Repair of Damaged Organs *in Vitro*

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Treatments that can be performed *ex vivo* following ischemia to accelerate cellular recovery and ameliorate reperfusion injury could have major impact. An acellular, near-normothermic perfusion was employed to deliver growth factors to ischemically damaged kidneys. During the treatment oxidative metabolism was sufficiently restored to support up-regulation of cellular processes with the potential to modulate both injury and repair proteins in damaged kidneys. The results suggest that growth factor administration, without concomitant inflammation, triggers pathways for new synthesis leading to cellular recovery rather than cell death.

Key words: Growth factor-mediated, renal ischemia, recovery

Introduction

Clinical organ transplantation has fallen victim to its own success. As the end-stage renal disease patient population being maintained with hemodialysis continues to grow, with the numbers of newly diagnosed patients each year exceeding healthcare group projections, there has been no substantial progress made in developing a comparable increase in the number of available allografts. Society may soon be faced with the public healthcare issue of sustaining the costs associated with transplantation when relatively few patients will ever have the opportunity to get an organ transplant. It is not so far-fetched to consider the possibility of one day having politicians touting 'a kidney for everyone, or a kidney for no one.'

Therefore, the main clinical problem facing society today is the discrepancy between the demand and supply of organs for transplantation.

It is crucial to the future of transplantation that a source of more kidneys be developed and perhaps the most obtainable in the short term will be the development of technology that can access severely warm ischemically damaged kidneys currently not considered for transplantation (1).

In the previous work we have successfully demonstrated the application of an acellular warm (32°C) perfusion technology (Exsanguineous Metabolic Support, EMS) in maintaining organ viability *ex vivo*. Following extended periods of EMS perfusion, transplanted organs demonstrated immediate life-sustaining function (2). The ability to maintain an intact organ in tissue culture-like conditions is predicated upon two major principles:

- adequate support of metabolism by providing all the necessary nutrients and substrates, including oxygen;
- maintenance of vascular integrity by preserving the normal barrier functions of the blood vessel wall.

We have also previously demonstrated the ability of warm perfusion to overcome an ischemic insult of as much as 2 h (3). In the present study we have extended this work to evaluate if the observed survival was attributable to new synthesis sustained by the continued oxidative metabolism during *ex vivo* perfusion.

We hypothesized that sufficient restoration of metabolism by EMS perfusion in combination with growth factor signaling (EMS + GF) could support the cellular mechanisms needed to repair ischemic damage while an organ is *ex vivo*.

The pharmacologic involvement of growth factor to accelerate regeneration in ischemically damaged kidneys is logical given that polypeptide growth factors are known to potentiate kidney differentiation and function, and have been shown to play an important role *in vivo* in the recovery of function following renal injury (4–6). For the purpose of the present study, both fibroblast growth factor (FGF) 1 and 2 were used. The rationale for using FGFs is their demonstrated receptor specific binding in the kidney, their role in regulating metabolic rate and transport processes and mitogenic effect on epithelial cells (7,8).

To test the feasibility of repairing ischemic damage *ex vivo*, we evaluated three critical features that would be involved in cellular recovery and repair:

1   interruption of the progression of damage;
2   restoration of metabolism and initiation of protein synthesis;
3   regeneration and repair.

In the present study we evaluated if active cellular repair could be achieved *ex vivo* in kidneys damaged by 2 h of warm ischemia. The ischemically damaged kidneys were warm perfused for 24 h in the presence and absence of FGFs. The effect of *ex vivo* stimulation of reparative processes on sequelae determining whether renal cells recovered or there was a continuation of the necrotic pathway, was assessed using a TUNEL assay.

The potential for new synthesis in a kidney during *ex vivo* warm perfusion was investigated. To circumvent the possibility that an endogenous cellular reservoir was in fact contributing the observed protein, an exogenous gene was employed. Human kidneys were transfected with adenovirus serotype five particles expressing the green fluorescent protein during 24 h of *ex vivo* EMS + GF perfusion. The potential for new synthesis was then determined by the expression of the reporter gene product in the kidneys following the requisite transfection, nuclear trafficking, transcription and protein synthesis.

