Increased Tissue Factor Activity of Monocytes/Macrophages Isolated From Canine Renal Allografts

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Kidney allografting was performed in a group of ten beagles, and viable leukocytes infiltrating the transplanted organs were isolated during episodes of acute rejection 5 or 6 days postoperatively. These infiltrate populations, consisting predominantly of lymphocytes and monocytes/macrophages, were found to have significantly increased amounts of procoagulant activity relative to control leukocytes isolated from circulating blood and lymph. Using nonspecific esterase staining in an agar microclot assay, procoagulant activity in the infiltrate leukocytes was found to reside in monocytes/macrophages rather than other coisolated cell types. By contrast, control monocytes from blood had no activity in this microclot assay. Procoagulant activity in the infiltrate cells was characterized as tissue factor. Increased amounts of this activator of the extrinsic pathway, as found in infiltrate monocytes/macrophages, may initiate clotting reactions and fibrin deposition within allografts.

Available evidence indicates that allograft rejection is a complex immunologic interaction largely mediated by leukocytes derived from blood that migrate extravascularly into transplants and exert tissue damage by mechanisms such as cell-mediated cytolysis, antibody-dependent cell-mediated cytolysis, and macrophage killing.1-3 However, it is clear that kidney allografts are destroyed not only by direct cytotoxic effects of lymphocytes and macrophages, but also by pathologic effects resulting from activation of the clotting system. Evidence that effects of blood coagulation are pathogenic during rejection is provided by findings of fibrin deposits in the capillaries, interstitial areas, and glomeruli of rejected kidneys. In addition, thrombosis of blood vessels within allografts and generalized thrombotic complications, such as pulmonary embolism and disseminated intravascular coagulation, occur in allograft recipients.4-14

Although there is ample evidence showing that activation of clotting occurs during rejection, precise sources of procoagulant activity triggering the clotting cascade in this type of inflammatory response remain to be identified. Our findings in the present experiments demonstrate that leukocyte populations isolated from kidney allografts have increased amounts of tissue factor activity, as compared to controls, from blood and lymph. Furthermore, monocytes/macrophages are identified as the source of this procoagulant in the infiltrate leukocyte populations.

Materials and Methods

Surgical Technique

Healthy outbred female beagle dogs (7–10 kg) with normal urinalysis, hemoglobins, and serum creatinines were used in this study. The dogs were anesthetized with 4% sodium thiamylal (1 ml/5 lb) and halothane. Following insertion of femoral arterial and venous catheters, normal saline (50 ml/kg plus 3 ml for each ml of estimated blood loss) was infused, and 1 g of ticarcillin was administered. Through a midline incision, the left renal artery, vein, and ureter were isolated. The dog was given intravenous heparin (2 mg/kg), furosemide 20 mg, and mannitol (6.25 g), and the left kidney was removed and flushed with 250 ml of cold Collin’s solution (electrolyte solution for kidney presentation, Travenol, Deerfield, IL) after 5 min of warm ischemia. The kidney was then either autotransplanted or taken to a similarly prepared dog for allografting. Hypothermia was maintained by placing the kidney in cold saline slush until blood flow was reestablished. Transplantation was performed by anastomosing the renal artery and vein to respective iliac vessels in an end-to-end fashion with 6-0 polypropylene sutures. Furosemide and mannitol were infused just prior to completion of the arterial anastomosis. A ureteroneocystostomy was performed by tunneling the ureter submucosally through the posterior bladder wall using an anterior cystotomy exposure. The midline incision was closed in layers. A second dose of ticarcillin was given, and the animal was awakened and returned to the vivarium recovery room.

Surgical exploration was performed on day 5 or 6 after transplantation, when allografts were removed for further study. Only animals with viable kidneys, as evidenced by blood flow identifiable through a patent renal artery and vein at the time of sacrifice, were included in the study, as thrombosed kidneys were found to be too necrotic for successful isolation of leukocytes. Previous experience with >25 beagle allotransplants in this laboratory invariably showed advanced rejection and kidney necrosis by the third postoperative week, indicating major histoincompatibility in the animal population studied.

