

Allogenic Stem Cells Transplantation in Rabbit Myocardial Infarction

S Jiang,¹MD, Kh H Haider,²PhD, Y Lei,³MD, Niagara,⁴BSc (Appl), Rufaihah,⁴BSc (Appl), EKW Sim,⁵FRCS

Introduction

It is generally accepted that cardiac myocytes lack the ability to regenerate after injury, which leads to permanent deficit in the number of functioning cardiomyocytes that contribute to the development and progression of heart failure. Cell therapy has emerged as a promising new strategy to supplement the deficient intrinsic repair mechanism of heart. Various types of cells, including fetal and adult cardiomyocytes and human skeletal myoblasts, have been used for cardiac transplantation. *In vitro* and *de milieu* dependent differentiation approaches are being pursued to generate cardiomyocytes from stem cells derived from different sources and have demonstrated great potential in treating heart failure. Stem cells are immature tissue precursor cells, capable of self-renewal and provision of replacement cells for many tissues. The present study involves transplantation of bone marrow-derived allogenic adult stem cells (BMCs) for cardiac repair. The results revealed that BMCs were able to survive in the infarcted myocardium in a rabbit model of myocardial infarction. The BMCs stimulated neovascularisation. An interesting feature of our findings is that some donor cells had transformed into endothelial cells and were seen as part of the blood vessel lumen.

Materials and Methods

Ten New Zealand white female rabbits (3~3.5 kg) were used in the study. The animals were divided into two groups: BMC-transplanted group (n = 6) and Dulbecco modified eagle's medium (DMEM) injected group (n = 4). All animals received humane care in accordance with the principles of research animals guidelines at the National University of Singapore.

The animals were premedicated with methohexitol (10 mg/kg im) and anaesthesia was maintained with isoflurane (2%) and 100% oxygen (2 L/min) using mechanical ventilation to maintain respiration. A left thoracotomy was performed on each animal and the heart was exposed. The first and second branches of the left circumflex coronary artery were ligated by suturing with prolene 4.0 and acute ischaemia was observed. The incision was closed and the animal was allowed to recover from anaesthesia. They were maintained on cephalosporin for 4 to 6 days.

Under general anaesthesia, 10 mL of bone marrow was aspirated using a bone marrow aspiration needle and cultured with 20% fetal bovine serum (FBS), 100 unit/mL penicillin G, 100 ug/mL streptomycin). The cells were incubated at 37°C in 5% carbon dioxide, and continually cultured for 4 weeks; the medium was changed every 2 to 3 days. As the haematopoietic stem cells do not attach themselves to the culture flask, they were removed with the culture medium changes.

To identify transplanted cells in the recipient myocardium, the cells were transfected with retrovirus encoding lac-z reporter gene for thrice. Briefly, 10 mL cell culture supernatant of FLYA4 cells containing 10⁵ viral particles/mL was filtered through a 0.22-um filter into each flask and incubated at 37°C in 5% CO₂. After 6 to 8

hours of transduction, another 10 mL of fresh DMEM was added and the cells were incubated for 24 hours. The transduction procedure was repeated over the next 3 days on 3 occasions. A day before transplantation, the BMCs were double labelled with BrdU. BrdU was diluted with DMEM at 1:600 and 15 mL of the diluted BrdU was added into the culture flasks and incubated for 24 hours at 37°C in 5% CO₂. The stromal cells were trypsinised using 0.05% trypsin, washed with PBS and 30 x 100⁶ cells were resuspended in 1 mL serum-free DMEM for transplantation.

At 2 to 3 weeks' post infarction, the animals were anaesthetised and the heart was exposed for the second time. BMCs suspended in 1 mL DMEM or DMEM without cells were injected into the centre and periphery of the infarct regions. The incision was sutured and the animals were maintained on cyclosporine at 5 mg/kg to prevent rejection of the donor cells. Antibiotics were administered for 5 to 7 days to prevent infection. At 1 week and 6 weeks post-transplantation, the animals were sacrificed, 3 each for the cell-transplanted group and 2 each for the DMEM-injected group. The hearts were harvested and processed for histological study.