Two critical regenerative pathways requiring new synthesis of proteins for recovery of cellular integrity following ischemic injury were evaluated. The junctional integrity protein, ZO-1 was used to determine the feasibility of recovering the cytoskeletal integrity lost as a consequence of ischemia. The time course for the reestablishment of tight cellular junctions was determined. Similarly, the up-regulation of proliferating cell nuclear antigen (PCNA) was used as a marker for DNA repair and recovery of synthetic functions.

The ability to repair the injury following 120 min of ischemic insult was determined by reimplantation of some of the kidneys following *ex vivo* treatment and using others to obtain the histochemical evidence supporting the occurrence of active cellular repair.

Methods

Animals and surgical protocol

Autotransplantation experiments were performed on 1-year-old foxhounds weighing 25–30 kg. All experiments were performed following the principles of laboratory animal care according to NIH standards. Animals demonstrated normal renal function prior to the start of the study. Kidneys were exposed through a midline incision and the left renal artery, vein and ureter were mobilized. Ten minutes prior to excision 200 mg/kg of mannitol was administered intravenously. The excised kidney was exposed to 120 min of WI (37°C) while remaining in the abdominal cavity.

Transplant studies

Kidneys were divided into two groups:

Group 1 ($n = 5$): 120 min WI followed by 24-h EMS perfusion without growth factor (GF) (32°C) and reimplantation.

Group 2 ($n = 5$): 120 min WI followed by 24-h EMS + GF perfusion (32°C) and reimplantation.

The mean anastomosis time was 25 min. Fifteen minutes prior to reperfusion, mannitol (200 mg/kg) and verapamil (0.1 mg/kg) were administered. Contralateral nephrectomy was performed prior to reperfusion of the *ex vivo* treated kidney.

Histochemical studies

Kidneys with 120 min of WI were subsequently treated with warm perfusion in the presence or absence of GF for 6, 18 or 24 h. Following treatment, fixative was perfused through the kidney via the cannulated renal artery and the kidneys were then used for histochemical analysis. The test groups were as follows:

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0 min WI (n = 5)
120 min WI (n = 5)
120 min WI followed by EMS without GF perfusion (32°C) for:
  6 h (n = 5)
  18 h (n = 5)
  24 h (n = 5)
120 min WI followed by EMS + GF perfusion (32°C) for:
  6 h (n = 5)
  18 h (n = 5)
  24 h (n = 5)

EMS perfusion

EMS technology (Breonics, Inc., Schenectady, NY, USA), was used for *ex vivo* warm perfusion at 32°C using a pressure controlled perfusion system including a siliconized membrane oxygenator and a pulsatile pump, retrofitted with controllers to maintain PaO_2, $PaCO_2$, pH and temperature. Perfusion was initiated with a systolic pressure of 50 mmHg and a circulating volume of 500 mL of the EMS medium (2). EMS medium was prepared with 0.1 mg of acidic-fibroblast growth factor (FGF) and 0.1 mg/mL of basic-FGF purified by heparin sepharose affinity chromatography from crude retinal derived extracts (BREONICS, Inc., Otisville, NY, USA). Medium was also made without the addition of growth factor. The acellular EMS medium was supplemented with pyridoxylated bovine hemoglobin (6 g%) (Ezon, Inc., Piscataway, NJ, USA) to provide adequate oxygen (PaO_2 of 200 mmHg) to support ongoing renal metabolism.

Transfection studies

Human kidneys procured for the purpose of transplantation and later determined to be nontransplantable by institutional criteria were stored in ViaSpan at 4°C (mean cold storage time of 38 h) prior to their release for research use. The kidneys were weighed, the renal artery cannulated and flushed with approximately 250 cc of the EMS medium warmed to 32°C to remove the hypothermic solution from the vasculature. Ad5.CMV5GFP serotype five particles expressing the Green Fluorescent Protein (GFP) (Quantum, Biotechnologies, Inc. Montreal Quebec, Canada) was administered into the perfusion path at a concentration of 1×10^6 VP/g of kidney and four human kidneys were perfused for 24 h. Frozen sections from five sites within each kidney were used to evaluate GFP expression. Two human kidneys that were perfused but not transfected served as controls.

