.03, 50 seconds, 0.21 ± 0.05, 60 seconds, 0.30 ± 0.07, 80 seconds, 0.42 ± 0.05, cured group: 0.05 ± 0.03; noncured group: 0.30 ± 0.04, respectively), and glucose-stimulated insulin release (heat shock stress; 0 seconds, 11.0 ± 2.6, 40 seconds, 2.51 ± 0.76, 50 seconds, 1.12 ± 0.14, 60 seconds, 1.13 ± 0.21, 80 seconds, 1.40 ± 0.41; cured group: 6.59 ± 1.78; noncured group: 1.35 ± 0.18, respectively) $P < .0001$, $< .0001$, $< .0001$, and .002: $\rho = .80$, .71, −.66, and .53, respectively. On the other hand, there was no correlation between islet transplantation outcome and insulin/DNA ratio (heat shock stress: 0 seconds, 0.73 ± 0.05, 40 seconds, 0.99 ± 0.13, 50 seconds, 0.86 ± 0.10, 60 seconds, 0.91 ± 0.06, 80 seconds, 1.12 ± 0.06; cured group: 0.78 ± 0.04; noncured group: 1.03 ± 0.05, respectively), and Trypan blue exclusion test (heat shock stress: 0 seconds, 100.0 ± 0.0, 40 seconds, 98.8 ± 0.6, 50 seconds, 99.3 ± 0.5, 60 seconds, 99.9 ± 0.1, 80 seconds, 94.9 ± 1.8; cured group: 99.5 ± 0.3; noncured group: 97.8 ± 0.8, respectively). The glucose-stimulated respiratory activity in islet preparations that could cure diabetic rats was significantly higher than in those unable to cure diabetes ($P < .0001$). Rat islets with glucose-stimulated respiratory activity more than 1.5 consistently cured diabetic rats, whereas rat islets with a value <1.5 hardly cured any rats ($P < .0001$) (Fig 1). Notably, the predictive rate for curing diabetic rats was 91% when glucose-stimulated respiratory activity was used.

DISCUSSION

It is well known that unexpectedly poor effects of grafts are still seen in the field of islet transplantation even using the current refined procedures. Most likely, this is attributed to suboptimal quality of the isolated islets.

It has been reported that the current methods of islet quality assessment have only a limited ability to predict

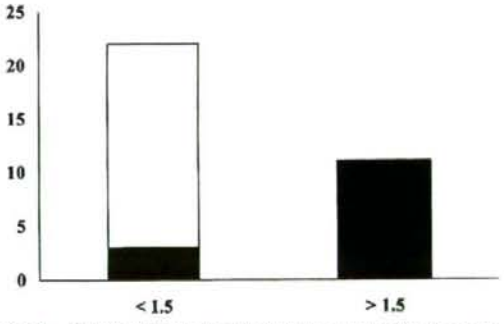

Fig 1. Streptozotocin-induced diabetic rats underwent intraportal transplantation with syngeneic islets that were exposed to various degrees of heat shock stress (0, 40, 50, 60, and 80 seconds). The X-axis indicates the glucose-stimulated respiratory activity, and the Y-axis indicates the number of animals. The black bar shows cured animals; the white bar shows noncured diabetic animals. Rat islets with glucose-stimulated respiratory activity >1.5 consistently cured diabetic rats, whereas rat islets with a value <1.5 hardly cured any rats ($P < .0001$).

outcomes after clinical transplantation.[1,9,10] In vivo bioassay has thus far been regarded as the most reliable assessment.[11,12] However, it is not clinically useful because several days are needed for evaluation. Therefore, we need establishment of reliable, rapid methods enabling accurate prediction of islet potency. This issue is crucial for Japan because only marginal organs from non–heart-beating donors are currently available for islet isolation.

In 2006, we reported that the ADP/ATP ratio was a useful predictive assay for isolated islets.[2] Although the ADP/ATP assay has many advantages as islet quality assessment, its limitation is the absence of a correlation with insulin release from the isolated islets, suggesting that it reflects islet viability rather than function.

As shown in the present study, glucose-stimulated respiratory activity strongly correlated with islet quality. This highly sensitive, noninvasive method made it possible to distinguish respiratory activity even in one islet by visualizing the reduction current in a simple form. Notably, the glucose-stimulated respiratory activity is expected to reflect not only islet viability but also function.

Taken together, measurement of the glucose-stimulated respiratory activity using SECM technique is a novel rapid, potent predictor of the outcome of clinical islet transplantation.

ACKNOWLEDGMENTS

We thank Takehiro Imura and Kozue Maya for their excellent technical assistance.

REFERENCES

1. Barnett MJ, McGhee-Wilson D, Shapiro AM, et al: Variation in human islet viability based on different membrane integrity stains. Cell Transplant 13:481, 2004

2. Goto M, Holgersson J, Kumagai-Braesch M, et al: The ADP/ATP ratio: a novel predictive assay for quality assessment of isolated pancreatic islets. Am J Transplant 6:2483, 2006

3. Yamashita K, Takagi M, Uchida K, et al: Visualization of DNA microarrays by scanning electrochemical microscopy (SECM). Analyst 126:1210, 2001

4. Oyamatsu D, Hirano Y, Kanaya N, et al: Imaging of enzyme activity by scanning electrochemical microscope equipped with a feedback control for substrate-probe distance. Bioelectrochemistry 60:115, 2003

5. Kasai S, Yokota A, Zhou H, et al: Immunoassay of the MRSA-related toxic protein, leukocidin, with scanning electrochemical microscopy. Anal Chem 72:5761, 2000

6. Yasukawa T, Uchida I, Matsue T: Microamperometric measurements of photosynthetic activity in a single algal protoplast. Biophys J 76:1129, 1999

7. Wennberg L, Song Z, Bennet W, et al: Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporine A, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days. Transplantation 71:1024, 2001

8. Brandhorst D, Brandhorst H, Zwolinski A, et al: Prevention of early islet graft failure by selective inducible nitric oxide synthase inhibitors after pig to nude rat intraportal islet transplantation. Transplantation 71:179, 2001

9. Eckhard M, Brandhorst D, Winter D, et al: The role of current product release criteria for identification of human islet preparations suitable for clinical transplantation. Transplant Proc 36:1528, 2004

10. Gerling IC, Kotb M, Fraga D, et al: No correlation between in vitro and in vivo function of human islets. Transplant Proc 30:587, 1998

11. Hering BJ, Kandaswamy R, Harmon JV, et al: Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 4:390, 2004

12. Ricordi C, Scharp DW, Lacy PE: Reversal of diabetes in nude mice after transplantation of fresh and 7-day-cultured (24 degrees C) human pancreatic islets. Transplantation 45:994, 1988

Xenotransplantation 2008: 15: 225–234
Printed in Singapore. All rights reserved
doi: 10.1111/j.1399-3089.2008.00482.x

XENOTRANSPLANTATION

Dissecting the instant blood-mediated inflammatory reaction in islet xenotransplantation

Goto M, Tjernberg J, Dufrane D, Elgue G, Brandhorst D, Ekdahl KN, Brandhorst H, Wennberg L, Kurokawa Y, Satomi S, Lambris JD, Gianello P, Korsgren O, Nilsson B. Dissecting the instant blood-mediated inflammatory reaction in islet xenotransplantation. Xenotransplantation 2008; 15: 225–234. © 2008 Wiley Periodicals, Inc.