The explanted heart tissue was embedded in freezing medium for cryosectioning. Sections of 6-8 um in thickness were cut and used for histochemical and immunohistochemical studies.

The tissue sections were fixed with 0.5% glutaraldehyde for 15 minutes at room temperature. After washing thrice with PBS for 10 minutes each, the slides were incubated with 5-bromo-3-indolyl-beta-D-galactosidase (x-gal) [40 mg/mL in N,N-dimethylformamide (DMF) in x-gal buffer containing 35 mM each of potassium ferricyanide, potassium ferrocyanide and 2 mM MgCl₂]. After 24 hours, the slides were rinsed in PBS and observed under microscope.

All sample slides were fixed in -20°C methanol for 10 minutes and rehydrated. The slides were blocked with 3% hydrogen peroxide thrice at room temperature and rinsed with PBS. After blocking with 1% BSA for 10 minutes, the slides were incubated with anti-BrdU primary antibodies, von Willebrand factor VIII or PBS in a moist chamber overnight at room temperature. The slides were incubated with secondary antibodies and after rinsing with PBS, the reaction was visualised with DAB substrate and photographed under microscope.

Results

BMCs that attached themselves to the base of the culture flasks proliferated within 3 to 4 days. The medium was changed every 2 to 3 days; the haematopoietic cells that did not attach to the flasks were washed away during the medium change (Fig. 1). The efficiency of lac-z transduction was 40% when stained with x-gal in vitro study. Fibrous tissue was observed in the infarcted region in both cell-transplanted heart and medium-injected heart. The transplanted cells were observed in transplanted heart tissue with positively stained BrdU and lac-z (Figs. 2, 3 and 5a). They survived in both infarct and peri-infarct regions. The medium-injected animal heart tissue stained negative for lac-z and BrdU expression (Fig. 5b). The donor cells

¹ Research Officer, National Heart Centre, Singapore,

² Research Scientist, Department of Surgery, National University of Singapore, Singapore

³ PhD Student, Department of Surgery, National University of Singapore, Singapore

⁴ Master Student, Department of Surgery, National University of Singapore, Singapore

⁵ Associate Professor, Department of Surgery, National University of Singapore, Singapore

Address of Correspondence: Dr S Jiang, Department of Surgery, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074.

were found following the tissue architecture. An interesting finding is that the donor BMCs were observed in the walls of the vasculature (Fig. 4). Immunostaining with von Willebrand factor VIII showed that the BMC-transplanted tissue showed more blood vessels compared to the DMEM-injected group (Figs. 6a and 6b).

Discussion

Myocardial transplants of cultured heart cells,¹⁻³ skeletal myoblasts,⁴⁻⁷ smooth muscle cells⁸ and bone marrow cells^{9,10} have been demonstrated to prevent heart failure after a myocardial injury. The advantages of bone marrow stromal cells include their easy availability and the possibility of differentiating them into cardiomyocytes that may form gap junctions and beat synchronously with the host myocardium.¹¹ In addition, they may differentiate into endothelial progenitor cells that may participate in neovascularisation after transplantation.¹² Tomita et al transplanted cultured bone marrow stem cells that were induced to differentiate into a myogenic phenotype with 5-azacytidine. This showed that the transplanted cells formed an island of cardiac-like tissue, induce angiogenesis and prevent thinning and dilatation of the infarct region. It also improved regional and global contractile functions.¹³ Orlic and associates transplanted bone marrow stem cells into an infarcted murine model. They showed a newly formed myocardium in the infarcted region. The newly developed tissue comprised proliferating myocytes and vascular structures.¹⁴ Kobayashi and coworkers also found that BMC implantation induced angiogenesis in a rat coronary artery ligation model.¹⁵ In our study, we showed that allogenic BMCs survived in the infarcted rabbit myocardium after transplantation. The donor cells survived both in infarct and normal myocardium. The lac-z and BrdU expression showed that they aligned following host myocardial architecture. vWFVIII staining demonstrated that the stromal cells secrete vascular endothelial growth factor; the transplantation of stromal cells accelerated neovascularisation.¹⁶ One limitation of our study is the small number of animals, which limited the evaluation of the cardiac function and statistics of our histological data. Further study is in progress to clarify the characteristics of transplanted cells.