Histochemical analysis

Following WI and perfusion, fixation consisted of perfusing each kidney with 100 cc of 2% paraformaldehyde-75 mM L-lysine-10 mM sodium periodate

(PLP). The flush fixed kidneys were stored in the PLP fixative for 24 h at 4°C and then washed and stored in phosphate buffered saline containing 0.02% azide. Prior to sectioning, the kidneys were equilibrated overnight at 4°C in 0.6 M sucrose-PBS. Kidneys were then embedded in paraffin blocks and 4-μm sections were cut from each kidney. The histochemical testing was performed in duplicate from five representative sites within each kidney using an indirect biotin-avidin immunoperoxidase assay with a DAKO Auto Immunochemistry Stainer (9,10). DNA fragmentation was detected using a TACS-XL kit (Trevigen, Inc., Gaithersburg, MD, USA). A consulting histopathologist evaluated the slides blindly. The results of the histochemical analysis were represented as the percent positive cells within five representative fields of each duplicate slide. The results of the blinded evaluations were represented as the mean from the duplicate slides from the five sites for each kidney and then a calculated mean and the standard deviation were determined for the five kidneys in each time point.

Posttransplant graft function

Blood samples for BUN and serum creatinine were taken each morning and analyzed using an ACE analyzer (Schiapparelli Biosystems, Inc., Fairfield, NJ, USA). Animals deemed to be in poor condition, with unlikely chance of recovery from the delayed graft function were classified as primary non-function (PNF). Canines with PNF were euthanized. Serum creatinine was considered normal with values below 2.0 mg/dL.

Results

Initiation of protein synthesis

The oxidative metabolism during the isolated kidney perfusion was evaluated for the ability to support de novo protein synthesis *ex vivo*. De novo synthesis was evaluated by transfecting four human kidneys during 24 h of *ex vivo* warm perfusion. Evaluation of the frozen sections made from the transfected kidneys following 24 h of perfusion demonstrated positive fluorescence limited to the intima of all the blood vessels examined (Figure 1A). The control human kidneys that were warm perfused but not transfected were negative (Figure 1B).

Histochemical studies

Interruption of the progression of damage: Normal kidneys evaluated for DNA fragmentation in the TUNEL assay were negative. Similarly, kidneys damaged by 120 min WI were also negative. In the ischemically damaged kidneys that were EMS perfused without the addition of growth factors, 10% ($\pm$2) of the epithelial cells stained TUNEL positive by 6 h of warm perfusion and by 24 h of perfusion the number of positive stained cells in the distal segment of the proximal tubules and the medullary ascending limb of the loop of Henle had increased to 50% ($\pm$5). When the ischemically damaged kidneys were warm perfused in the presence of growth factors, TUNEL staining was negative at all time points ($p < 0.05$).

Restoration of cytoskeleton integrity—ZO-1: In normal dog kidneys ZO-1 protein expression at the peripheral membrane was observed with only faint positive cytoplasmic staining for ZO-1 seen in the cortex, outer medulla and inner medulla that was limited to only 2% ($\pm$0.5) of the tubules (Figure 2A). Two hours of ischemic insult resulted in diffuse cytoplasmic staining of ZO-1 due to its dispersion within the cytoplasm (Figure 2B). Expression of ZO-1 in the kidneys with positive cytoplasmic staining was detected in both the cortex and outer medulla, with many positive tubules demonstrating intense ZO-1 staining resulting in 80% ($\pm$5) of tubules staining positive.

