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

**Rapid Publication**

**Human Pancreatic Duct Cells Exert Tissue Factor–Dependent Procoagulant Activity**

**Relevance to Islet Transplantation**

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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 pancreatic tissue 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 digestes 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 µg/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 µl) was incubated at 37°C for 1 min with 100 µl of normal citrated plasma before the initiation of clotting by the addition of 100 µl of 25 mmol/l CaCl2. 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.
**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, Andrey, 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 deparaffinized 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′-GGTCAGAAGGATTCTATG-3′; antisense primer, 5′-GGTGTCACACATATGCTGG-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′-GGGAATCTGAGAATATTCTACATCA-3′; TF antisense primer, 5′-TAGCCAGGAT

![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×). FITC, fluorescein isothiocyanate.](image-url)
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) obtained from human pancreata. As shown in Table 1, we obtained from human pancreata. As shown in Table 1, we

**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.

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**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 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 10^3 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
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 aTf, which encodes the molecule-soluble form (12). It has been suggested that aTf could be involved in the propagation of the coagulation in blood and lead to the formation of macroscopic thrombi (12). Expression of aTf 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 originally described (5) as a consequence of TF expression by endocrine cells.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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**TABLE 2**

<table>
<thead>
<tr>
<th>Duct cell number†</th>
<th>Anti-TF</th>
<th>Clot†</th>
<th>Platelets (×10³/μL)</th>
<th>Fibrinogen (mg/dl)</th>
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<tr>
<td>0</td>
<td>–</td>
<td>0/7</td>
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<td>246 ± 14.2</td>
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<tr>
<td>10²</td>
<td>–</td>
<td>0/3</td>
<td>173 ± 31.1</td>
<td>251 ± 21.0</td>
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<td>10³</td>
<td>5/5</td>
<td>9 ± 4.2</td>
<td>&lt;20</td>
<td></td>
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<tr>
<td>+</td>
<td>0/3</td>
<td>154 ± 22.3</td>
<td>214 ± 32.2</td>
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Data are means ± SE. *Cultured primary duct cells (10⁶ or 10⁷ 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 agitation at 37°C 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 agitation at 37°C 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 agitation at 37°C 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 agitation at 37°C 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 agitation at 37°C 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 agitation at 37°C were added to 5 ml whole blood incubated in tubing loops under agitation at 37°C.