Abstract: Background: A massive destruction of transplanted tissue occurs immediately following transplantation of pancreatic islets from pig to non-human primates. The detrimental instant blood-mediated inflammatory reaction (IBMIR), triggered by the porcine islets, is a likely explanation for this tissue loss. This reaction may also be responsible for mediating an adaptive immune response in the recipient that requires a heavy immunosuppressive regimen.
Materials and methods: Low molecular weight dextran sulfate (LMW-DS) and the complement inhibitor Compstatin were used in a combination of in vitro and in vivo studies designed to dissect the xenogeneic IBMIR in a non-human primate model of pancreatic islet transplantation. Adult porcine islets (10 000 IEQs/kg) were transplanted intraportally into three pairs of cynomolgus monkeys that had been treated with LMW-DS or heparin (control), and the effects on the IBMIR were characterized. Porcine islets were also incubated in human blood plasma in vitro to assess complement inhibition by LMW-DS and Compstatin.
Results: Morphological scoring and immunohistochemical staining revealed that the severe islet destruction and macrophage, neutrophilic granulocyte, and T-cell infiltration observed in the control (heparin-treated) animals were abrogated in the LMW-DS-treated monkeys. Both coagulation and complement activation were significantly reduced in monkeys treated with LMW-DS, but IgM and complement fragments were still found on the islet surface. This residual complement activation could be inhibited by Compstatin in vitro.
Conclusions: The xenogeneic IBMIR in this non-human primate model is characterized by an immediate binding of antibodies that triggers deleterious complement activation and a subsequent clotting reaction that leads to further complement activation. The effectiveness of LMW-DS (in vivo and in vitro) and Compstatin (in vitro) in inhibiting this IBMIR provides the basis for a protocol that can be used to abrogate the IBMIR in pig-human clinical islet transplantation.

Masafumi Goto,[1] Jenny Tjernberg,[2] Denis Dufrane,[3] Graciela Elgue,[2] Daniel Brandhorst,[2] Kristina Nilsson Ekdahl,[2] Heidi Brandhorst,[2] Lars Wennberg,[4] Yoshimochi Kurokawa,[5] Susumu Satomi,[6] John D. Lambris,[7] Pierre Gianello,[3] Olle Korsgren[2] and Bo Nilsson[2]

[1]Tohoku University International Advanced Research and Education Organization, Tohoku University, Sendai, Japan, [2]Department of Radiology, Oncology and Clinical Immunology, Division of Clinical Immunology, The Rudbeck Laboratory, University Hospital, Uppsala, Sweden, [3]Université Catholique de Louvain, Faculty of Medicine, Experimental Surgery Unit, Brussels, Belgium, [4]Department of Transplantation Surgery, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden, [5]Tohoku University Innovation of New Biomedical Engineering Center, Tohoku University, Sendai, Japan, [6]Division of Advanced Surgical Science and Technology, Tohoku University, Sendai, Japan, [7]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Medical School, Philadelphia, PA, USA

Key words: adult porcine islet – compstatin – instant blood-mediated inflammatory reaction – low molecular weight dextran sulfate – xenotransplantation

Address reprints request to Bo Nilsson, Department of Radiology, Oncology and Clinical Immunology, Division of Clinical Immunology, The Rudbeck Laboratory, University Hospital, Uppsala, Sweden (E-mail: bo.nilsson@klinimm.uu.se)

Received 27 April 2008;
Accepted 29 May 2008

Introduction

Clinical islet transplantation is a promising treatment for type I diabetic patients. The improved protocol introduced by Shapiro et al. in 2000 [1] has greatly improved the results of this approach, but despite these advances, islets derived from more than one donor pancreas are still generally required to cure an individual diabetic patient. This requirement has drawn

attention to the limited availability of human islets for transplantation and sparked interest in the use of islets from alternative sources, particularly the pig [2].

One obstacle to be surmounted before porcine islets can be used in clinical islet xenotransplantation is the injurious instant blood-mediated inflammatory reaction (IBMIR) that elicits massive cell destruction when porcine islets are exposed to fresh human blood [3]. The xenogeneic IBMIR is characterized by activation of platelets and the coagulation and complement systems. This activation is accompanied by infiltration of the islets by polymorphonuclear lymphocytes (PMNs) [3].

The occurrence of this deleterious IBMIR is supported by studies demonstrating that porcine islets are immediately destroyed when transplanted intraportally into the liver of non-human primates [4,5]. Kirchhof et al. [6] reported that most of their porcine islet xenografts (22–73%) were substantially damaged after 24 h when transplanted into non-immunosuppressed monkeys. The grafts exhibited cell destruction, with deposition of coagulation and complement components and platelets, supporting the contention that the IBMIR contributes to the islet damage in this model. Further support for the importance of the IBMIR comes from the observation that although porcine islets can successfully survive in the liver of diabetic monkeys for more than 100 days [7,8], very high quantities of islets (25 000 and 50 000 IEQs/kg BW, respectively) are needed to produce normoglycemia in the monkeys, indicating that there is a substantial loss of transplanted tissue.

Using in vitro and small-animal models, we have previously demonstrated that low molecular weight dextran sulfate (LMW-DS) effectively inhibits the activation of the coagulation and complement systems and the infiltration of leukocytes into the islets during xenogeneic islet transplantation [9]. In this study, we have used LMW-DS together with Compstatin, a new peptide complement inhibitor that is suitable for use in clinical islet xenotransplantation [10], to dissect the IBMIR in in vivo (LMW-DS) and in in vitro (LMW-DS and Compstatin) xenotransplantation models. The results of these studies have broadened our understanding of the innate immune events that might be expected to occur in clinical islet xenotransplantation and have provided the basis for a protocol for abrogating the IBMIR during clinical transplantation with porcine pancreatic islets.

Materials and methods

Animals

Retired breeder pigs, weighing approximately 200 kg, were used as donors for all experiments. Cynomolgus monkeys (*Macaca fascicularis;* 3- to 6-yr old; 4–6 kg) were used as recipients. All procedures using pigs were approved by the Swedish Council on Medical Ethics. Cynomolgus monkeys were housed according to the guidelines of the Belgian Ministry of Agriculture and Animal Care. All procedures using monkeys were approved by the local Ethical Committee for Animal Care of the Université Catholique de Louvain.