Ischemically damaged kidneys that were warm perfused in the presence of growth factors demonstrated a normalizing trend in the cytoskeleton. Following 6 h of warm perfusion, the widespread disruption of the skeleton remained essentially unchanged with strong cytoplasmic staining. However, by 18 h of perfusion the number of positive cytoplasmic stained cells was reduced to 32% ($\pm$8).
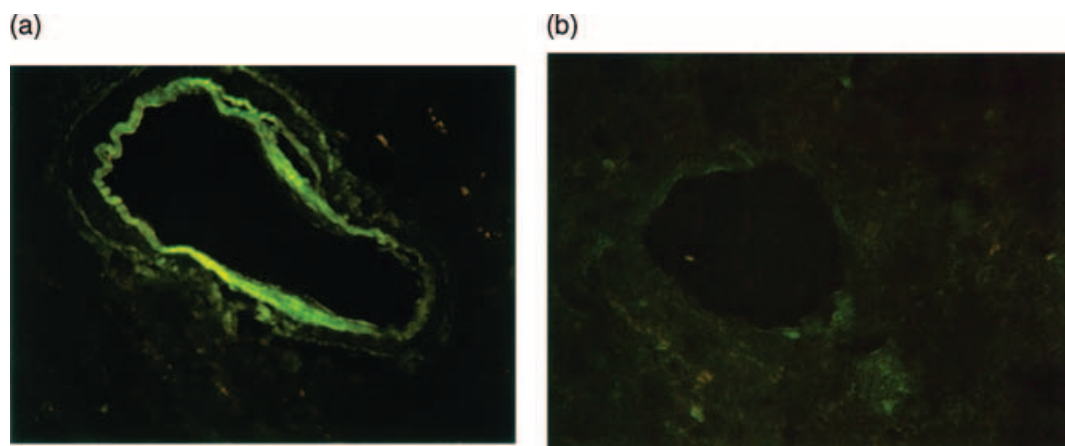

Figure 1: Transfection studies. Positive reporter gene expression—human kidney displaying positive fluorescence along blood vessel intima following transfection with 1×106 VP/g of Ad5.CMV5GFP during 24 h of *ex vivo* perfusion (A). Negative control—human kidney that was perfused but not transfected (B).
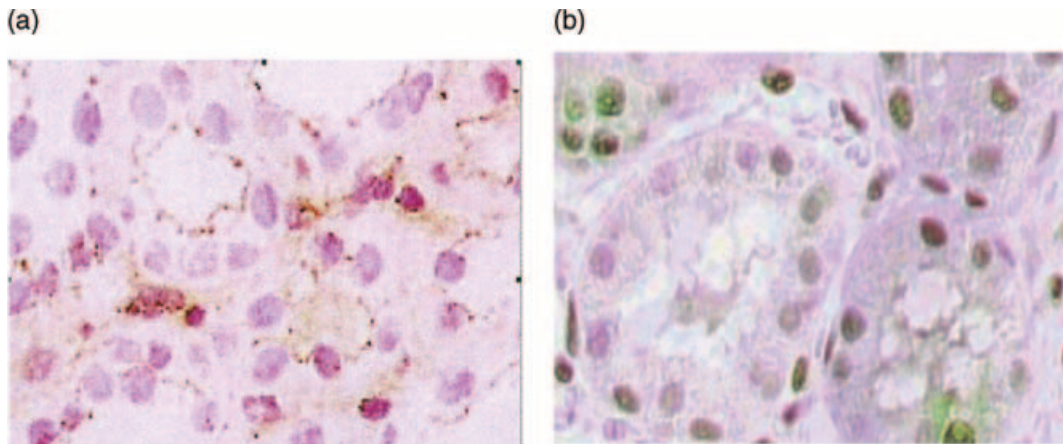
Figure 2: ZO-1 expression studies. ZO-1 staining as seen in a normal undamaged kidney (A). ZO-1 staining in a kidney subjected to 120 min of WI (B).

