Organ Procurement in Experimental Pancreas Transplantation with Minimal Microcirculatory Impairment

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Key Words

Pancreas transplantation · Ischemia-reperfusion injury · Organ procurement · Intravital fluorescence microscopy · Microcirculation · Apoptosis

Abstract

Background: Ischemia-reperfusion injury has been shown to deteriorate microcirculation in experimental pancreas transplantation. However, minor concern was taken on the impact of organ procurement in this condition. We examined the impact of a standardized technique of organ procurement on microcirculation and apoptosis in experimental pancreas transplantation. **Methods:** Male Lewis rats were divided into three groups: sham-operated animals without dissection of the pancreas served as controls (n = 5); animals undergoing nearly total process of organ procurement with the pancreas pedunculated on the aorta and the hepatoduodenal ligament (n = 7), and animals receiving pancreaticoduodenal transplantation. Pancreatic grafts were preserved for 6 h in cold University of Wisconsin solution (n = 7). At 1 and 2 h reperfusion and in time-matched controls, microcirculation was assessed by means of intravital fluorescence microscopy. Tissue samples were obtained after 2 h measurement and DNA breaks of acinar cells were detected by in situ nick end-labeling (TUNEL assay). The apoptotic index (apoptotic cells per high-

power fields; hpf) was quantified by microscopic counting of at least 50 hpf. **Results:** Assessment of functional capillary density (FCD) in animals undergoing subtotal process of organ procurement revealed a slight non-significant decrease at 1 and 2 h compared with controls. In addition, leukocyte sticking to postcapillary venules (LAV) as well as the apoptotic index were found slightly increased after organ procurement compared with controls (p > 0.05). However, after pancreas transplantation the apoptotic index and the LAV were significantly increased and the FCD significantly decreased compared with both groups of non-transplanted animals (p < 0.01). **Conclusions:** Our validated technique of organ procurement does not negatively impact microcirculation and apoptosis in experimental pancreas transplantation.

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Introduction

Pancreas transplantation is currently the method of choice in therapy of insulin-dependent type I diabetes with end-stage renal failure. However, success is limited by the development of graft pancreatitis that occurs with an incidence in the range from 17 to 35% leading to a considerable morbidity and mortality [1, 2]. Experimental studies have shown the relevance of ischemia-reperfusion injury in the development of graft pancreatitis after

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pancreas transplantation [3, 4]. However, minor concern was taken on the impact of organ procurement on post-transplant deterioration of organ function or even organ failure. Recent studies in experimental and clinical liver transplantation indicate that graft function is considerably reduced by dissection or manipulation during organ harvest [5–7]. The pancreas is known to be highly susceptible to manipulation with the development of traumatic pancreatitis. Therefore, great concern has to be taken that manipulation in transplant models may not lead to artifacts that are caused by the operative procedure and are misinterpreted as consequences of ischemia-reperfusion injury. For experimental pancreas transplantation, Lee was the first who outlined the impact of organ procurement on post-transplant pancreatitis. Based on empiric observations, he established the ‘no touch’ technique to avoid harmful manipulation of the gland [8]. However, until now there is no experimental evidence for this technique.

It is now accepted that microcirculatory derangements play a pivotal role on the pathogenesis of ischemia-reperfusion injury and also of acute pancreatitis [3, 9–11]. Most obvious functional changes are a decrease of nutritive capillary perfusion and increase in leukocyte-endothelial interaction in the early period after reperfusion. Different investigations have previously described pancreatic microcirculation impairments by means of intravital fluorescence microscopy (IVM) [10, 12–14]. Moreover, there is increasing evidence that ischemia-reperfusion injury evokes apoptotic cell death in organ transplantation [15–18]. Thus, both impairment of microcirculation and increased apoptotic cell death are established markers for ischemia-reperfusion injury [19] and may as well be triggered by the operative procedure. The aim of the present study was to determine the contribution of organ procurement to IVM-measured total ischemia-reperfusion injury and apoptosis in experimental pancreas transplantation. In addition, operative techniques of pancreas transplantation which are based on the investigations made by Lee et al. [8] have been further developed in our institution and are described in detail in this study.