Leukocyte Procurement from Blood, Lymph, and Kidney Tissues

One week prior to surgery, blood was sampled from each animal. At the time of sacrifice, thoracic duct lymph as well as the normal and transplanted kidneys were obtained. Blood (60 ml) was collected from the jugular vein in a syringe containing 6 ml of citrate anticoagulant, divided equally into 15-ml tubes, and centrifuged at 22°C for 10 min at 220 g. Theuffy coat was aspirated into a 50-ml tube containing cold minimal essential medium (MEM) with 1% bovine serum albumin (BSA), 6 g/liter HEPES buffer, and 1.5 g/liter EDTA. Following centrifugation, the
buffy coat was removed and diluted to 50 ml with cold MEM and kept on ice while other tissues were processed. Lymph (20 ml) was collected from a thoracic duct cannula into a 50-ml tube containing 2 ml citrate anticoagulant. The lymph was diluted to 50 ml with cold MEM and kept on ice.

After ligation of the renal artery, vein and ureter, kidneys were removed from donor animals and chilled on ice. The entire removed kidney was decapsulated and cut into small pieces (≤0.5 cm). Infiltrate leukocytes were isolated by teasing the kidney tissue in a petri dish using a 20-gauge hypodermic needle attached to a syringe. Proteolytic enzymes were not used. The teased tissue in MEM was pressed and washed through a fine wire mesh. Cells filtered through the mesh were chilled on ice and further purified as described below. As the entire kidney was used for leukocyte extraction, the numbers of leukocytes recovered, as described in Results for allografts (and controls), indicate total yields obtained per transplant.

Throughout the remainder of the isolation procedure, blood, lymph, and kidney were processed in the same manner. To remove any large clumps, preparations were suspended in the 50-ml tubes and allowed to settle for 1 min. The supernatants were decanted into fresh tubes and poured over 20-ml nylon-wool columns. Cell eluates were quickly collected and washed twice in cold MEM. The resultant cell pellets were suspended in 40 ml of MEM/0.5 ml of pellet volume. Each 40 ml of this cell suspension was underlaid with 10 ml of 8% Ficoll-Hypaque (refractive index: 1.3570–1.3575) and centrifuged at 27°C and 1,700 rpm (700 g) for 30 min.

Leukocyte bands at the gradient interface were aspirated and washed with saline, and resultant cell pellets were suspended in 1 ml MEM (without EDTA)/0.2–0.3 ml of pellet volume. One milliliter of this suspension was layered over a continuous Percoll (Pharmacia, Piscataway, NJ) gradient and centrifuged at 2,000 rpm (1,000 g) for 20 min at room temperature. Cell band positions were measured in centimeters from the meniscus, and density was determined using a calibration curve prepared with colored density marker beads (Pharmacia) centrifuged on control gradients. The bands were collected by aspiration with a Pasteur pipette and washed in cold MEM without EDTA for 10 min at 1,000 rpm (250 g) at 4°C. Supernatants were discarded and cell pellets were then suspended in MEM to a 0.5 ml total volume. Cell counts were determined using a Coulter Counter and concentrations adjusted to 2 x 10^6 cells/ml. Viability was determined by trypan blue exclusion. The percent of mononuclear cells was counted by using Wright's stained smears, and the percent monocytes/macrophages was determined by nonspecific esterase staining.15

Preparation of Percoll Gradients

A Pharmacia GM 1 gradient maker was used to prepare the continuous density Percoll gradients. Percoll (22.5 ml), with 2.5 ml of 10X Hanks’ balanced salt solution (HBSS) containing 1% BSA and 60 g/liter HEPES buffer, was placed into the right cup of the gradient maker, and 25 ml of 1X HBSS with 0.1% BSA and 6 g/liter HEPES was placed into the left cup. The gradients were collected in clear glass centrifuge tubes (Bellco, Vineland, NJ) and had a final volume of 50 ml.

Clotting Assay for Measurement of Procoagulant Activity Units

Leukocyte suspensions (2 x 10^6 cells/ml) were dispensed in 0.1-ml amounts into a 12 x 75 ml clear plastic tube in a 37°C water bath. To this was added 0.1 ml of 0.025 M CaCl₂, dissolved in saline, followed by 0.1 ml of citrated dog plasma. Added phospholipid had no effect on the assay.15 Recalcification times of buffer banks were always >500 sec. Clotting times were converted into procoagulant activity units using a calibration curve with a slope of −0.29, prepared as previously described.15 Curves made with serial dilutions of cells and dog brain tissue factor prepared in our laboratory were parallel. In this assay, 4.6 mg of a commercially available dried rabbit brain standard (thromboplastin C, Dade, Miami, FL), reconstituted according to the manufacturer’s specifications, gave a clotting time of 16 sec. Duplicate samples yielded clotting times differing by ≤6%.