Table 1: Histochemical studies: TUNEL assay, ZO-1 staining and PCNA staining in kidneys with 0 min WI; 120 min WI; 120 min WI followed by EMS w/o GF (6,18, 24 h); and 120 min WI followed by EMS + GF (6, 18, 24 h). n = 5 in each group

Groups (n = 5)	0 min WI	120 min WI	EMS w/o GF			EMS + GF		
			120 min WI + 6 h EMS	120 min WI + 18 h EMS	120 min WI + 24 h EMS	120 min WI + 6 h EMS	120 min WI + 18 h EMS	120 min WI + 24 h EMS
TUNEL	0	0	10 ± 2	38 ± 2	50 ± 5	0	0	0
ZO-1	2 ± 0.5	80 ± 5	84 ± 7	78 ± 3	68 ± 10	76 ± 5	32 ± 8	8 ± 3
PCNA	2 ± 0.3	4 ± 2	10 ± 2	27 ± 4	33 ± 5	23± 3	57 ± 8	88 ± 7

Values are expressed as percent (%) of positive cells within five representative fields of each duplicate slide. Results are calculated as mean and the standard deviation.
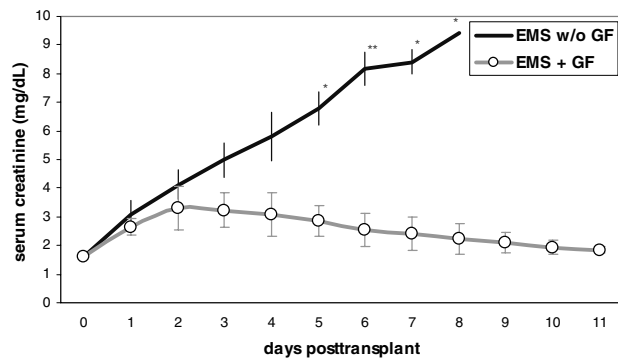

Figure 3: Posttransplant function. Serum creatinine concentration following reimplantation of canine kidneys subjected to 120 min of WI followed by 24 h of EMS warm perfusion without growth factors (Group 1; n = 5) and 24 h of EMS warm perfusion with growth factors (Group 2; n = 5). Values are expressed as the mean with standard deviation for each experimental group. * = euthanized.

By 24 h of EMS perfusion with growth factors ZO-1 staining was equivalent to the normal kidneys with 8% (±3) positive cells, suggesting a trend towards recovery of junctional integrity.

In the absence of growth factor during *ex vivo* warm perfusion the recovery of cytoskeletal integrity was impaired. ZO-1 staining at 6 and 18 h showed little change in staining pattern in comparison to the kidneys that were immediately fixed following 120 min of warm ischemia. By 24 h of warm perfusion in the absence of growth factor 68% (±10) of the tubules within the nephron stained positive for cytoplasmic ZO-1. The time-dependant recovery of the cytoskeleton in the ischemically damaged kidneys that were warm perfused in the presence of growth factor in comparison to the kidneys that were not exposed to growth factor reached statistical significance (p < 0.05).

Cellular repair—PCNA: PCNA in normal kidneys was marginally expressed with 2% (±0.3) of cells staining positive. Following a 2-h ischemic insult to the kidneys, no significant increase in expression was observed in glomeruli and in the outer medulla.

In kidneys damaged by 120 min WI that were subsequently warm perfused with exogenous growth factor for 6 h up-regulation of PCNA expression was observed with 23% (±3) of cells staining positive. Increased expression of this repair marker was observed at 18 and 24 h of EMS perfusion with growth factors, widespread staining was observed with 88% (±7) of the cells staining positive. The strongest staining pattern was encountered

in the outer medulla. In the ischemically damaged kidneys that were warm perfused without exogenous growth factor only 33% ($\pm$5) of the renal cells stained were PCNA positive following 24 h *ex vivo*. These results suggest that up-regulation of PCNA during EMS perfusion in the presence of exogenous growth factors occurred in a time-dependant fashion, providing the first evidence of active cellular repair *ex vivo* in ischemically damaged kidneys. Therefore, during the EMS perfusion it appears that oxidative metabolism was sufficient to support the up-regulation of cellular processes with the potential to modulate both injury and repair proteins.