Conclusion

Allogenic bone marrow stem cells can survive in both normal and infarcted regions of the heart. They can transform into endothelial cells and stimulate neovascularisation.

REFERENCES

- Li RK, Weisel RD, Mickel DA, Jia ZQ, Kim EJ, Sakai T, et al. Autologous porcine heart cell transplantation improved heart function after a myocardial infarction. *J Thorac Cardiovasc Surg* 2000; 119:62-8.
- Soonpa MH, Koh GY, Klug MG, et al. Formation of nascent intercalated discs between grafted fetal cardiomyocytes and host myocardium. *Science* 1994; 264:98-101.
- Leor J, Patterson M, Quinones MJ, et al. Transplantation of fetal myocardial tissue into the infarcted myocardium of rat. A potential method for repair of infarcted myocardium? *Circulation* 1996; 94(Suppl II):332-6.
- Atkins BZ, Lewis CW, Kraus WE, Hutcheson KA, Glower D, Taylor DA. Intracardiac transplantation of skeletal myoblasts yields two populations of striated cells in situ. *Ann Thorac Surg* 1999; 67:124-9.
- Chiu RC, Zibaitis A, Kao RL. Cellular cardiomyoplasty: myocardial regeneration with satellite cell transplantation. *Ann Thorac Surg* 1995; 60:12-8.
- Van Meter CH, Claycomb WC, Delcarpio JB, et al. Myoblast transplantation in the porcine model: a potential technique for myocardial repair. *J Thorac Cardiovasc Surg* 1995; 110:1442-8.
- Talor DA, Atkins BZ, Hunspreugs P, Jones TR, Reedy MC, Hutcheson KA, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med* 1998; 4:929-33.
- Li RK, Jia ZQ, Weisel RD, Merante F, Mickel DA. Smooth muscle cell transplantation into myocardial scar tissue improves heart function. *J Mol Cell Cardiol* 1999; 31:513-22.
- Tomita S, Li RK, Weisel RD, Mickel DAG, Ja ZQ. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation*. 1999; 100(19 suppl II):247-56.
- Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* 1999; 100(Suppl II):247-56.
- Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 1999; 103:697-705.
- Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in

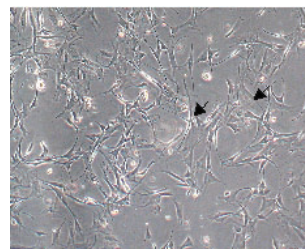


Fig. 1.

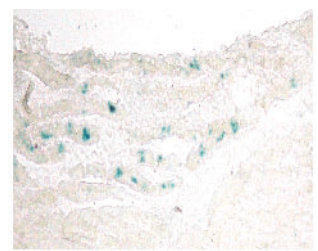


Fig. 2.

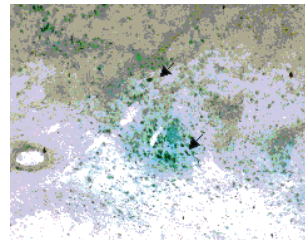


Fig. 3.

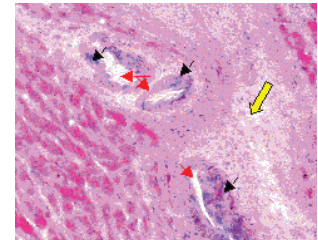


Fig. 4.

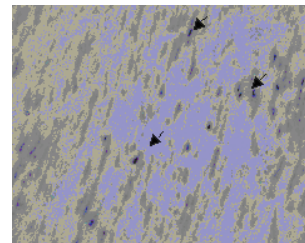


Fig. 5a.

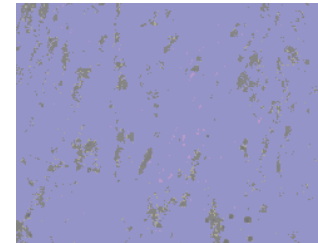


Fig. 5b.