Islet isolation

Isolation of porcine islets was performed as previously described [11], with minimal modifications. Purified islet fractions were pooled and cultured at 37 °C in a humidified atmosphere with 5% CO_2 in CMRL 1066 medium (Biochrom, Berlin, Germany) supplemented with 20% heat inactivated porcine serum, 2 mM *N*-acetyl-L-alanyl-L-glutamine, 10 mM N-2-hydroxyethylpiperazine-N1-2-ethanesulfonic acid (HEPES), 100 IU/ml penicillin, 100 μg/ml streptomycin (Biochrom), and 20 μg/ml ciprofloxacin (Bayer, Leverkusen, Germany).

Evaluation of porcine islet quality

The in vitro function and viability of the porcine islets were assessed after overnight culture as described above. Islet viability determined by trypan blue exclusion assay and insulin release defined as the ratio of stimulated (16.5 mM glucose) to basal (1.65 mM glucose) insulin release, were performed as previously described [11]. For assays of islet insulin content, 1-ml samples were washed with distilled water, then sonicated (Labsonic; Braun, Melsungen, Germany) for 30 s. A 200-μl aliquot of each sample was subjected to acid–ethanol extraction (0.18 M HCl) and used for insulin measurement. Another 100-μl aliquot was dried at 60 °C overnight for consecutive fluorometric DNA assays [12], using calf thymus DNA type I (Sigma, Deisenhofen, Germany) as a standard; 24-h insulin secretion: Immediately after a medium change, 500-μl samples of the medium were taken in duplicate from the remaining Petri dishes for determination of insulin accumulation in the medium, to calculate the 24-h insulin secretion by the islets. Transplantation of islets into nude mice was performed as previously described [11].

Islet transplantation

Before each experiment, the monkeys were sedated with 6 mg/kg Zoletil® 100 (Virbac S.A., Carros, France) intramuscularly, and general anesthesia was maintained with inhalation of 1–3% enflurane. During the experiment, electrocardiogram, blood pressure, and pulse were continuously monitored. The pig islets were suspended in 10 ml of transplant medium (Ringer acetate; Braun) with 25% (w/v) human albumin and 5 mM glucose and injected slowly into the portal vein over the course of 5 min. The animals were treated in pairs, with each pair being given porcine islets from the same donor. One recipient in each pair received LMW-DS (monkeys M5, M7, and M9) and the other heparin as a control (monkeys M6, M8, and M10):

1. Intravenous infusion of LMW-DS (MW 5000; Sigma Chemicals, St Louis, MO, USA) was performed via an indwelling catheter placed in the jugular vein or via a catheter in the portal vein. In the LMW-DS-treated groups, dextran with a molecular weight of 1 kDa (Promiten, Pharmalink AB, Upplands Väsby, Sweden) was injected i.v. just before islet transplantations to avoid the risk of anaphylactoid reactions triggered by LMW-DS. After the injection of Promiten, the monkey received a bolus dose of LMW-DS (1.5 mg/kg) i.v. prior to islet infusion, followed by 3.0 mg/kg LMW-DS given together with the porcine islets (10 000 IEQs/kg of recipient BW). The transplantation was followed by a continuous i.v. infusion of LMW-DS (1.0–1.5 mg/kg/h) for up to 24 h.
2. In the heparin-treated groups, the monkeys received a continuous i.v. infusion of heparin (35 U/kg of BW, heparin LEO, 5000 U/ml; LEO Pharma Nordic, Malmö, Sweden) for 24 h, beginning immediately prior to islet infusion.

Blood samples

All blood samples from the monkeys were drawn from a femoral vein catheter at 0, 15, 30, 45, 60, 75, 90, 120 min and 24 h after transplantation. Blood was also drawn from healthy human blood donors into 7-ml tubes containing citrate, EDTA, or 500 μg of hirudin, a specific inhibitor of thrombin (Refludan; Pharmion Ltd, Cambridge, UK). To obtain plasma, the samples were centrifuged at 4500 *g* for 5 min. If not immediately analyzed for activated partial thromboplastin time (APTT), the samples were stored at 70 °C.

Analyses of blood and plasma samples

Activated partial thromboplastin time measurements were performed as previously described [13]. Platelet counts and differential leukocyte counts were obtained using a Coulter-AcT-diff analyzer (Beckman Coulter, Miami, FL, USA) and EDTA-treated blood. Plasma levels of thrombin–antithrombin (TAT) were quantified using commercially available EIA kits (TAT; Behringswerke, Marburg, Germany). C3a generation was measured in plasma according to the method of Nilsson Ekdahl et al. [14], and sC5b–9 was analyzed using a modification of the enzyme immunoassay described by Nilsson Ekdahl et al. [14] and Mollnes et al. [15].

Plasma interleukin-6 (IL-6), tumor necrosis factor-α (TNFα), IL-1β, and C-reactive protein (CRP) were measured using a commercial ELISA kit (Immulite IL-6, Immulite TNFα, Immulite IL-1β, and Immulite High Sensitivity CRP, respectively; Diagnostic Products Corporation, Los Angeles, CA, USA).

Histological and immunohistochemical staining

The monkey livers bearing transplanted adult porcine islet grafts were retrieved 24 h after transplantation, at a time when the major part of the IBMIR has generally occurred [3]. Some tissue samples were snap-frozen in isopentane and stored at −70 °C. Other samples were fixed with 4% *p*-formaldehyde overnight, and then embedded in paraffin. The samples were sectioned and subsequently used for morphological scoring after hematoxylin eosin staining.

Immunohistochemical staining was carried out using guinea pig anti-insulin (DAKO, Carpenteria, CA, USA), mouse anti-human neutrophil elastase (DAKO), mouse anti-human CD68 (DAKO), mouse anti-human MAC 387 (Abcam, Cambridge, UK), mouse anti-human CD56 (Monosan, Stockholm, Sweden), rabbit anti-human CD3 (DAKO), mouse anti-human CD20 (DAKO), rabbit anti-human IgG and IgM (DAKO), mouse anti-human CD41 (DAKO), mouse anti-human C3c (QUIDEL, San Diego, CA, USA), or goat anti-human C9 (Serotec Ltd Scandinavia, Oslo, Norway).

Treatment of porcine islets with human plasma

Approximately 1000 pig islets/40 μl of plasma (typically 5000 islets in 200 μl) were incubated in human hirudin-treated plasma in heparinized test tubes. Five different islet preparations and five

different plasma preparations were used in these experiments. In some experiments, hirudin-treated plasma was pre-incubated with 20 μM (final concentration) of the potent Compstatin analog, Ac-ICV(1-MeW)QDWGAHRCT-NH_2 [16], for 15 min at 37 °C before the islets were added. The mixture of islets and plasma was then incubated, with gentle shaking, at 37 °C for up to 30 min. After centrifugation, the islets were immediately prepared for complex object parametric analyzer and sorter (COPAS) analysis and confocal microscopy.

