

Human Pancreatic Duct Cells Exert Tissue Factor–Dependent Procoagulant Activity Relevance to Islet Transplantation

Claire Beuneu,^{1,2} Olivier Vosters,^{1,2} Babak Movahedi,^{2,3} Myriam Rimmelink,⁴ Isabelle Salmon,⁴ Daniel Pipeleers,^{2,3} Olivier Pradier,¹ Michel Goldman,^{1,2} and Valérie Verhasselt^{1,2}

Activation of the coagulation cascade contributes to early graft loss and intraportal thrombotic events in clinical islet transplantation. Although these complications were shown to be related to the presence of tissue factor in human islet preparations, the contribution of duct cells, which represent a major contaminant of clinical islet isolates, has not been specified so far. Herein, we used flow cytometry, immunohistochemistry, RT-PCR, and functional coagulation assays to demonstrate that duct cells exert a potent factor VII–dependent procoagulant activity related to their expression of tissue factor. Both the classical membrane-bound and the recently described soluble form of tissue factor were shown to be synthesized by duct cells. We conclude that contaminating duct cells contribute to early β -cell damage after islet transplantation through their involvement in tissue factor–mediated thrombotic and inflammatory events. *Diabetes* 53:1407–1411, 2004

Early loss of functional β -cells after islet transplantation represents a major limitation of this therapeutic modality for type 1 diabetes, even with the most efficient protocols currently available (1,2). Recent observations by Korsgren, Nilsson, and coworkers (3–5) provided convincing evidence that this complication is related, at least in part, to activation of the coagulation cascade by islet isolates. Indeed, the procoagulant state induced by islet transplantation can culminate in portal vein thrombosis (6). The demonstration that

endocrine cells express tissue factor (TF) provided a plausible cause for the thrombotic reaction elicited by human islet suspensions (5). However, it has been established (7,8) that islet suspensions prepared for clinical transplantation contain a significant proportion (up to 40%) of nonendocrine duct cells. Indeed, even in the most successful report by Shapiro et al. (2), percentages of β -cells in clinical preparations never exceeded 52%, with a mean percentage of 24%. The contaminating duct cells have already been suggested to directly contribute to β -cell damage through their production of nitric oxide (9). Herein, we assessed the impact of duct cells on the coagulation system to address their possible involvement in the thrombotic reaction elicited by islet isolates.

RESEARCH DESIGN AND METHODS

Cell preparations. Human pancreatic duct cells were isolated from pancreata obtained from adult heart-beating organ donors as previously described (9). The organs were procured by European hospitals affiliated with the Eurotransplant Foundation (Leiden, the Netherlands) and sent to the Human β -Cell Bank in Brussels (Belgium) for the preparation of isolated fractions. Collagenase digests were separated by Ficoll gradient purification into an islet fraction and an exocrine fraction. The exocrine fraction was cultured in serum-free medium for at least 4 days, leading to a preparation enriched in duct cells. As recently reported (10), the purity of these duct cell preparations was routinely >90–95% as assessed by cytokeratin 19 (CK19) staining. The culture medium consisted of HAM's F10 with 6 mmol/l glucose, supplemented with 0.5% BSA, 0.1 mg/ml streptomycin, 125 units/ml penicillin, and 2 mmol/l L-glutamine. Thereafter, cells were either maintained in a serum-free medium (cultures in suspension) or dissociated in a calcium-free medium and cultured with 10% FCS to obtain monolayers. Human adenocarcinoma pancreatic duct cell line (CAPAN-2) was cultured as previously described (11). In one set of experiments, β -cells and duct cells were directly isolated using fluorescence-activated cell sorting, as previously described (9). As control, peripheral blood mononuclear cells (PBMCs) from healthy volunteers were activated during 6 h with 1 μ g/ml lipopolysaccharide from *E. coli* (serotype 0128:B12; Sigma Chemicals, Bornem, Belgium).

Procoagulant activity assay. Procoagulant activity (PCA) was determined in duplicates by a single-stage clotting assay on culture supernatants or on cell extracts realized in PBS by repetitive freezing. Each sample (100 μ l) was incubated at 37°C for 1 min with 100 μ l of normal citrated plasma before the initiation of clotting by the addition of 100 μ l of 25 mmol/l CaCl_2 . Clotting time was recorded with a KC10 apparatus (Amelung, Lemgo, Germany), and PCA (in milliunits per milliliter) was determined by reference to a standard curve generated with a commercial rabbit thromboplastin (BioMérieux, Marcy l'Etoile, France). The amount of thromboplastin that yielded a clotting time of 12.4 s was assigned a value of 1 unit. To determine the role of the TF/factor VII pathway in the PCA, experiments were performed with factor VII–deficient plasma (Dade Behring, Anderlecht, Belgium). The number of cells in the extracts was determined by counting or DNA measurement.