Microclot Assay in Agar for Identification of Leukocytes With Procoagulant Activity

This assay is modified from Levy et al.16 Dog plasma (1.8 ml) and 1.8 ml of 0.025 M CaCl₂ were placed in a test tube at room temperature. Leukocytes (0.5 ml of 2 x 10^6 cells/ml) were added and followed immediately by 1.5 ml of boiled agar mixture at 37°C. (The agar was prepared by combining 0.9 g agar, 0.94 g CaCl₂, and 50 ml saline and boiling the ingredients continuously for 4 hr.) After quick but thorough mixing, the mixture was dispensed in 1-ml portions into 10 x 35 mm plastic retainer rings fastened onto 75 x 50 mm glass microscope slides. The slides were incubated at 37°C for 10 min, then placed on ice and immediately overlaid with heparinized saline (20 U/ml) at 4°C. At the time of stopping the coagulation reaction, approximately 5%-10% of leukocytes had pericellular clots, as determined by inspecting the cell preparations with inverted phase microscopy before further processing or staining. The agar was soaked overnight in cold MEM, pH 7.0 and 7.4, followed by a 2-hr soaking with saline to remove the MEM. The slides were then fixed with phosphate-buffered 45% acetic and 25% formalin for 30 sec at 4°C. After soaking again in cold distilled water overnight, the slides were stained for nonspecific esterase, followed by a methyl green counterstain.15 Finally, the agar was covered with cellulose acetate strips, placed in an evacuation chamber until dry, and then inspected microscopically under oil immersion for enumerating percent leukocytes in clots and percent of cells staining for nonspecific esterase.

Characterization of Procoagulant Activity

Procoagulant activity was identified as tissue factor using congenitally monozygotic human fetal plasma as substrates for one-stage assays and by inhibition with concanavalin A and phospholipase C, as previously described.15 Due to the weak procoagulant activity of canine leukocytes in human compared to homologous plasma (shown in Table 2), lysates of the infiltrate leukocytes were concentrated approximately tenfold before assay in human plasma by placing these preparations in dialysis tubing covered with Carbowax PEG 20,000 (Fischer Scientific, Raleigh, NC) for several hours at 4°C.

Statistical Methods

Dogs were randomly assigned to allograft and autograft groups. The Friedman method of analysis of variance and the Mann-Whitney U contrast test were used. Data are shown as means ± SE, unless otherwise indicated in text, tables, or figures.

RESULTS

Morphological, Cytochemical, and Density Characterization of Leukocytes Isolated From Rejected Allografts

Experiments were carried out to measure and characterize the procoagulant activity of leukocytes infiltrating allografted kidneys. Mononuclear leukocyte populations were isolated, as described in Materials
and Methods, from canine renal allografts that were surgically removed approximately 1 wk after transplantation into 10 recipient beagles. Control cells were isolated preoperatively on the day of transplantation from circulating blood using the same dogs, and additional controls were obtained from thoracic duct lymph 1 wk postoperatively at the time of allograft removal. Using continuous Percoll density gradients, mononuclear leukocytes were consistently isolated from each of the 10 renal allografts as a discrete band with a density of 1.069 g/ml. An average of 17.8 ± 5.6 x 10^6 infiltrate leukocytes per allotransplant was recovered in this band. Control leukocytes isolated from blood using the same density gradient technique were isolated as three bands (densities 1.063, 1.075, and 1.085 g/ml, Table 1). Cells from lymph were isolated as one band (density 1.075 g/ml, Table 1).

Cytomorphological and histochemical analyses were performed on infiltrate cells and controls. Viability after Percoll purification averaged greater than 97% (Table 1). As determined by nonspecific esterase and Wright’s staining, the single band of cells consistently isolated from allografts contained 80.9% mononuclear cells, including 49.5% lymphocytes and 31.4% monocytes/macrophages; the remaining cells in this population were neutrophils (Table 1). It can be seen that cell types and proportions in control preparations isolated from blood in the band having a density of 1.063 g/ml were highly similar to those in the single allograft infiltrate band, especially with regard to the percent monocytes/macrophages. By comparison, the other two bands from blood showed increased numbers of lymphocytes and neutrophils. Control populations from lymph were composed almost entirely of lymphocytes.

**Measurements of Expressed and Total Procoagulant Activities**

Amounts of expressed procoagulant activity, as measurable in assays of viable cells in the mononuclear leukocyte band isolated from allografts, were compared to the expressed activity of viable control leukocytes isolated from blood and lymph. Allograft infiltrate leukocytes expressed a median of 257.5 procoagulant activity U/2 x 10^6 cells (range 113–826 U). This was 60–135-fold higher than median amounts of activity expressed by the three leukocyte subpopulations isolated from blood and by leukocytes isolated in the single band from lymph, as shown by taking antilog of the procoagulant activity units given in log format in Fig. 1. These differences between infiltrate cells and controls were statistically significant (see "p" statistic, Fig. 1).