Methods

All animal experiments were in accordance with current German law on the protection of animals and were approved by the local governmental animal care and use committee. All microsurgical procedures were performed under a microscope with $\times 5$ magnification (Zeiss, Jena). Highly inbred male Lewis rats weighing 290–350 g (University of Rostock, Department of Pathology, Rostock, Germa-

ny) were used throughout the study. After overnight fasting but free access to water the animals were anesthetized by intraperitoneal injection of 60 mg/kg body weight pentobarbital together with administration of 5 μ g atropine subcutaneously. Anesthesia was maintained using 0.2–0.6 vol% isoflurane (Forene; Abbott GmbH, Wiesbaden, Germany) in N₂O-O₂ (35% oxygen) utilising a Sullas 808 vaporizer (Draeger AG, Lübeck, Germany). The animals were placed in the supine position on a heating pad to maintain body temperature between 36.0 and 37 °C. Polyethylene catheters (ID 0.50 mm; Portex, Hythe, UK) were inserted into the left carotid artery and right internal jugular vein of both the donor and the recipient for continuous monitoring of arterial blood pressure (Sirecust 1261; Siemens, Munich, Germany), for continuous substitution of saline solution (4 ml/h) and for intravenous injection of fluorescent dyes.

Experimental Protocol

Animals were randomly assigned to three different groups as follows. Nontransplanted animals (sham, $n = 5$) with only exteriorization of the pancreas served as controls. Group Tx ($n = 7$) received pancreatic grafts stored for 6 h in 4 °C UW solution. Nontransplanted animals (Procurement group, $n = 7$) undergoing subtotal process of organ harvest were studied to assess the impact of organ procurement on pancreatic microcirculation. In these animals, step 1–4 of organ procurement was performed as described below. However, the dissection of the aorta was accomplished without ligation of both renal arteries to avoid electrolyte imbalance during observation. There was no disruption of pancreatic blood flow during the course of preparation. Thus, neither pancreatic tissue of the Procurement group nor control animals was subjected to ischemia-reperfusion injury.

Donor

Operative procedure of organ harvest was divided into 5 major steps.

Step 1: The abdomen was entered via a midline incision extending from the xyphoid process to the pubic symphysis. After retracting the stomach onto the chest wall, the greater omentum was freed from the pancreas. The pancreas was meticulously prepared from the pyloric region with the right gastroepiploic vessels being ligated and divided beyond the border of the gland. The short gastric vessels and the left gastric vessels were divided after ligation. Thereafter, the proximal duodenum was ligated and cut off from the pylorus. The distal part of the esophagus was electrocauterized and divided. The stomach was completely removed and the vessels of the splenic hilum were ligated.

Step 2: The colon was retracted to the caudal abdominal cavity and the ligament of Treitz was cut together with the mesenteric band of the descending colon. The transverse colon was detached from the pancreas to expose the middle colonic vessels and the mesenteric root. After dividing the middle colic vessels, the mesenteric root was dissected and ligated distal to the junction with the inferior pancreaticoduodenal vessels. The jejunum was cut off distally the duodenojejunal flexure. After ligation of the rectum, the whole intestine except the duodenum was removed giving an excellent exposure of the abdominal cavity for further preparation.

Step 3: The pancreas was turned to the right and the aorta was prepared from the ilio-lumbal vessels to the diaphragm with ligation of the left renal vessels and the lumbar branches of the aorta. After turning the pancreas to the left, the preparation was continued by dissection of the right renal artery without ligation.

Step 4: At the liver hilum, the hepatic artery was ligated and cut together with the bile duct followed by dissection of the portal vein to its bifurcation.

Step 5: The aorta was clamped with a microclip (Medorn) below the superior mesenteric artery and ligated proximally to the ilio-lumbal vessels. After intravenous injection of 100 IU heparin, a catheter (G 18) was inserted into the distal aorta close to its distal ligature. Up to this point, neither any warm ischemia nor disruption of blood supply of the pancreas had occurred. Finally, the left renal artery and the aorta were ligated proximal to the celiac artery and immediately thereafter, the clamp on the aorta was removed to accomplish perfusion by cold (4°C) University of Wisconsin (UW) solution until the pancreas appeared totally bloodless. With this technique, the warm ischemia time was below 15 s. In order to provide sufficient outflow of the preservation solution during inflow an incision in the proximal part of the portal vein was made. After finishing perfusion, the aorta and the portal vein were closed by a microclip and the portal vein was cut at the liver hilum at its bifurcation in a fashion described by Lee et al. [20]. The duodenum was then flushed by cold saline solution via the distal stump. Finally, the duodenopancreas was wrapped in gauze and stored in 4°C University of Wisconsin solution. Animals undergoing organ harvest without ischemia (Procurement group) were only subjected to steps 1–4.