Function in ex vivo repaired kidneys

The up-regulation of PCNA, accompanied by the down-regulation of ZO-1 that occurred by 24 h of *ex vivo* acellular perfusion in ischemically damaged kidneys in the presence of exogenous growth factors was found to correlate with outcomes posttransplantation. Reimplantation of these 'repaired' kidneys resulted in life-sustaining function (Group 2). EMS warm perfusion without the addition of exogenous growth factors (Group 1) resulted in nonviable kidneys.

Discussion

The ischemic injury cascade is recognized to consist of a prelethal phase followed by a lethal phase where proteolytic enzymes are activated and the injury becomes irreversible (11). The prelethal phase of ischemia is characterized by the sudden interruption of oxidative phosphorylation with loss of the adenine compound pool, loss of cell volume regulation and disruption of the cytoskeletal integrity. By definition this prelethal phase of the ischemic cascade represents potentially reversible injury, with the potential of recovering normal function. A compounding feature when ischemically damaged tissue is reperfused with blood is secondary damage, which is commonly referred to as reperfusion injury. Any recovery following a prelethal cell injury involves the physiologic processes involved in normal wound repair consisting of the primary steps of coagulation and inflammation, along with a migratory/adhesion phase.

In the present study we evaluated if a repair process supported by new synthesis could be accomplished *ex vivo* from a prelethal phase of ischemic injury. Any repair achieved *ex vivo* would be distinct from the inflammatory and coagulation responses associated with *in vivo* repair. Rather, any recovery in function *ex vivo* would be more analogous to the cellular recovery that occurs with restoration of metabolism during cell culture. Previously, we have reported that a period of *ex vivo* warm perfusion in an exsanguineous environment resulted in reestablishment of oxidative metabolism, restoration of cell volume regulation and recovery of vascular functions (12). In another study, we evaluated if it was feasible to overcome severe

ischemic injury by reestablishing oxidative metabolism during *ex vivo* warm perfusion. (3).

A goal of this study was determining the consequence of the injury: whether sequelae led to eventual cell death or whether the restored metabolism with corresponding growth factor activation of the tyrosine kinases would instead initiate a reparative pathway. The results provide evidence that the metabolic rate restored in ischemically damaged kidneys during the *ex vivo* period was of sufficient magnitude to support de novo protein synthesis as assessed by the expression of a transfected reporter gene product. Following *ex vivo* gene transfection, nuclear trafficking with transcription and synthesis occurring during the 24-h *ex vivo* period resulted in localized expression of the gene product limited to the luminal surfaces within the vasculature.

The association of increasing PCNA-positive cells with increasing duration of perfusion time with exogenous growth factors demonstrates that new synthesis associated with active cell repair also occurred during the perfusion period. In our original studies with ischemically injured kidneys a crude growth factor preparation was used as a component of the perfusate at a concentration of 20 mg/L. In the present study purified FGF-1 & 2 were each used at a concentration of 100 μg/L with the intent of identifying the regulatory role, establishing efficacy and eventually optimizing the growth factor components. The results of this study demonstrates the important role growth factors play in triggering pathways of cellular recovery based upon new synthesis rather than continuation of the injury cascade resulting in cell death in ischemically damaged kidneys. The absence of exogenous growth factor in EMS perfusion resulted in impaired recovery of synthetic functions.

PCNA, an acidic nuclear protein, is required for both DNA replication and repair (13). Following a prelethal duration of warm ischemic insult, a variety of genes are associated with PCNA expression, such as the tumor suppressor gene p53, c-JUN Na terminal kinase (JNK) and structure specific nuclease Fen1 involved in DNA replication. It is well recognized that PCNA is absent or weakly expressed in normal resting cells and this concept was confirmed in the normal canine kidneys. The presence and distribution of PCNA is known to fluctuate according to the cell cycle, where strongest expression is observed in actively proliferating cells.