From the ¹Laboratory of Experimental Immunology and Institute for Medical Immunology, Université Libre de Bruxelles-ULB, Erasme Hospital, Brussels, Belgium; the ²Juvenile Diabetes Research Foundation Center for β -Cell Therapy in Europe, Brussels, Belgium; the ³Diabetes Research Center, Vrije Universiteit Brussels-VUB, Brussels, Belgium; and the ⁴Laboratory of Pathology, Université Libre de Bruxelles-ULB, Erasme Hospital, Brussels, Belgium.

Address correspondence and reprint requests to Michel Goldman, MD, PhD, Laboratory of Experimental Immunology, Université Libre de Bruxelles, Hôpital Erasme, 808 Route de Lennik, B-1070 Brussels, Belgium. E-mail: mgoldman@ulb.ac.be.

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asTF, alternatively spliced tissue factor; CK19, cytokeratin 19; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PCA, procoagulant activity; TF, tissue factor.

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TABLE 1
PCA of pancreatic cell preparations in plasma

| | PCA (mU/10 ⁶ cells)* | | |
|----------------------------|---------------------------------|--------------|--------------|
| | Experiment 1 | Experiment 2 | Experiment 3 |
| Pancreatic cell isolates | | | |
| Endocrine fraction† | | | |
| Normal plasma | 66 | 30 | 67 |
| Exocrine fraction | | | |
| Normal plasma | 295 | 123 | ND |
| Cultured duct cells | | | |
| Normal plasma | 235 | 201 | 270 |
| Factor VII–depleted plasma | 1 | 1 | 2 |
| CAPAN-2 cell line | | | |
| Cell extract | | | |
| Normal plasma | 839 | 688 | 579 |
| Factor VII–depleted plasma | 13 | 10 | 8 |
| Supernatant‡ | | | |
| Normal plasma | 97 | 106 | 74 |

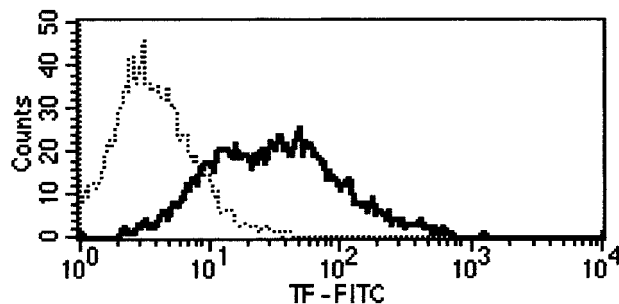
*PCA was assessed in plasma as described after addition of cell extracts or CAPAN-2 cell line supernatant; †the endocrine fraction contained 46, 49, and 52% of endocrine cells in experiments 1, 2, and 3, respectively; ‡PCA values in CAPAN-2 supernatant were corrected for supernatant volume and number of cells per well to allow comparison with cell extracts values. ND, not determined.

Flow cytometry analysis. Cells were washed in PBS supplemented with 1% BSA and 10% pooled human serum and incubated for 20 min at 4°C with the fluorescein isothiocyanate–conjugated IgG1 monoclonal antibody (mAb) against TF no. 4508CJ (American Diagnostica, Andresy, France) or the corresponding isotype-matched control mAb (Becton Dickinson, Erembodegem, Belgium). Cell fluorescence was analyzed using a FACScan flow cytometer (Becton Dickinson).

Immunohistochemistry studies. Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded human pancreatic specimens. Five-micron-thick sections were cut onto coated slides and deparaffined by routine techniques. After antigen retrieval in EDTA and microwave pretreatment, the sections were incubated with a mouse anti-TF mAb (IgG1 n4509; American Diagnostica) or a mouse anti-CK19 mAb (clone RCK108; Dako, Merelbeke, Belgium) at 1/100 dilution. Negative controls were obtained by omitting the primary antibody. Labeling was revealed by the streptavidin-biotin-peroxidase complex (Dako).