Recipient

Heterotopic pancreaticoduodenal transplantation (Tx group) with systemic venous drainage was performed using a modification of the technique described in detail by Lee et al. [8]. The abdominal cavity was again entered via a midline incision and the intestine was retracted to the left of the rat. After dissection of the aorta and the inferior vena cava the animal was turned 90° with the head showing to the left side of the surgeon. In accordance with Lee et al. [20], gonadal vessels were ligated and divided if they arose in a position that could jeopardize the graft anastomosis. The graft was placed in the recipient's left flank on a aluminum foil with the head pointing to the right and the tail pointing to the left side of the rat. Thereafter, the head of the pancreatic graft was placed on its tail providing an excellent access to the donor aortic conduit and portal vein. The aorta was cross-clamped distal to its junction with the renal artery and an end-to-side anastomosis between the aortic segment of the graft and the recipient infrarenal aorta was performed. Thereafter, the arterial blood flow of the recipient was re-established, while the donor aorta remained clamped. Systemic venous drainage was performed by end-to-side anastomosis between the infrarenal caval vein and the donor portal vein. The incision in the anterior aortic wall was only slightly larger than the diameter of the donor aorta, whereas the opening of the recipient's caval vein was about twice to three times the size of the diameter of the donor portal vein. Both anastomoses were carried out as running sutures. After placing a stay suture in the left corner of the anastomosis, a 9-0 nylon suture (Serag Wiessner, Naila, Germany) was tied in the right corner. The posterior wall of both anastomoses was completed from the intraluminal side followed by extraluminal suturing of the anterior wall. This technique overcomes the need for repositioning the graft and the recipient animal as if both walls of the anastomosis were sutured from the outside (fig. 1). After finishing, the clamp on the donor portal vein was released first followed by the arterial clamp. All grafts immediately returned to their normal pink color with the arterial stump pulsating. Finally, an end-to-side duodenojejunostomy for enteric drainage was performed with running sutures using 8-0 nylon (Serag Wiessner, Naila, Germany).

Fig. 1. Re-establishing of arterial blood flow by end-to-side anastomosis of the recipient infrarenal aorta and the aortic conduit of the donor including the donor celiac and superior mesenteric artery. The back wall was sutured from the intraluminal side, the front wall from the extraluminal side employing 9-0 nylon running sutures.

The pancreatic tail was gently exteriorized and the adjacent spleen was fixed with tissue adhesive (Histoacryl, Braun Melsungen, Germany) on a specially designed mounting panel for intravital microscopy. The mounting panel permitted the examination of the pancreatic graft omitting any tension of the vessels. To exclude exposure to ambient air, to prevent drying and to avoid temperature changes of the graft the pancreatic surface was covered with oxygen-impermeable transparent films and constantly superfused with 37°C Ringer's lactated solution. Temperature of the pancreatic tail was continuously measured by a temperature-sensitive probe (LICOX; GMS, Kiel, Germany). All measurements were undertaken in recipient animals with systolic arterial blood pressure above 90 mm Hg. Short episodes of hypotension immediately after reperfusion were treated by bolus substitution of increments of 0.4 ml saline solution according to changes in systemic hemodynamic parameters. Reperfusion of grafts of the Tx group and investigation of time-matched controls were allowed for 2 h. Thereafter, animals were sacrificed by exsanguination.