Preparation of islets for flow cytometry and confocal microscopy

Ten microliters of fluorescein isothiocyanate (FITC)-labeled antibody recognizing one of the following proteins was added to 5000 islets (corresponding to approximately 10×10^6 cells) in 100 μl of phosphate-buffered saline (PBS) according to the manufacturer's recommendations for single cells: C1q (1.0 g/l; AbCam), C3c (3.2 g/l, for detection of C3b and iC3b; DakoCytomation, Glostrup, Denmark), C4 (1.3 g/l; DakoCytomation), C9 (2.6 g/l; DakoCytomation), mannose-binding lectin (MBL) (0.7 g/l; DakoCytomation), IgG (2.6 g/l; DakoCytomation), or IgM (4.0 g/l; DakoCytomation). Irrelevant mouse IgG1 (0.1 g/l; DakoCytomation) was used as a negative control. For all immunostaining experiments, the islets were incubated, while gently rotating on ice, for 30 min in the presence of an individual antibody. After being washed with PBS, the islets were treated with 1% formaldehyde (Apoteket, Gothenburg, Sweden) and kept on ice until analyzed.

Complex object parametric analyzer and sorter analysis

The fluorescence-stained islets were analyzed using a COPAS (Union Biometrica, Somerville, MA, USA), which is a large particle-based flow cytometry instrument [17]. For each experiment, 1000 islets were analyzed using a 488/514 multi-line laser, and positive cells were sorted out for further analysis by confocal microscopy. The COPAS flow cytometry data were analyzed using CellQuest Pro software (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Data were reported as mean fluorescent intensity (MFI).

Confocal microscopy

One to two hundred hand-picked, stained islets were contained in a drop of PBS in a small Petri dish and protected from light before examination in the confocal microscope (Zeiss 510 Meta confocal; Carl Zeiss, Jena, Germany). Examination of the stained islets was performed using the 488-nm laser at 10 times magnification. Counter staining with 4′,6-diamidino-2-phenylindole was used to visualize the nuclei of living islet cells.

Complement inhibition assay

One hundred microliters of 10% human serum (v/v), diluted in veronal buffer with 1 mM Ca^{2+}, 0.3 mM Mg^{2+}, 1% (w/v) bovine serum albumin, and 0.05% (v/v) Tween 20, was incubated in the presence of serially diluted LMW-DS and/or Compstatin in wells of microtiter plates for 30 min at 37 °C. The wells were then washed with PBS containing 0.05% (v/v) Tween 20, and the bound C3 fragments were detected using 100 μl of horseradish peroxidase-conjugated anti-C3c (Dako AS, Glostrup, Denmark).

Statistical analysis

All values are expressed as mean ± SEM and were compared using Student's unpaired *t*-test or using the Mann–Whitney test for unpaired samples. Values of $P < 0.05$ were considered statistically significant.

Results

Islet quality

The viability of the adult porcine islets (APIs) used in this study was 96, 100, and 97%, respectively. The stimulation index in the static glucose stimulation (SGS) test was 1.29, 1.84, and 1.40, and the mean insulin content was 613, 149, and 685 μU/IEQs, respectively. Adult porcine islets used in each experiment cured diabetic athymic mice. When we assessed the possible detrimental effect of LMW-DS by incubating APIs from three different pancreata in the presence (100, 1000, or 2500 mg/l) or absence of LMW-DS, we found no adverse effect of LMW-DS on insulin release at any of the concentrations tested (data not shown).

Influence of LMW-DS on blood cell counts, liver and renal function, and cytokine induction in transplanted monkeys

One of the transplanted control monkeys (M6) treated with heparin died 2 h after transplantation due to severe hypoglycemia. The platelet and leukocyte counts and the creatinine levels were kept within normal ranges throughout the experiments with one exception: The granulocyte count

tended to increase 2 h after transplantation in the heparin-treated group (3.9 ± 0.5 vs. 9.6 ± 1.6) compared to that of the LMW-DS-treated group (6.0 ± 0.9 vs. 7.3 ± 1.4). There was also a tendency towards an increase in the liver enzymes at 24 h after islet transplantation in the heparin-treated monkeys [heparin vs. LMW-DS: aspartate aminotransferase (AST), 434.7 ± 126.4 vs. 288.0 ± 130.4; alanine aminotransferase (ALT), 207.7 ± 68.7 vs. 116.8 ± 47.7]. No bleedings or other adverse reactions were observed.

Influence of LMW-DS on cytokine induction was examined using three healthy monkeys. Only a slight increase in the IL-6 levels was seen 24 h after administration of LMW-DS in two out of three healthy monkeys (maximum 27 μg/l). However, LMW-DS did not trigger an increase of plasma IL-1β, TNFα, or CRP (not shown).

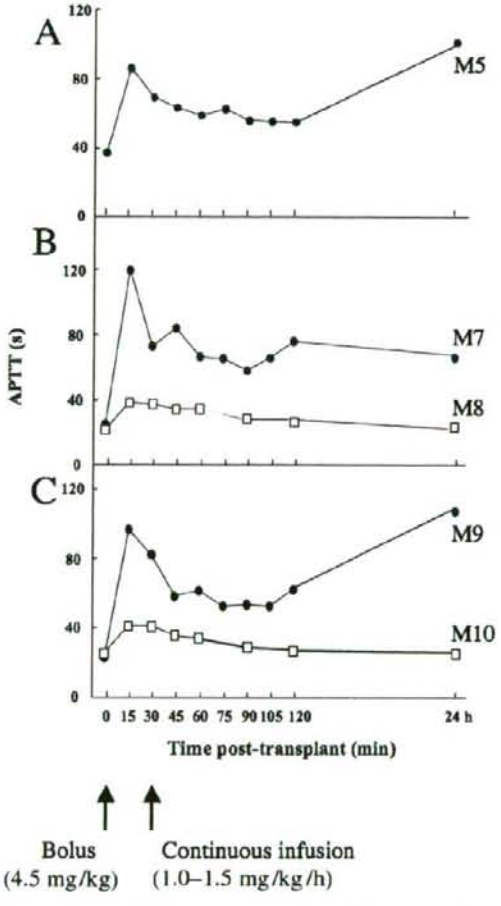

Fig. 1. Plasma APTT values in transplanted diabetic monkeys (M5 and M7–M10) treated with heparin (squares) or LMW-DS (circles).