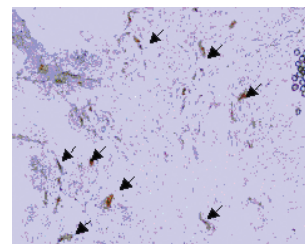


Fig. 6a.

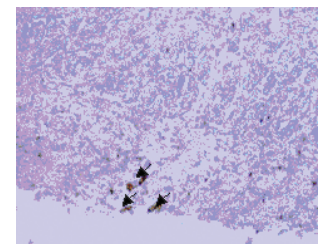


Fig. 6b.

Fig. 1. BMC in vitro culture at two weeks after bone marrow harvesting.

Fig. 2. BMC in the periphery of the infarcted region expressing lac-z reporter gene at 6 weeks after transplantation.

Fig. 3. Photomicrograph showing survival of lac-z expressing BMCs in the infarcted myocardium 1 week after transplantation.

Fig. 4. The transplanted BMCs transdifferentiated into endothelial cells and form the walls of the vasculature. The cells stained positive with x-gal formed the wall of blood vessel. Lac-z positive tissue counter stained with haematoxylin.

Fig. 5. The BMC-transplanted tissue stained for BrdU using anti-BrdU antibody.

(a) BMC of 6 weeks post-transplantation.

(b) DMEM-injected tissue stained negative for BrdU.

Fig. 6. The (a) BMC-transplanted tissue stained with factor VIII antibody for vascular density measurement. The BMC-transplanted tissue showed more neovascularisation. (b) DMEM-injected tissue.

- physiological and pathological neovascularization. *Cardiovasc Res* 1999; 85:221-8.
13. Tomita S, Mickel DA, Weisel RD, Jia ZQ, Laura CT, Yasmin A, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg* 2002; 123:1132-40.
 14. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow mesenchymal stem cells regenerate infarcted myocardium. *Nature*. 2001; 410:701-5
 15. Kobayashi T, Hamano K, Li TS, Katoh T, Kobayashi S, Matsuzaki M, et al. Enhancement of angiogenesis by the implantation of self bone marrow cells in a rat ischemic heart model. *J Surg Res* 2000; 89:189-95.
 16. Bautz F, Rafii S, Kanz L, Mohle R. Expression and secretion of vascular endothelial growth factor A by cytokine-stimulated hematopoietic progenitor cells: possible role in the hematopoietic microenvironment. *Exp Hematol* 2000; 28:700-6.

Special Poster Presentation

The Effect of Sialidase Inhibitors on Hyaluronan-mediated Escape from Apoptosis Induced by Anti-cancer Drugs in Gastric Cancer

P Wang,¹MBBS (China), M Look,²MBBS, FRCS (Edin), FRCS (Glas), SP CHEW,³FAMS, MBBS, FRCS

Introduction

Gastric adenocarcinoma is one of the leading causes of cancer death in Singapore. Surgery remains the main modality for cure, but this is no longer effective once metastasis occurs. Systemic chemotherapy by anti-cancer drugs is often useful, but the response is unpredictable. Moreover, the degree of differentiation of the gastric cancer also seems to affect the prognosis and pattern of metastasis. The reasons for these are unclear.

Metastasis is a multi-step cascade initiated when malignant cells break free from the primary tumour and enter the bloodstream, lymphatics or peritoneal cavity. Adhesion receptors on the surface of cancer cells play an important role in tumour cell migration, invasion and metastasis. A number of specific cell surface associated molecules that mediate cell-matrix and cell-cell interactions have been characterised. Cell-cell interactions are known to protect cells from apoptosis and may help to explain the chemoresistance of solid tumours.

CD44, an integral membrane glycoprotein expressed in many types of cells, is the principal transmembrane hyaluronate receptor and may be a determinant of metastatic and invasive behaviour. The sialylation process of CD44 plays a role in the conjugation of cells to hyaluronan (HA). HA is an important mediator of cancer apoptosis. The aim of our study is to observe the effect of HA on gastric cancer cell apoptosis induced by anti-cancer drugs and the influence of sialylation of cell surface adhesion molecules on this process.