RT-PCR. mRNA was extracted from 0.2×10^6 duct cells or 0.5×10^6 PBMCs using the MagNA Pure mRNA extraction kit on the MagNA Pure instrument (Roche Applied Science, Brussels, Belgium) following the manufacturer's instructions. One-step RT-PCR was performed on a Lightcycler instrument (Roche Applied Science) with primers synthesized at Biosource Europe (Nivelles, Belgium). RT-PCR for TF or β -actin was realized with the following primers: TF: sense primer, 5'-TGAATGTGACCGTAGAAGATGA-3'; antisense primer, 5'-GGAGTTCTCCTTCCAGCTCT-3'; and β -actin: sense primer, 5'-GGTCAGAAGGATTCCATG-3'; antisense primer, 5'-GGTCTCAAACATGATCTGGG-3'. Products were separated by electrophoresis on 2% agarose and visualized with ethidium bromide. We also developed a real-time RT-PCR method using the following primers and probes: TF sense primer, 5'-GGGAATTCAGAGAAATATTCTACATCA-3', TF antisense primer, 5'-TAGCCAGGAT

A



B

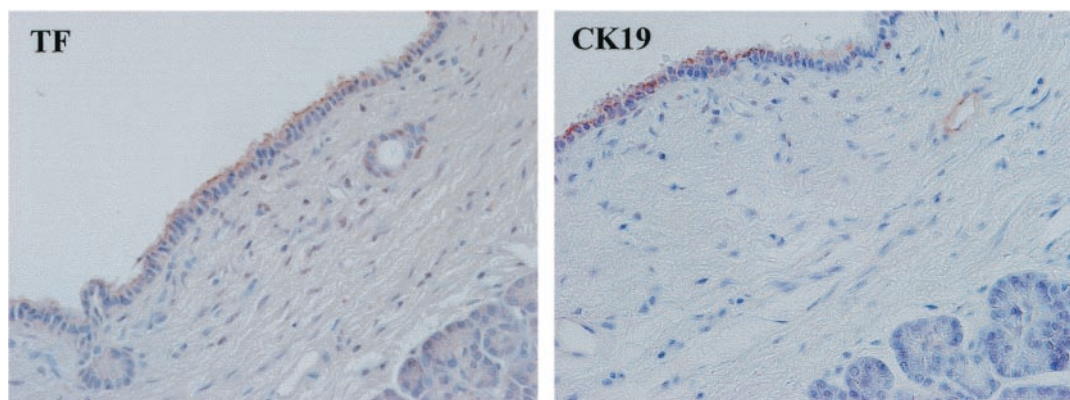


FIG. 1. Primary duct cells express TF. *A*: Primary duct cells cultured in monolayers were dissociated and analyzed by flow cytometry after staining by either anti-TF IgG mAb (thick line) or corresponding control isotype mAb (dotted line). A similar staining was found in four independent experiments. *B*: Immunohistochemical analysis for TF and CK19 was performed on serial sections of formalin-fixed, paraffin-embedded, human normal pancreatic specimens. *Left panel*: TF staining of epithelial cells. *Right panel*: CK19 staining on a consecutive section (original magnification 400 \times). FITC, fluorescein isothiocyanate.

GATGACAAGGA-3'; alternatively spliced TF (asTF) sense primer, 5'-TCT-TCAAGTTCAGGAAAGAAATATTCT-3'; asTF antisense primer, 5'-CCAGGATGATGACAAGGATGA-3'; and probe, 5'-TGGAGCTGTGGTATTTGTGGTCA-3'.

Tubing loop model. A whole-blood experiment protocol was adapted from a model previously described (5). Loops made of polyvinylchloride tubing (inner diameter 6.3 mm and length 390 mm) and treated with a Corline heparin surface were purchased from Corline (Uppsala, Sweden). Loops were supplemented with cell samples in 150 μ l PBS or with PBS alone before blood addition. When indicated, samples were preincubated at room temperature for 10 min with a neutralizing mAb against TF (IgG1 no. 4509) or PBS and added to the loops after two washing steps. Nonanticoagulated blood (5 ml) from healthy volunteers was added to each loop. To generate a blood flow of about 45 ml/min, loop devices were placed on a platform rocker inside a 37°C incubator. Blood samples were collected into EDTA (4.1 mmol/l final concentration) and citrate (12.9 mmol/l final concentration) tubes before and 30 min after perfusion began. Platelets were counted using a CellDyn 4000 automate (Abbott Laboratories, Abbott Park, IL), and fibrinogen was determined using a Behring coagulation system (Dade Behring).