PCNA expression was first noted in the nuclear compartment in ischemically damaged kidneys following 6 h of warm perfusion with the EMS medium. The PCNA expression increased with perfusion time and by 24 h of perfusion widespread staining was observed throughout the nephron. This represents an accelerated time frame than has previously been reported for *in situ* studies. *In vivo*, the up-regulation of PCNA can be detected 24 h after restoration of blood flow to ischemic tissue, with

increased intensity in subsequent days (14). The acceleration in PCNA up-regulation observed in this study may be attributable in part to activation of tyrosine kinases via signal transduction mediated by the growth factors that appeared to interrupt the injury cascade leading to cell death. By treating the kidneys *ex vivo* prior to vascular reflow, amelioration of the secondary reperfusion injury may have occurred, also contributing to the accelerated recovery in the growth-factor-treated kidneys. In contrast, the lack of significant up-regulation in PCNA expression in the damaged kidneys that were warm perfused without exogenous growth factor correlated with nonviable outcomes when kidneys were reimplanted.

PCNA expression has also been shown to be associated with a reduction in subsequent DNA fragmentation (15). There was an absence of TUNEL positive cells in the kidneys that were warm perfused with exogenous growth factor supplementation while positively stained cells were observed without growth factor treatment.

In terms of cytoskeletal integrity, the injury marker ZO-1 is normally found exclusively at the plasma membrane, localized to the points of cell-to-cell contact. Following an injury that disrupts or damages the cytoskeleton, ZO-1 gets diffused within the cytoplasm (16). The cytoplasmic dispersion of ZO-1 can be used as an injury marker and its reassociation with the tight cellular junctions is indicative of recovery of cytoskeletal integrity. ZO-1 was found to be translocated to the cytoplasm following 2 h of ischemic insult and remained largely dispersed after subsequent warm perfusion in the absence of growth factor. In contrast, the cytoplasmic staining diminished in a time-dependant manner during EMS perfusion with exogenous growth factors. By 24 h of EMS perfusion the canine kidneys were essentially negative for ZO-1 with staining equivalent to normal kidneys.

Tight cellular junctions provide barrier functions that help to separate the luminal and interstitial compartments in epithelial tissues. These cellular junctions also serve as barriers differentiating apical and basolateral domains of plasma membranes. It has been suggested that disruption of the cytoskeleton is the cause of cell surface alterations mediated by ischemia (17,18). Therefore, in order to reestablish tight junctional integrity, the cytoskeleton must likewise be reestablished for normal cell adhesion, polarization of the cell membranes and signal transduction (19,20).

These results suggest that following ischemic insult the successful resuscitation of oxidative metabolism using a near-normothermic perfusion, in combination with exogenous growth factors potentiating tyrosine kinase activation, initiates reparative cell processes without concomitant inflammation. The initiation of reparative pathways may serve as trigger for new synthesis leading to cellular recovery rather than continuation of the necrotic pathway. Cellular repair of ischemic tissues prior to reestablishing

blood flow appears to ameliorate secondary reperfusion injury and provide enhanced opportunities for recovery of normal function.

The ability to support cellular reparative processes in ischemically damaged allografts *ex vivo* would enable a major expansion of the organ donor pool by making it feasible to routinely recover organs from donors beyond the small ICU-based pool. The routine utilization of kidneys from non-heartbeating donors instead of today's dependence on the heartbeating donor would help to provide a solution to the organ shortage.

The work described here represents the potential of a warm perfusion technology in its infancy. Active metabolism while an organ is maintained *ex vivo* presents the opportunity for pretransplantation treatment regimens such as immunomodulation, and the development of effective gene transfer technologies with corresponding protein expression. In conclusion, this work provides further evidence of the feasibility of *ex vivo* warm perfusion using an acellular technology based upon the principles of cell culture. The real visionary of this technology was one of the founding fathers of clinical organ preservation technology, Professor Dr. Folkert Belzer, who in 1982 predicted that the future of organ preservation would be at warmer, more physiologic temperatures.

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