Materials and Methods

Two gastric cancer cell lines (poorly differentiated MKN45 and moderately differentiated MKN74) were cultured and subjected to anti-cancer drug induced apoptosis and the HA inhibition test. 2×10^6 cells of exponential-phase culture were treated with etoposide (a topoisomerase inhibitor) and 5FU (a uracil analogue) at various concentrations for 12 hours at 37°C, in 5%CO₂. Apoptosis was measured by the propidium iodide stain method and the Hoechst 33258 stain method. The experiment was repeated with HA inhibition, with varying concentrations of HA from 5 to 25 µg/mL.

Next, CD44 expression in the cell lines was measured by indirect immunostaining under fluorescence microscope and flow cytometry. This was repeated after treatment with siastatin B between

10 and 100 µg/mL.

Next, the HA adhesion test was performed by coating flat-bottom plates with hyaluronate at 1 mg/mL and heat-denatured bovine serum albumin in phosphate-buffered saline at 10 mg/mL overnight at 4°C. 1×10^5 cells in a single-cell suspension were added and adhesion allowed to proceed for 1 hour at 4°C. The plates were inverted and unattached cells were aspirated. The plates were washed with phosphate buffered saline and viable cells were counted. The effect of two sialidase inhibitors, siastatin B and asialo-fetuin, on the HA adhesion test was studied at various concentrations.

Results

Apoptosis induced by the anti-cancer drugs was observed in a dose-dependant manner for both cell lines. Etoposide at 20 µg/mL and 5FU at 10 µg/mL induced apoptosis in 50% of culture cells. In the HA inhibition test, HA at concentrations between 5 and 25 µg/mL effectively prevented apoptosis induced by 20 µg/mL of etoposide in MKN45 cells ($P < 0.05$). This effect was not observed in MKN74 cells or in 5FU-induced apoptosis.

CD44 positive rate was 60% to 70% in MKN45 cells, but this could not be demonstrated in MKN74 cells. CD44 expression in MKN45 cells decreased after treatment with siastatin B between 10 and 100 µg/mL ($P < 0.05$), but not to asialo-fetuin.

In the HA adhesion test, sialidase inhibition by siastatin B and asialo-fetuin was found to decrease the gastric cell adhesion to HA in MKN45 cells ($P < 0.05$), but not in MKN74 cells.

Discussion

Apoptosis, or programmed cell death, is an orderly and genetically controlled process initiated either by ligand binding to cell surface receptors or by cell toxins via a pathway that targets the mitochondria. Most anti-cancer drugs act by activating an endogenous biochemical process for apoptosis. Malignant cells that develop defects in the genes that control apoptosis may render themselves resistant to chemotherapy.

CD44 is a family of transmembrane glycoprotein receptors for HA and this affinity can be reduced by alternative splicing and glycosylation. Cell surface CD44 overexpression can promote tumour cell survival in invaded tissues and its suppression can induce

¹ MSc Research Student, Department of Surgery, Tan Tock Seng Hospital, Singapore

² Consultant and Head Upper Gastrointestinal Surgery, Director, Centre for Advanced Laparoscopic Surgery, Department of Surgery, Tan Tock Seng Hospital, Singapore

³ Senior Consultant and Head Hepatobiliary Surgery, Department of Surgery, Tan Tock Seng Hospital, Singapore

Address of Correspondence: Dr Melvin Look, Department of Surgery, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433.

apoptosis of the invading tumour cells. There are only a few reports on the relationship between HA and apoptosis, and some of these are contradictory. Our findings suggest that adhesion to HA inhibited apoptosis induced by etoposide for the poorly differentiated MKN45 gastric cancer cells. This effect was not seen for 5FU. It has been reported that etoposide-induced apoptosis is inhibited by CD44 overexpression, possibly through a CD44-mediated signalling pathway. On the other hand, 5FU is known to decrease proliferation status and induce apoptosis, possibly via the Fas pathway. The different pathways of action for different anti-cancer drugs and their relationship with CD44 and HA may, therefore, account for our findings.