RESULTS

PCA of pancreatic cell preparations in human plasma. In a preliminary set of experiments, we compared the PCA in human plasma of several cell preparations obtained from human pancreata. As shown in Table 1, we first found that both the endocrine and exocrine fractions induced significant PCA. When the exocrine fraction was cultured in conditions resulting in duct cell enrichment (>95%), PCA increased to reach values three- to sixfold above that of the endocrine fraction. The PCA activity of the enriched duct cell preparations was factor VII-dependent since it was not observed in factor VII-depleted plasma. In accordance with these findings obtained on primary duct cells, we observed that the CAPAN-2 pancreatic duct cell line exerted a factor VII-dependent PCA that was also present in the culture supernatants of this cell line.

TF expression by duct cells. The factor VII dependence of the duct cell-associated PCA strongly suggested the involvement of TF. TF expression by primary duct cells was first documented by flow cytometry (Fig. 1A). Additional immunohistochemical studies were conducted to confirm the constitutive expression of TF on duct cells *in vivo*. First, we confirmed a positive staining of islets for TF (data not shown). As shown in Fig. 1B, a clear staining for TF was observed on cells that express CK19, a marker specific for epithelial cells. The staining for TF was cytoplasmic and concentrated at the apical side.

Using RT-PCR, we confirmed that both the CAPAN-2 duct cell line and primary duct cells express TF mRNA (Fig. 2A). Interestingly, both the classical form and the recently described alternatively spliced variant (12) of TF mRNA were detected in the duct cell extracts. For control, we documented the upregulation of TF mRNA expression in PBMCs upon activation by bacterial lipopolysaccharide. In this cell type, the classical form of TF mRNA was clearly induced, whereas asTF was barely detected. In additional experiments, we used a real-time RT-PCR method to quantify TF and asTF mRNA expression in β -cells and duct cells directly purified by using flow-activated cell sorting. As shown in Fig. 2B (which is representative of three independent experiments), the expression of both TF and asTF mRNA was higher in duct cells (87% pure) than in β -cells (74% pure) from the same donor.

PCA of primary duct cells in whole blood. To investigate the PCA of duct cells in a model closer to the *in vivo* situation, we adapted the tubing loop system that was used to demonstrate the thrombotic reaction induced by