Measurements were also performed using cells lysed before assay to determine total amounts of procoagulant activity in the mononuclear cell populations studied above (Fig. 2). This assay provides a measure of the sum of expressed cell surface activity as shown in Fig. 1 and any intracellular activity released after lysis. The median total procoagulant activity in allograft infiltrate cells was 458 U/2 x 10^6 leukocytes (range

Table 1. Characteristics of Leukocyte Cell Fractions Isolated From Renal Allografts, Blood, and Lymph*

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Renal Allografts</th>
<th>Fractionated Blood</th>
<th>Thoracic Duct Lymph</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Percent viability</td>
<td>97.4 ± 0.5</td>
<td>97.2 ± 0.6</td>
<td>97.2 ± 0.6</td>
</tr>
<tr>
<td>Percent mononuclear</td>
<td>80.9 ± 6.3</td>
<td>90.9 ± 2.4</td>
<td>73.4 ± 3.2</td>
</tr>
<tr>
<td>cells (Wright’s stain)</td>
<td>(nonspecific esterase stain)</td>
<td>31.4 ± 7.6</td>
<td>33.4 ± 5.3</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>Specific gravity (g/ml)</td>
<td>1.069</td>
<td>1.063</td>
</tr>
</tbody>
</table>

*The fractions shown correspond to the same cell populations studied in Figs. 1 and 2.
plasmas deficient in the single clotting factors indicated. Factors VII, X, II, and V were specifically required for shortening of clotting times by the activity of these cells in dog plasma. (Respective times were 303 sec and 500 sec after inhibition as compared to 116 sec before inhibition of a representative leukocyte sample.)

Cytologic Assay of Procoagulant Activity

Allograft infiltrate leukocytes were further analyzed in a microscopic clotting assay using nonspecific esterase staining to identify classes of leukocytes with procoagulant activity in this population. After suspension in agar and incubation for 10 min in the presence of plasma and CaCl₂ (see Materials and Methods), an average of 8.6% of the isolated infiltrate leukocytes was found to be surrounded by microclots. Thus, only a minority of cells had sufficient procoagulant activity to initiate clot formation. Of the isolated cells, 2.6% ± 0.7% were observed to be individually surrounded by clots, and 6.2% ± 4.0% were in clots containing 2 or 3 leukocytes. In the individual cell microclots, 91.4% ± 5.0% of leukocytes were identified as monocytes/macrophages by nonspecific esterase staining (Fig. 3), and similarly, in clots containing 2 or 3 leukocytes, 94.8% ± 5.1% of the cells were found to be monocytes/macrophages. Thus, the great majority of the infiltrate leukocytes in clots were of monocyte/macrophage lineage, indicating that these cells were the likely source of procoagulant activity in infiltrate populations. By contrast, the same microcbot assay did not reveal coagulation around leukocytes from blood and lymph, indicating that monocytes and other isolated control cells had levels of procoagulant activity too low for detection by this cytologic assay. Due to the undetectable levels of procoagulant activity in the cytologic assays of controls, procoagulant activity in these populations could not be directly attributed to particular cells, such as all or some of the monocytes.

Control Kidneys

Controls were carried out to determine whether findings with allografts were specific for kidneys with rejection. Using techniques similar to those applied to studies of the allograft recipients, leukocytes were isolated from only 2 of 26 autologous nontransplanted kidneys and 5 of 10 autografts. In these 7 controls with isolated leukocytes (4.5 ± 1.6 × 10⁶/kidney), kidney biopsies showed inflammatory changes and leukocyte

| Table 2. Characterization of the Leukocyte Infiltrate Procoagulant as Tissue Factor |
|---------------------------------|---|---|---|---|---|---|---|---|
|                                | Dog | Human | VII | X | II | V | XII | XI | IX | VIII |
| Leukocytes                     | 92  | 239   | >500| >500| >500| >500| 227 | 248 | 221 | 214  |
| Thromboplastin standard (dog brain) | 74  | 220   | >500| >500| >500| >500| 225 | 227 | 249 | 256  |