LMW-DS concentrations in transplanted monkeys

Previous studies showed a strong correlation between APTT and the concentration of LMW-DS [13]. Plasma APTT was therefore used to follow the blood concentration of LMW-DS in the transplanted monkeys (Fig. 1). The APTT in monkeys treated with heparin at concentrations routinely used in clinical islet transplantation (i.e. 500–1000 IU/l) was kept constant at 25–40 s throughout the whole study period. The APTT in monkeys treated with LMW-DS reached around 100 s at 15 min after islet infusion, but gradually decreased during 2 h after islet transplantation. After 24 h, the APTTs in monkeys M5, M7, and M9 were 101, 66, and 107 s, respectively. Thus, both M5 and M9 had higher concentrations of LMW-DS compared with M7.

Inhibition of the IBMIR by LMW-DS during pig islet xenotransplantation

Low molecular weight dextran sulfate, unlike heparin, diminished both the coagulation and the complement cascade activation in two sets of monkeys. The increase of coagulation marker TAT was effectively inhibited by LMW-DS (Fig. 2). The complement activation parameters C3a and sC5b–9 were also suppressed by LMW-DS in both treated monkeys compared to the controls during the study period (Fig. 2). In M5, TAT was totally suppressed while C3a was more difficult to evaluate without the corresponding control (M6). In this animals, C5b–9 was not assessed due to an insufficient amount of plasma samples.

Histological evaluation of grafted pig islets after intraportal transplantation into monkeys treated with LMW-DS or heparin

Morphological aspects of islet grafts were scored semi-quantitatively according to the representative examples shown in Fig. 3. As summarized in Table 1, histology of the transplanted grafts were well kept in the monkeys treated with LMW-DS in both settings of experiments. However, the beneficial effects of LMW-DS were more pronounced in M5 and M9 compared with M7. Indeed, the completely preserved islets (score 0 in all categories) were encountered in 37.2 and 44% of the LMW-DS treated animals M5 and M9 (LMW-DS treated monkeys), respectively, but in only 22% of the control M10.

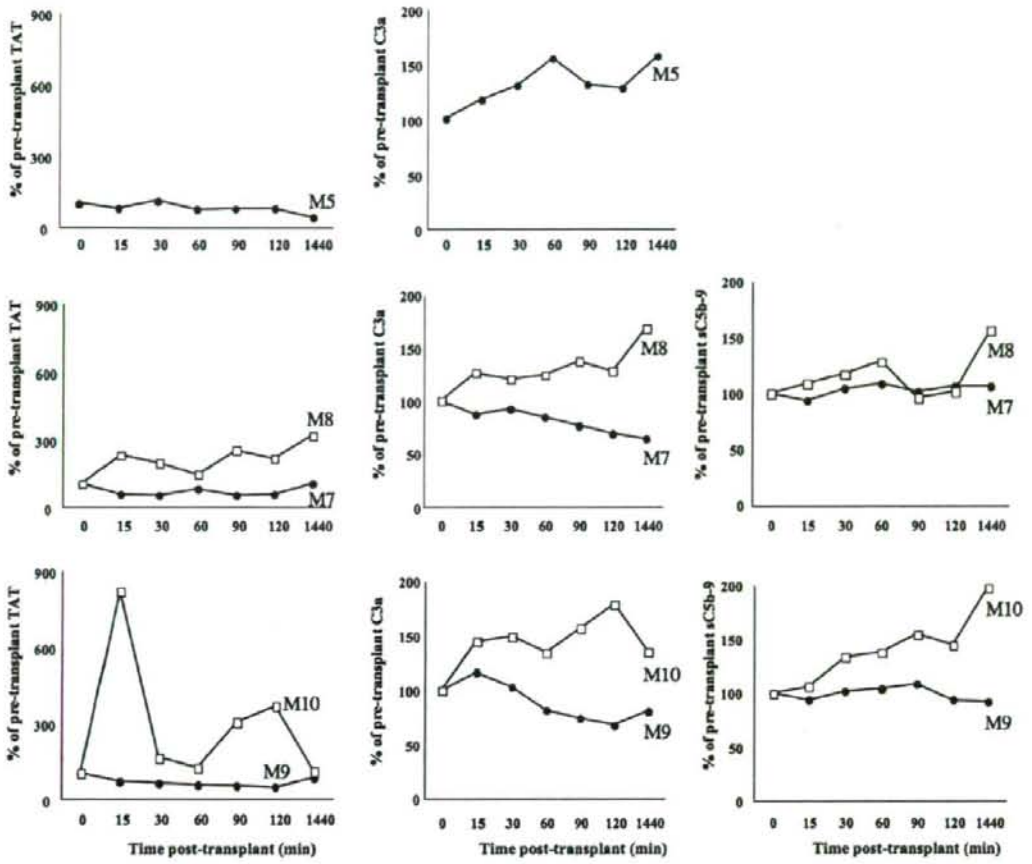

Fig. 2. EDTA blood was drawn from a femoral vein catheter of the transplanted monkeys treated with heparin (squares) or LMW-DS (circles) at varying time points after porcine islet xenotransplantation. TAT, C3a, and sC5b–9 levels were assessed and expressed as percentage of the pre-transplant values.

Immunohistochemical staining of grafted pig islets after intraportal transplantation into monkeys treated with LMW-DS or heparin

The immunohistochemical findings from the grafts were summarized in Fig. 4 and Table 2. As expected, most parameters involved in innate immune responses were active after 24 h post-islet transplantation in the controls M8 and M10. In particular, CD68+ macrophages, and neutrophil elastase positive PMNs were abrogated in the monkeys treated with LMW-DS compared with the controls given heparin. Also, CD41+ platelets tended to be lower in the LMW-DS treated animals. CD56+ natural killer cells were found only occasionally. Unlike the soluble complement markers there was no clear inhibition of complement activation as reflected in deposition of C3 fragments and C9 on the surface of the islets. Furthermore, IgM antibodies were found on islet both in LMW-DS and heparin-treated animals. Most of parameters reflecting specific immune responses were yet silent. However, CD3+ T-cell infiltration was already seen in the islet grafts of the controls M8 and M10. Notably, this infiltration was effectively suppressed by LMW-DS.