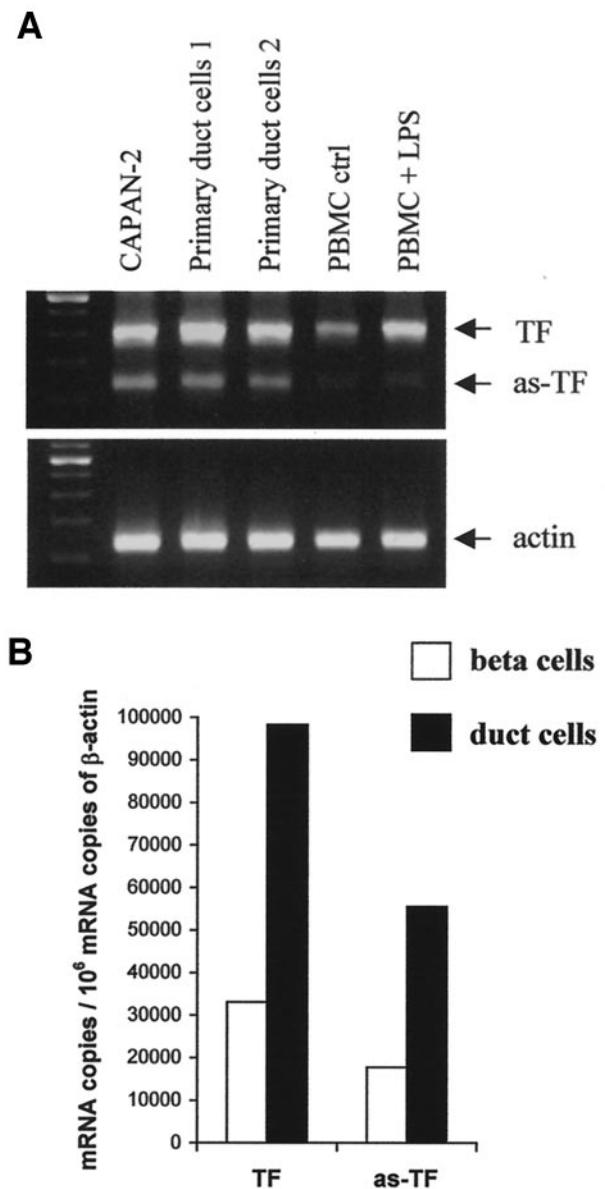


FIG. 2. Duct cells express the two forms of TF mRNA. **A:** mRNA extracted from CAPAN-2 cells, cultured primary duct cells, or resting or lipopolysaccharide-activated PBMCs were analyzed by RT-PCR for TF and β -actin. One representative experiment out of three is shown. TF indicates the size of classical TF mRNA (407 bp) encoding the membrane form; asTF indicates the size (247 bp) of the alternatively spliced variant encoding the soluble form. **B:** Quantification of TF and asTF mRNA using real-time RT-PCR in β -cells (74% pure) and duct cells (87% pure) from the same donor. One representative experiment out of three is shown. Results are expressed per 10⁶ mRNA copies of β -actin.

islet preparations (5). This model is based on the injection of nonanticoagulated blood in plastic loops in which the inner surface is coated with heparin to prevent contact-dependent blood coagulation, thus allowing the investigation of coagulation in the presence of platelets. Blood coagulation was assessed by macroscopic examination for the presence of clots and by monitoring platelet counts and fibrinogen levels, which drop as a consequence of coagulation activation. As shown in Table 2, we found that as few as 10³ duct cells added to 5 ml of blood were sufficient to induce clot formation within 30 min. This was associated with a dramatic drop in platelet counts and a

TABLE 2
TF-dependent PCA of cultured duct cells in whole blood

| Duct cell number* | Anti-TF | Clot† | Platelets ($\times 10^3/\mu\text{l}$) | Fibrinogen (mg/dl) |
|-------------------|---------|-------|---|--------------------|
| 0 | – | 0/7 | 196 \pm 15.9 | 246 \pm 14.2 |
| 10 ² | – | 0/3 | 173 \pm 31.1 | 251 \pm 21.0 |
| 10 ³ | – | 5/5 | 9 \pm 4.2 | <20 |
| | + | 0/3 | 154 \pm 22.3 | 214 \pm 32.2 |

Data are means \pm SE. *Cultured primary duct cells (10^2 or 10^3 cells) were added to 5 ml whole blood incubated in tubing loops under agitation at 37°C. When indicated, duct cells were pretreated with anti-TF antibody. Clot formation, platelet counts, and fibrinogen levels were monitored after 30 min incubation in the absence or presence of duct cells; †number of clotted samples/number of experiments.

complete consumption of fibrinogen. In three independent experiments, pretreatment of duct cells with an anti-TF mAb prevented the formation of visible clots and reduced the drop in platelet counts and fibrinogen levels.

DISCUSSION

The demonstration that the CAPAN-2 duct cell line expresses TF is in line with previous studies (13) showing the presence of TF in pancreatic ductal adenocarcinoma. As far as the normal pancreas is concerned, TF expression was previously documented (14) during embryogenesis, but not in adult tissue. Development of more sensitive immunostaining techniques using different anti-TF antibodies probably explains the recent demonstration (5) of TF expression in human islets. In our study, we confirmed this observation and also provided evidence for TF expression by duct cells in normal pancreas (Fig. 2). The latter finding is consistent with the expression of TF by normal epithelial cells in breast and kidney (15,16).

Interestingly, duct cells were shown to express the classical TF mRNA, encoding the membrane form of TF as well as asTF, which encodes the molecule-soluble form (12). It has been suggested that asTF could be involved in the propagation of the coagulation in blood and lead to the formation of macroscopic thrombi (12). Expression of asTF by duct cells might thus contribute, possibly along with the release of TF in microparticles (17), to the PCA present in the supernatants of duct cell cultures.

The relevance of our observations to islet transplantation is suggested by experimental observations (18) in rats showing the formation of thrombi shortly after transplantation, particularly around the nonendocrine tissue that contaminates islet preparations. Furthermore, there is also clinical evidence (6) that insufficient islet purification can promote development of portal vein thrombosis. In allogeneic as well as autologous islet transplants, the regular use of heparin might contribute to limit these thrombotic complications. Activation of coagulation by TF could not only result in thrombotic events but also elicit an inflammatory reaction involving upregulation of adhesion molecule expression and chemokine production (19,20). In the context of islet transplantation, the so-called instant blood-mediated inflammatory reaction likely contributes to early β -cell loss (5). It could also enhance graft immunogenicity, thereby promoting transplant rejection (21). Our findings demonstrate that contaminating duct cells can contribute to this post-islet transplant blood-mediated

inflammatory reaction originally described (5) as a consequence of TF expression by endocrine cells.