Intravital Fluorescence Microscopy

IVM was performed using a modified Nikon-Eclipse E600-FN epifluorescence microscope (Nikon GmbH, Düsseldorf, Germany) with a 100-watt mercury vapor lamp HB-10103AF-Hg. Filter blocks FITC (excitation 465–495 nm, emission >515 nm) and G-2A (excitation 510–560 nm, emission >590 nm) were used for epi-illumination. A universal immersion objective (water and oil, X2010.45, Plan Fluor; Nikon GmbH, Düsseldorf, Germany) provided a magnification of approximately ×650 on the video screen (WV-BM1700, diagonal 41 cm; Panasonic, Osaka, Japan). Observations were recorded

by means of a charge-coupled device video camera (RS-170 Monochrome CCD Camera; Cohu, San Diego, Calif., USA) and transferred to an S-VHS video recorder (AG-4700EY; Panasonic, Osaka, Japan) for off-line evaluation. A time-code generating interface (TCI 70; Alpermann + Velte, Wuppertal, Germany) was installed between the camera and the video recorder. For contrast enhancement of microvessels 0.2 ml of 0.4% bovine serum albumin labelled with FITC (FITC-albumin; Sigma, Taufkirchen, Germany) and for *in vivo* staining of leukocytes 0.2 ml 0.01% rhodamine 6G (Sigma, Taufkirchen, Germany) were administered. Each observation field was recorded for 30 s.

Assessment of Microcirculation

Quantitative analysis of the microcirculation of pancreatic exocrine tissue included the determination of functional capillary density (FCD) as well as the measurement of the number of leukocytes sticking in postcapillary venules (LAV). As described previously by Hoffmann et al. [13], randomly selected non-overlapping regions of the exposed pancreatic tail were scanned 1 and 2 h after the onset of reperfusion in the Tx group and in time-matched controls as well as organs of the 'Procurement' group. FCD, which is defined as the length of all red blood cell-perfused capillaries per observation area (cm/cm^2), was determined by off-line analysis of 10 randomly chosen exocrine observation areas ($350 \times 200 \mu\text{m}$) per time point with the method described by Schmid-Schoenbein et al. [21]. A square-type grid system (7×4 squares, square side representing $50 \mu\text{m}$) was superimposed to the video screen. FCD was calculated by counting the number of intersections between the grid and the capillaries as follows: $\text{FCD} (\text{cm}/\text{cm}^2) = \pi/2 \times (\text{number of points of intersection} / (2 \times \text{number of squares of the grid system} \times \text{length of the edge of the grid}))$. LAV was quantified by analyzing three postcapillary venules (diameter $>20 \mu\text{m}$ and length $<80 \mu\text{m}$) at each time point. Adherent leukocytes ($\text{number}/\text{mm}^2$) were defined as leukocytes sticking to the vessel wall for at least 30 s. LAV was calculated in accordance with the equation $\text{LAV} (\text{number}/\text{mm}^2) = \text{number of sticker} / \pi \times \text{length of the vessel} \times \text{diameter of the vessel}$.

In situ Nick-End Labeling of Fragmented DNA

Identification of apoptotic cells by detection of DNA strand breaks was performed using an *in situ* cell death detection kit (TUNEL POD; Roche, Mannheim, Germany). The method uses terminal deoxynucleotidyl transferase (TdT) for *in situ* nick end-labeling of free 3'-OH ends in genomic DNA with deoxyuridine triphosphate (dUTP) (TUNEL assay). The TUNEL reaction was performed following the manufacturer's instruction manual. Briefly, after deparaffinization, the tissue sections were digested by incubation with $20 \mu\text{g}/\text{ml}$ proteinase K (Roche, Mannheim, Germany) for 15 min at room temperature (RT). After washing with PBS, sections were covered with 3% peroxidase block solution for 5 min at RT to inactivate endogenous peroxidase. After 2 rinses in PBS, tissue sections were immersed on ice in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Thereafter, the sections were incubated in TUNEL reaction mixture containing terminal deoxynucleotidyl transferase enzyme (TdT) at 37°C in a 5% CO_2 humidified chamber for 60 min. The reaction was terminated by a 30-min incubation in a stop/wash buffer. For visualization of incorporated digoxigenin-11-dUTP, sections were incubated with peroxidase-conjugated antidigoxigenin for 30 min at RT. Visualization was achieved using DAB substrate solution. Finally, the sections were counterstained by hematoxylin. Positive controls were treated with DNase ($100 \mu\text{g}/\mu\text{l}$; Boehringer, Mann-

heim, Germany). Negative controls were obtained by omitting the TdT in the reaction mixture. Apoptotic cells were counted from >50 fields under a light microscope using a magnification of $\times 400$ by an observer blinded to the source of the sections. Apoptotic cells were counted only after identification of typical morphological criteria like chromatin condensation and cell fragmentation. An apoptotic index (AI) was defined as the number of apoptotic cells counted per high power field (hpf).