*Results are given for assays of infiltrate leukocytes in one-stage coagulation tests in normal dog and human substrate plasmas, and in human plasmas deficient in the single clotting factors indicated. Factors VII, X, II, and V were specifically required for shortening of clotting times by the leukocytes and thromboplastin standard, demonstrating that the clotting pathway used by these cells and dog brain tissue factor are the same. Preincubating leukocytes in the tissue factor inhibitors concanavalin A or phospholipase C for 10 min before assay consistently reduced the procoagulant activity of these cells in dog plasma. (Respective times were 303 sec and >500 sec after inhibition as compared to 116 sec before inhibition of a representative leukocyte sample.)
Fig. 3. The microclot assay used to identify cells with procoagulant activity in the leukocyte populations studied is shown. In the assay, plasma, and calcium were added to leukocytes suspended in agar. Procoagulant activity is detected as pericellular fibrin formation. This cell, within the fibrin network, was isolated from a renal allograft and showed typical brown-red coloration in a nonspecific esterase stain, indicating that clotting was initiated by a monocyte/macrophage.

infiltration (acute tubular necrosis and/or interstitial nephritis), although the numbers of infiltrate cells seen histologically in these controls were smaller than in allografts with rejection. (The remaining 29 control kidneys were histologically normal, except for mild inflammatory changes in autografts.) Because of the low cell yields and findings of inflammation, macrophage populations from normal and pathologic controls could not be compared to those from allografted kidneys regarding procoagulant activity amounts, characterization, and leukocyte source. Nevertheless, these negligible to low cell yields from controls demonstrate that the large numbers of infiltrate leukocytes harvested from allografted kidneys were induced by rejection rather than by any inflammation resulting from transplantation per se or kidney disease endemic to the animal population studied.

DISCUSSION

Histologic examination of rejected kidneys has demonstrated a spectrum of coagulation abnormalities within affected organs, including vascular and extravascular fibrin deposition and intrarenal thrombosis. Additional evidence of clotting system activation during rejection has been provided by studies showing coagulation abnormalities in the plasma of allograft recipients, including clotting factor deficiencies and increased levels of fibrinopeptide A and fibrinolytic products. Although there can be little doubt that blood coagulation contributes to the pathology of rejection reactions, mechanisms initiating clotting in this type of inflammatory response have not been clarified.

It has been suggested that the platelet contributes to coagulopathies of rejection, since in histologically examined allografts, platelet thrombi can be found in capillary lumens together with fibrin. Another possibility is that, during rejection, coagulation may also be initiated by mononuclear leukocytes. Lymphocyte-macrophage mixtures have been shown to produce tissue factor and other clotting factors following incubation in vitro. Moreover, in the case of tissue factor, generation in vitro by mononuclear leukocytes is enhanced by immunologic stimuli closely resembling those present in kidney allotransplant recipients. These include allogeneic leukocytes, antigen–antibody complexes, cells sensitized with antibody, immunizing antigens, lymphokines, and complement cleavage products. Further, in a human case study, leukocytes isolated from a kidney allograft were found to have tissue factor activity when assayed after in vitro culture for several days.

Although previous work suggests that leukocytes may activate clotting during rejection, there has been no reported investigation indicating whether or not such cells infiltrating allografts have procoagulant activity. Therefore, in the experiments described here, leukocytes were isolated from the parenchyma of functioning kidney transplants, and the procoagulant activity of these cells was assayed in a one-stage test without using preliminary culture or stimulation steps to induce generation of cellular procoagulant activity in vitro. These isolated infiltrate leukocytes were found to have activity characterized as tissue factor, the initiator of clotting through the extrinsic pathway utilizing factors VII, X, II and V. Further, amounts of tissue factor in the infiltrate cells were found to be significantly increased relative to controls from blood and lymph. Using a microclot assay, the infiltrate cell procoagulant activity was observed to be localized to monocytes/macrophages, rather than coisolated lymphocytes or neutrophils. Unlike these infiltrate monocytes/macrophages, blood and lymphatic monocytes (as well as the other leukocytes coisolated from blood and lymph) lacked procoagulant activity detectable in the microclot assay.

Monocytes/macrophages comprise approximately 25% of mononuclear leukocytes migrating from blood to renal allografts during rejection. As shown here, these cells isolated from kidney allografts have enhanced ability to activate coagulation via the extrinsic pathway compared to similar classes of cells from the circulation. The availability of tissue factor on the
surgery
account for coagulation in rejected allografts near surfaces of infiltrate monocytes/macrophages in
increased amounts compared to the lower procoagulant activity of circulating precursors may, in part, account for coagulation in rejected allografts near lymphoid infiltrates and adjacent vessel walls, glomeruli, and other structures. Additionally, induction of local fibrin deposition by macrophage surface tissue factor may influence the mobility or function of these cells during the rejection process.

REFERENCES

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