Binding of complement components to porcine islets after incubation in human plasma

After incubation in hirudin-treated plasma, the porcine islets were stained with FITC-conjugated antibodies recognizing IgG, IgM, C1q, C3b/iC3b, C4 fragments, C9, and MBL. Large particle flow cytometry and confocal microscopy demonstrated that antibodies against IgG, IgM, C1q, C4, and C3

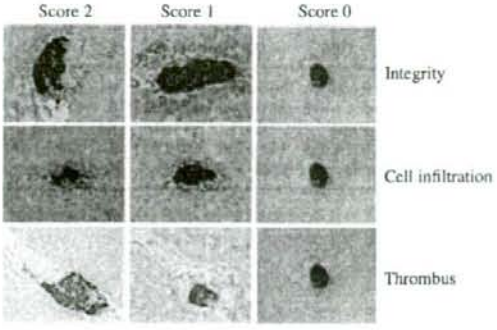

Fig. 3. Visual examples of the morphological scoring system used to quantify different aspects of the IBMIR. Hematoxylin eosin-stained porcine islet grafts retrieved 24 h after intraportal xenotransplantation from diabetic monkeys treated with LMW-DS or heparin were used. A summary of all transplanted monkeys is presented in Table 1.

bound strongly to the islets, but the binding of MBL and C9 was less prominent (Fig. 5A and B). C3b/iC3b fragments were detected on the islets after only 5 min, and the binding of C3b/iC3b continued to increase over time. Addition of Compstatin significantly reduced the binding of C3b/iC3b to the islets (Fig. 5C). Confocal microscopy analyses confirmed these results (not shown).

Inhibition of complement activation by LMW-DS and Compstatin

Ten percentage (v/v) human serum was incubated in wells of microtiter plates in the presence of LMW-DS and/or Compstatin for 30 min at 37 °C (Fig. 6). In the presence of Compstatin there was no effect below 0.5 μM of the compound, but at higher concentrations Compstatin gradually inhibited complement activation. At 5 μM total inhibition was achieved. LMW-DS inhibited complement activation only marginally between 10 and 100 mg/l, but the effect was more pronounced at concentrations above this level. There was no indication of interaction between the drugs regarding this effect on complement activation in serum.

Discussion

We have previously shown that LMW-DS efficiently prevents clotting that occurs in both allogeneic and xenogeneic IBMIR triggered by APIs both in vitro and in vivo in a small animal model [9,18]. Here, we confirm that LMW-DS is efficient also in a primate model mimicking the clinical setting. The effect of LMW-DS was compared with that of heparin, which is routinely used in clinical islet transplantation. LMW-DS was proved to be

Table 1. Summary of the morphological score (as depicted in Fig. 3) of the islets grafts in recipient monkeys M5 and M7–M10

Monkey number	Treatment	Integrity	Thrombus	Cell infiltration	Percentage of score 0[a] (%)	APTT at 24 h after transplantation (s)
M5	LMW-DS, n = 113	0.66 ± 0.04[b]	0.26 ± 0.03	0.90 ± 0.07	37.2	101
M7	LMW-DS, n = 134	0.93 ± 0.06	0.52 ± 0.06	1.08 ± 0.06	26.1	66
M8	Heparin, n = 149	1.05 ± 0.05	0.62 ± 0.06	1.17 ± 0.06	20.1	24
P-value[c]		0.13	0.28	0.32	0.23	
M9	LMW-DS, n = 134	0.63 ± 0.05	0.37 ± 0.05	0.85 ± 0.06	44.0	107
M10	Heparin, n = 125	0.95 ± 0.06	0.54 ± 0.06	1.25 ± 0.07	22.4	25
P-value[d]		<0.0001	<0.05	<0.0001	<0.001	

[a]Percentage islets with no signs of IBMIR (score 0); [b]Values are expressed as mean ± SEM; [c]P-values for M7 and M8; [d]P-values for M9 and M10. APTT, activated partial thromboplastin time; IBMIR, instant blood-mediated inflammatory reaction; LMW-DS, Low molecular weight dextran sulfate.

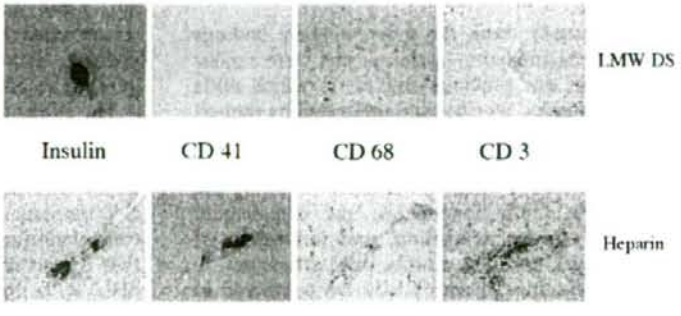

Fig. 4. Immunohistochemical staining of porcine islet grafts retrieved 24 h after intraportal xenotransplantation from diabetic monkeys treated with LMW-DS or heparin. The figure shows representative expression of insulin and of CD41 (platelets), CD68 (macrophages), and CD3 (T cells) in the grafts. A summary of all transplanted monkeys is presented in Table 2. Magnification 200×.

Table 2. Summary of the immunohistochemical staining (as depicted in Fig. 4) of the islet grafts in recipient monkeys receiving LMW-DS or heparin

Treatment	CD41	C3c	C9	Neutrophil elastase	CD68	MAC 387	CD56	CD3	CD20	IgG	IgM
LMW-DS	(−)-(++)	(−)-(++)	(−)-(+++)	(−)-(++)	(−)-(++)	(−)-(++)	(−)-(+)	(−)-(++)	(−)-(+)	(−)	(−)-(+)
(n = 21)	0.59 ± 0.19[a]	0.80 ± 0.37	1.50 ± 0.31	0.42 ± 0.23	1.31 ± 0.21	0.90 ± 0.28	0.10 ± 0.10	0.63 ± 0.20	0.20 ± 0.13	0	0.25 ± 0.25
Heparin (n = 18)	(−)-(+++)	(−)-(++)	(−)-(+++)	(−)-(++)	(++)-(+++)	(+)-(+++)	(−)-(+)	(−)-(+++)	(−)-(++)	(−)	(−)-(++)
	1.60 ± 0.51	0.63 ± 0.26	1.67 ± 0.33	1.08 ± 0.23	2.17 ± 0.11	2.11 ± 0.26	0.22 ± 0.15	1.90 ± 0.35	0.40 ± 0.27	0	0.60 ± 0.24
P value	0.056	0.69	0.71	0.04	0.002	0.01	0.48	0.006	0.83	–	0.36

[a]Values are expressed as mean ± SEM. LMW-DS, Low molecular weight dextran sulfate.