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REFERENCES

- Ryan EA, Lakey JR, Rajotte RV, Korbitt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM, Warnock GL, Larsen I, Shapiro AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710–719, 2001
- Shapiro AM, Lakey JR, Ryan EA, Korbitt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
- Bennet W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, Brandhorst H, Bretzel RG, Elgue G, Larsson R, Nilsson B, Korsgren O: Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? *Diabetes* 48:1907–1914, 1999
- 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* 51:1779–1784, 2002
- Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, Ostraat O, Salmela K, Tibell A, Tufveson G, Elgue G, Nilsson EK, Korsgren O, Nilsson B: Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* 360:2039–2045, 2002
- Shapiro AM, Lakey JR, Rajotte RV, Warnock GL, Friedlich MS, Jewell LD, Kneteman NM: Portal vein thrombosis after transplantation of partially purified pancreatic islets in a combined human liver/islet allograft. *Transplantation* 59:1060–1063, 1995
- Ling Z, Pipeleers DG: Prolonged exposure of human beta cells to elevated glucose levels results in sustained cellular activation leading to a loss of glucose regulation. *J Clin Invest* 98:2805–2812, 1996
- Keymeulen B, Ling Z, Gorus FK, Delvaux G, Bouwens L, Gruppung A, Hendriecx C, Pipeleers-Marichal M, Van Schravendijk C, Salmela K, Pipeleers DG: Implantation of standardized beta-cell grafts in a liver segment of IDDM patients: graft and recipients characteristics in two cases of insulin-independence under maintenance immunosuppression for prior kidney graft. *Diabetologia* 41:452–459, 1998
- Pavlovic D, Chen MC, Bouwens L, Eizirik DL, Pipeleers D: Contribution of ductal cells to cytokine responses by human pancreatic islets. *Diabetes* 48:29–33, 1999
- Vosters O, Beuneu C, Nagy N, Movahedi B, Aksoy E, Salmon I, Pipeleers D, Goldman M, Verhasselt V: CD40 expression on human pancreatic duct cells: role in nuclear factor-kappa B activation and production of proinflammatory cytokines. *Diabetologia*. In press
- De Breuck S, Lardon J, Rooman J, Bouwens L: Netrin-1 expression in fetal and regenerating rat pancreas and its effect on the migration of human pancreatic duct and porcine islet precursor cells. *Diabetologia* 46:926–933, 2003
- Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y: Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med* 9:458–462, 2003
- Kakkar AK, Lemoine NR, Scully MF, Tebbutt S, Williamson RC: Tissue factor expression correlates with histological grade in human pancreatic cancer. *Br J Surg* 82:1101–1104, 1995
- Luther T, Flossel C, Mackman N, Bierhaus A, Kasper M, Albrecht S, Sage EH, Iruela-Arispe L, Grossmann H, Strohle A, Zhang Y, Nawroth PP, Carmeliet P, Loskutoff DJ, Muller M: Tissue factor expression during human and mouse development. *Am J Pathol* 149:101–113, 1996
- Luther T, Flossel C, Albrecht S, Kotsch M, Muller M: Tissue factor

- expression in normal and abnormal mammary gland (Letter). *Nat Med* 2:491-492, 1996
16. Sugawara T, Yamabe H, Osawa H, Kaizuka M, Shirato K, Nakamura M, Tamura M, Okumura K: Tissue factor pathway inhibitor production by human proximal tubular epithelial cells in culture. *Thromb Res* 110:141-147, 2003
 17. Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Fallon JT, Badimon JJ, Hember J, Riederer MA, Nemerson Y: Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci U S A* 96:2311-2315, 1999
 18. Franklin WA, Schulak JA, Reckard CR: The fate of transplanted pancreatic islets in the rat. *Am J Pathol* 94:85-95, 1979
 19. Dugina TN, Kiseleva EV, Chistov IV, Umarova BA, Strukova SM: Receptors of the PAR family as a link between blood coagulation and inflammation. *Biochemistry (Mosc)* 67:65-74, 2002
 20. Bokarewa MI, Morrissey JH, Tarkowski A: Tissue factor as a proinflammatory agent. *Arthritis Res* 4:190-195, 2002
 21. Le Moine A, Goldman M: Contributions of innate immunity to allograft rejection and survival. *Curr Opin Organ Transplant* 8:2-6, 2003