Statistical Analysis

All data were analyzed using the GraphPad Prism software (version 3.00). Results are expressed as mean values $\pm$ SEM. Values of $p < 0.05$ were considered significant. All data were first proven to fit the assumption of normality. Differences between groups were tested by an analysis of variance (ANOVA), followed by a post hoc comparison using the Tukey's method.

Results

Reperfusion was successful in all transplanted animals with a systolic arterial blood pressure above 90 mm Hg. Short episodes of hypotension immediately after reperfusion were treated by bolus substitution of saline solution as stated above. None of the transplanted animals had to be excluded due to explicit bleeding from anastomosis or bleeding from the pancreatic graft.

Microcirculation

Process of organ procurement (Procurement group) resulted in a slight non-significant decrease of functional capillary density (FCD; cm/cm^2) at 1 h (407 ± 12 vs. 441 ± 12 ; $p > 0.05$) and 2 h measurements (401 ± 18 vs. 455 ± 24 ; $p > 0.05$) compared with sham-operated animals (fig. 2a). In parallel, we found leukocyte sticking to postcapillary venules (LAV; cells/mm^3) not significantly increased in animals undergoing subtotal organ harvest compared with sham-operated animals (33 ± 7 vs. 14 ± 9 at 1 h, $p > 0.05$; 57 ± 16 vs. 20 ± 20 at 2 h measurements, $p > 0.05$) (fig. 2b). However, among transplanted animals, FCD was significantly ($p < 0.01$) decreased to 353 ± 15 at 1 h and to 315 ± 21 at 2 h reperfusion compared with both groups of nontransplanted animals. In addition, LAV was significantly ($p < 0.01$) increased to 116 ± 23 at 1 h and to 198 ± 33 at 2 h reperfusion. As a result of continuous measurement of arterial blood pressure in both the recipient and the donor we were able to exclude any alterations of microcirculation evoked by hypotension.

TUNEL Staining

Apoptosis was noted in both groups of nontransplanted animals (sham: $\text{AI} = 0.16 \pm 0.03$ cells/hpf; con-

Fig. 2a, b. Quantitative analysis of the microcirculation of exocrine pancreatic tissue after whole pancreas transplantation assessed by means of intravital fluorescence microscopy. Sham = Animals with only exteriorization of the pancreas (n = 5); Procurement = nontransplanted animals undergoing subtotal process of organ harvest (n = 7) and Tx = animals subjected to pancreas transplantation with grafts stored for 6 h in 4°C UW solution (n = 7). **a** Functional capillary density (FCD). **b** Permanently (>30 s) adherent leukocytes (sticker) in postcapillary venules (LAV) at 1 and 2 h reperfusion and in time-matched controls as well as organs of the 'Procurement' group. Data presented as means $\pm$ SEM; analysis of variance (ANOVA) and Newman-Keuls multiple post hoc comparison test. # p < 0.001 compared with controls; * p < 0.01 compared with the Procurement group.

trol: AI = 0.23 ± 0.02 cells/hpf; $p > 0.05$; fig. 3). Grafts subjected to cold ischemia and subsequent reperfusion displayed an apoptotic index of 0.42 ± 0.08 cells/hpf at 2 h reperfusion that was significantly higher than in both groups of nontransplanted animals ($p < 0.01$).

Discussion

Pancreas transplantation has evolved over the past decade. However, there are still numerous unsolved problems with this issue. Experimental studies are the background for investigations concerning surgical, immunological, and microcirculatory problems in pancreas trans-

plantation. Nowadays, rats are commonly used for experimental research. Major advantages are lower costs, no need for sterile technique, simple induction of experimental diabetes and availability of inbred strains [22]. During the last years, major concern was taken on understanding and abrogation of ischemia-reperfusion injury in organ transplantation. For the pancreas, microcirculatory impairment play a pivotal role in different post-transplant pathological conditions including graft pancreatitis, infection and vascular thrombosis, which contribute to postoperative morbidity, graft failure and mortality [23]. They are characterized by a variety of pathophysiologic conditions including nutritive capillary perfusion failure, cell activation with release of various mediators, expression of