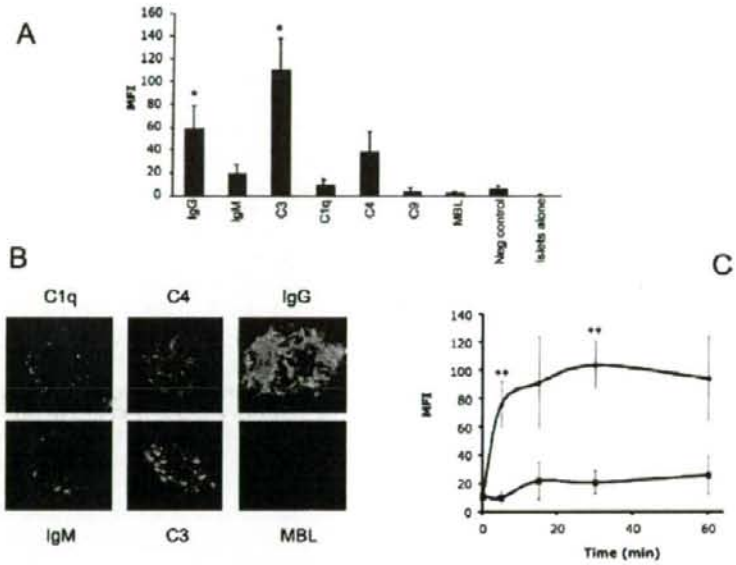

Fig. 5. Porcine islets incubated in hirudin-treated plasma for 30 min. The islets were stained for IgG (n = 5), IgM (n = 5), C3b/iC3b (n = 5), C1q (n = 3), C4 (n = 5), C9 (n = 3), and MBL (n = 3). As negative control, an antibody recognizing mouse IgG was used (n = 5). The islets were analyzed by (A) large particle flow cytometry and (B) confocal microscopy. In (C), the deposition of C3b/iC3b on the islet in the absence and presence of Compstatin is presented after analysis by large particle flow cytometry (n = 5; statistical evaluation was performed at 5 and 30 min where n = 7; $^{*}P < 0.05$ and $^{**}P < 0.01$).

far more efficient in inhibiting the IBMIR than heparin. These data confirm those of Rood et al. [19] who recently demonstrated longer porcine islet survival in non-human primates treated with LMW-DS.

In this study, both the morphological findings and the measurements in the plasma were similar to those in our previous studies, in which APIs were surrounded by clots and infiltrated by numerous leukocytes immediately after contact with fresh blood seen in the tubing loop model and our small animal model [9]. Most parameters reflecting the IBMIR, i.e. both coagulation and complement cascades, platelet deposition, and infiltration of macrophages and neutrophils, were attenuated in the monkeys treated with LMW-DS compared to the controls. There was also a tendency that increases in granulocyte count and liver enzymes were attenuated. One control monkey (M6) died of hypoglycemia, suggesting a strong IBMIR. Notably, T-cell infiltration observed in some of the transplanted islet grafts was also effectively suppressed, demonstrating that also the adaptive immune responses are attenuated by LMW-DS.

The effects of LMW-DS on the adaptive immune system may be explained by the effects on complement activation as complement is also of great importance in bridging innate immunity and specific immune responses. In allogeneic whole organ transplantation, C3 is one of the essential factors that trigger rejection in mice [20–22] and humans [23]. It is therefore reasonable to expect that

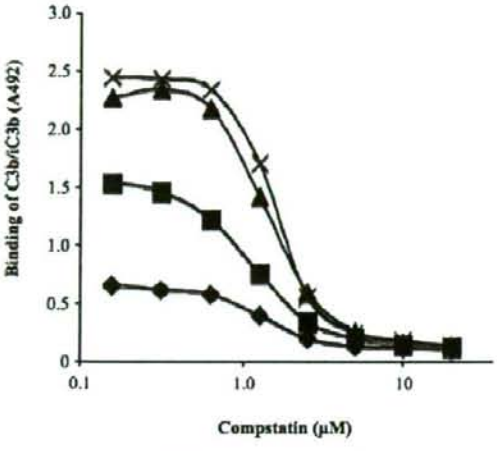

Fig. 6. Binding of C3b/iC3b to the surface of microtiter wells after incubation with 10% serum in the presence of increasing doses of Compstatin for 30 min at 37 °C. 0 (cross), 10 (triangle), 100 (squares), and 1000 (diamond) mg/l of LMW-DS was present in the wells.

complement activation will trigger a profound adaptive immune response raised against the graft, necessitating an unwarrantedly heavy immunosuppressive regimen. Previous studies support such a hypothesis [4,5].

As shown in Table 1, the islet grafts in M9 that reached an APTT of 107 s 24 h after transplantation, demonstrated well-preserved morphology suggesting that this dose of LMW-DS would be preferable. In a recently performed phase I study in normal individuals, we have shown that this concentration can be reached without an increased risk of bleeding or side effects (manuscript under preparation). This makes treatment with LMW-DS during xenogeneic islet transplantation an attractive alternative. It should be noted that a specific concentration of LMW-DS gives different APTT in blood from different individuals both in vitro and in vivo, probably due to that different allotypes of certain coagulation factors interact with LMW-DS differently.

In our previous studies, we showed that complement activation induced in xenogeneic IBMIR occurs secondarily to coagulation activation; a reaction which is also seen in allogeneic IBMIR [9,24] and which is elicited by chondroitin sulfate released by activated platelets [25]. This explains why complement activation in this study was suppressed in parallel with the reduction of coagulation activation at substantially lower concentrations (15–35 mg/l) of LMW-DS than used in other studies aiming for a inhibition of hyper acute rejection [26,27]. However, unlike complement activation in the fluid phase, immunohistochemical analyses showed that complement deposition was still seen on the islet grafts in the monkeys treated with LMW-DS. These reactions were analyzed in detail in vitro using large particle flow cytometry and confocal microscopy to be able to clarify the mechanism of activation. The experiments were performed using human plasma to directly translate the findings to clinical islet xenotransplantation. Pig islets incubated in human plasma revealed an almost instantaneous binding of IgM and IgG antibodies and complement components already after 5 min. This rapid activation was completely inhibited by Compstatin. It is possible that the instantaneous insulin dumping in a non-human primate model previously reported by Bennet et al. [3] and also observed in monkey M6 is explained by this antibody-mediated reaction. The severity of this reaction, which was totally abrogated by the recombinant complement inhibitor CR1, is reflected in the fact that the release of insulin corresponded to about 40% of the insulin in the transplanted islets.

One way to fully inhibit complement activation is to increase the dose of LMW-DS, but as shown in our in vitro experiments, doses between 10 and 100 mg/l have only minor effects on complement activation alone. Moreover, higher doses of LMW-DS are likely to give side effects. It is therefore obvious that LMW-DS must be combined with a specific complement inhibitor such as Compstatin to block the immediate destructive immunoglobulin-triggered complement activation found both in vitro and in vivo [10,28]. The in vitro studies show that LMW-DS and Compstatin do not interact in human serum.

Taken together, it possible to propose a model of how the different components of IBMIR interact in xenogeneic combinations: (1) Immediately when porcine islets come in contact with human blood there is an instantaneous binding of IgG and IgM antibodies to the islet surface which triggers a deleterious complement activation; (2) This is followed by a clotting reaction with accompanying complement activation. Based upon the experimental data presented, LMW-DS combined with a specific complement inhibitor is an attractive alternative to control the detrimental innate immune responses that are postulated to occur in forthcoming intraportal pre-clinical and clinical islet xenotransplantation trials. We are at the moment in progress to produce sufficient amounts of Compstatin to perform studies in the NHP model with Compstatin combined with LMW-DS.