Fig. 3. Apoptotic index (apoptotic cells per high-power fields) of acinar cells in pancreatic tissue. Grafts were harvested after the second measurement of microcirculation. Sham = Animals with only exteriorization of the pancreas (n = 5); Procurement = non-transplanted animals undergoing subtotal process of organ harvest (n = 7); Tx = animals subjected to pancreas transplantation with grafts stored for 6 h in 4°C UW solution (n = 7). Apoptotic cells were identified by typical morphological signs after in situ staining using TUNEL assay method. Baseline apoptosis was found among control animals and was slightly increased in animals subjected to subtotal organ harvest. Data presented as means $\pm$ SEM; analysis of variance (ANOVA) and Newman-Keuls multiple comparison test (# $p < 0.01$ compared with controls; * $p < 0.05$ compared with the Procurement group).

adhesion molecules and loss of endothelial integrity with formation of edema [14, 24]. There are only few studies published which focused on IVM-measured microcirculation in pancreas transplantation using a small animal model [12, 25, 26]. However, none of these studies determined the impact of organ procurement on post-transplant impairment of microcirculation. This is noteworthy since appropriate organ procurement of the pancreas in rats is complex and requires a lot of expertise. Recent studies have demonstrated that in situ manipulation of liver grafts during organ harvest by touching, retracting and moving liver lobes induces a Kupffer cell dependent injury which dramatically reduces survival after liver transplantation [27, 28]. In addition, in clinical transplantation as well as in a porcine model, there is increasing evidence that dissection of the liver during harvest reduces graft function after transplantation [6, 7].

However, for experimental pancreas transplantation the contribution of organ procurement to total ischemia-reperfusion injury had still to be determined. Since early deterioration of microcirculation after reperfusion is crucial for development of graft pancreatitis [3], we and others focused on the first 2 h after reperfusion [12, 25, 26, 29]. With regard to sham-operated animals and transplanted grafts we found our results in conformity with data presented by Vollmar et al. [25], who investigated pancreatic microcirculation after 6 h of cold preservation in HTK solution.

In our institution, pancreas transplantation in rats has been established over 6 months of daily practice. This

training period, in which no experimental data have been acquired, was necessary to get familiar with handling anesthesia, technique of microsurgically performed vascular anastomoses and organ harvest. Regarding the latter, details have been described in literature before [8, 20, 22, 30]. However, there is no standard of rational organ procurement. In this study, we present a standardized technique of 5 different operative steps that enables a reliable method of organ harvest also for beginners. These different steps can be entitled with 'gastrectomy' (step 1), 'exenteration' (step 2), 'dissection of the aorta' (step 3), 'dissection of liver hilus' (step 4) and 'graft perfusion' (step 5). Gastrectomy encompasses the total freeing of the pancreatic tail including removal of the stomach and of the greater omentum with ligation of the splenic vessels. We completely finish every above mentioned step during the course of organ harvest without returning to the same site later. This is time saving and minimizes the need for further handling of the organ. Major concern has to be taken to avoid any direct manipulation of the graft as already been advocated by the so called 'no touch technique' of Lee et al. [8]. The removal of the whole intestine except the duodenum in the early stage of operation enables an excellent exposure of the aorta and caval vein for further preparation (step 2). In contrast to Lee, we did not dissect the mesenteric root to divide the portal vein and the superior mesenteric artery separately [20]. Both structures can be ligated together. Moreover, we recommend not removing the spleen until transplantation is finished. It serves as a grip for handling of the graft during all

steps of operation. In addition, during the whole process of organ harvest we suggest to keep the pancreas wrapped in soaked gauze to prevent drying of the organ. With careful preparation, anesthesia and organ harvest will take about 90 min even with an experienced surgeon.

Our study revealed only a slight non-significant deterioration of microcirculation and increase in apoptosis in

animals undergoing subtotal process of organ procurement compared with sham operated animals. Therefore, with appropriate organ procurement, the microcirculatory impairments and the increase of apoptosis of the transplanted graft are mainly related to ischemia-reperfusion injury rather than organ harvest.

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