Acknowledgments

This work was supported by National Institute of Health Grants 5U01-A1065192, GM-62134, GM069736, and AI068730, Juvenile Diabetes Research Foundation, 1-2004-18, Swedish Research Council Grants 5647, and 15244.

References

1. Shapiro AM, Lakey JR, Ryan EA et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343: 230.
2. Rood PP, Buhler LH, Bottino R, Trucco M, Cooper DK. Pig-to-nonhuman primate islet xenotransplantation: a review of current problems. Cell Transplant 2006; 15: 89.
3. Bennet W, Sundberg B, Lundgren T et al. Damage to porcine islets of Langerhans after exposure to human blood *in vitro*, or after intraportal transplantation to cynomologus monkeys: protective effects of sCR1 and heparin. Transplantation 2000; 69: 711.
4. Buhler L, Deng S, O'Neil J et al. Adult porcine islet transplantation in baboons treated with conventional immunosuppression or a non-myeloablative regimen and CD154 blockade. Xenotransplantation 2002; 9: 3.
5. Cantarovich D, Blancho G, Potiron N et al. Rapid failure of pig islet transplantation in non human primates. Xenotransplantation 2002; 9: 25.
6. Kirchhof N, Shibata S, Wukstrom M et al. Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. Xenotransplantation 2004; 11: 396.
7. Cardona K, Korbutt GS, Milas Z et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med 2006; 12: 304.
8. Hering BJ, Wukstrom M, Graham ML et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 2006; 12: 301.
9. Goto M, Johansson H, Maeda A, Elgue G, Korsgren O, Nilsson B. Low molecular weight dextran sulfate prevents the instant blood-mediated inflammatory reaction induced by adult porcine islets. Transplantation 2004a; 77: 741.
10. Nilsson B, Larsson R, Hong J et al. Compstatin inhibits complement and cellular activation in whole blood in two models of extracorporeal circulation. Blood 1998; 92: 1661.
11. Brandhorst H, Brandhorst D, Hering BJ, Bretzel RG. Significant progress in porcine islet mass isolation utilizing liberase HI for enzymatic low-temperature pancreas digestion. Transplantation 1999; 68: 355.
12. Kissane JM, Robins E. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J Biol Chem 1958; 233: 184.
13. Johansson H, Goto M, Dufrane D et al. Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. Am J Transplant 2006; 6: 305.
14. Nilsson Ekdahl K, Nilsson B, Pekna M, Nilsson UR. Generation of iC3 at the interface between blood and gas. Scand J Immunol 1992; 35: 85.
15. Mollnes TE, Riesenfeld J, Garred P et al. A new model for evaluation of biocompatibility: combined determination of neoepitopes in blood and on artificial surfaces demonstrates reduced complement activation by immobilization of heparin. Artif Organs 1995; 19: 909.
16. Katragadda M, Magotti P, Sfyroera G, Lambris JD. Hydrophobic effect and hydrogen bonds account for the improved activity of a complement inhibitor, compstatin. J Med Chem 2006; 49: 4616.
17. Fernandez LA, Hatch EW, Armann B et al. Validation of large particle flow cytometry for the analysis and sorting of intact pancreatic islets. Transplantation 2005; 80: 729.
18. Goto M, Groth CG, Nilsson B, Korsgren O. Intraportal pig islet xenotransplantation into athymic mice as an in vivo model for the study of the instant blood-mediated inflammatory reaction. Xenotransplantation 2004b; 11: 195.
19. Rood PP, Bottino R, Balamurugan AN et al. Reduction of early graft loss after intraportal porcine islet transplantation in monkeys. Transplantation 2007; 83: 202.
20. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. Nat Med 2002; 8: 582.
21. Chowdhury P, Zhou W, Sacks SH. Complement in renal transplantation. Nephron Clin Pract 2003; 95: c3.
22. Peng Q, Li K, Patel H, Sacks SH, Zhou W. Dendritic cell synthesis of C3 is required for full T cell activation and development of a Th1 phenotype. J Immunol 2006; 176: 3330.
23. Brown KM, Kondeatis E, Vaughan RW et al. Influence of donor C3 allotype on late renal-transplantation outcome. N Engl J Med 2006; 354: 2014.
24. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. Diabetes 2002; 51: 1779.
25. Hamad O, Nilsson Ekdahl K, Nilsson P et al. Complement activation is triggered by chondroitin sulfate released by thrombin receptor activated platelets. J Thrombosis Haemostasis 2008, in press.
26. Fiorante P, Banz Y, Mohacsi PJ et al. Low molecular weight dextran sulfate prevents complement activation and delays hyperacute rejection in pig-to-human xenotransplantation models. Xenotransplantation 2001; 8: 24.
27. Thomas H, Maillet F, Letourneur D, Jozefonvicz J, Fischer E, Kazatchkine MD. Sulfonated dextran inhibits complement activation and complement-dependent cytotoxicity in an in vitro model of hyperacute xenograft rejection. Mol Immunol 1996; 33: 643.
28. Soulika AM, Khan MM, Hattori T et al. Inhibition of heparin/protamine complex-induced complement activation by Compstatin in baboons. Clin Immunol 2000; 96: 212.

Brain death in combination with warm ischemic stress during isolation procedures induces the expression of crucial inflammatory mediators in the isolated islets

Yukihiko Saito[1], Masafumi Goto[1,2], Kozue Maya[2], Norihiko Ogawa[1], Keisei Fujimori[3], Yoshimochi Kurokawa[4], and Susumu Satomi[1]

[1]Division of Advanced Surgical Science and Technology, Tohoku University, Sendai, 980-8574, Japan

[2]Tohoku University International Advanced Research and Education Organization, Tohoku University, Sendai, 980-8575, Japan

[3]Medical Safety Management Office, Tohoku University, Sendai, 980-8574, Japan

[4]Tohoku University Innovation of New Biomedical Engineering Center, Tohoku University, Sendai, 980-8574, Japan

This study has been supported by grants from Innovation Plaza Miyagi of JST (Japan Science and Technology Agency), the Japanese Grant-in-Aid for Scientific Research (B), the Nakajima Foundation, and Takeda Foundation.

Address for correspondence:

Masafumi Goto, M.D., Ph. D.

Tohoku University International Advanced Research and Education Organization,

Tohoku University

2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan

Tel : +81 22 717 7895

Fax : +81 22 717 7899

E-mail : gotokichi@aol.com

Total word count : 2454

Abstract word count : 289 (<300)

Tables : 0

Figures : 5