CD44 is acidic largely due to sialic acid. Sialic acids are a family of amino sugars that are commonly found as terminal oligosaccharide residues on glycoproteins and glycolipids. Glycosylation of CD44 itself can affect HA binding ability by negative regulation of its recognition of HA. Certain cells which had sialic acid removed by neuraminidase from their cell surface acquire the ability to bind HA. Analogues of sialic acid, such as neuraminidase inhibitors, can be potential anti-adhesion drugs. Our postulate that sialidase inhibitors can block the adhesion of gastric cancer cells to HA was verified in our HA inhibition test. This effect was achieved with siastatin B and asialo-fetuin for the poorly differentiated MKN45 cancer cells, but not in the moderately differentiated MKN74 cells. Siastatin B, a

multi-functional azasugar isolated from streptomyces culture, is an inhibitor of sialidase, while asialo-fetuin is a glycoprotein substrate for sialidase.

The differences in behaviour of the two cell lines in our experiment could be explained by their adhesive properties. The impact of our experiment was only limited to the low-adhesion, poorly differentiated MKN45 cell line, and not in the high-adhesion, moderately differentiated MKN74 cell line. Desialylation is higher in low-adhesion cell lines and may represent a potential target for therapy.

It was very interesting to find that siastatin B decreased the CD44 expression of MKN 45 cells in our experiment. It is generally thought that the variable glycosylations or splice variants determined its HA adhesion. CD44 is stable in the glycosylation process. Therefore, our findings suggest that HA adhesion might be important for CD44 splitting and, finally, CD44 expression.

Conclusion

Adhesion to HA may be an important mechanism for escape from apoptosis induced by anti-cancer drugs. This effect seems to be limited to poorly differentiated gastric cancer cells rather than moderately differentiated ones. Highly effective and specific sialidase inhibitors may modulate this effect. Our findings are preliminary but important, as it may lead to a novel and promising biologic therapy for increasing the chemosensitivity of anti-cancer drugs for gastric cancer.

Special Poster Presentation

Blood-Brain Barrier Disruption Following Traumatic Brain Injury: Roles of Tight Junction Proteins

I Ng,¹MBBS, FRCS, E Yap,²BSc, WL Tan,²BSc, NY Kong,³BDS, PhD

Introduction

The endothelial cells within the central nervous system (CNS) form a continuous wall joined by tight junctions with a high reflective coefficient and electrical resistance that prevents virtually all water-soluble substances from entering the CNS. This property, known as the blood-brain barrier (BBB), is pivotal in the maintenance of a stable and controlled microenvironment in the neuronal parenchyma by protecting against neurotoxic compounds and ionic concentration variations.¹ The tight junction consist of proteins, such as occludin,² claudin-1,^{3,4} ZO-1,⁵ ZO-2^{6,7} and ZO-3,⁸ which play important structural and accessory roles in the normal physiological function of the BBB.³

Intracranial hypertension is a major cause of secondary brain injury.⁹⁻¹¹ It may occur as a result of mass effect from a space-occupying lesion (haematoma or tumour) and/or brain swelling (vasogenic and cytotoxic),¹²⁻¹⁴ and contributes significantly to morbidity and mortality.

The contribution of BBB disruption following traumatic brain injury (TBI) to the development of intracranial hypertension is uncertain and is poorly understood at the molecular level. The proposed mechanisms of vasogenic oedema following TBI and intracranial hypertension include opening of tight junctions, enhanced vesicular transport and damage of endothelial membranes. In this study, we attempted to study the roles of tight junction protein (occludin, claudin-1, ZO-1, ZO-2 and ZO-3) as a result of TBI.

Materials and Methods

Fluid Percussion Injury

35 male Sprague-Dawley rats were randomly assigned to sham-operated group and lateral fluid percussion injured group. They were further divided into 3 subgroups for different experimental procedures: 15 for brain water content, 10 for immunohistochemistry analysis of tight junction proteins and 10 for horseradish peroxidase (HRP) staining procedures.

The lateral fluid percussion injury was induced in the rats based on

¹ Consultant Neurosurgeon, Director of Neurocritical Care Unit and Head of Neurotrauma and Cerebrovascular Surgery Program, Department of Neurosurgery, National Neuroscience Institute, Singapore

Assistant Professor of Neurosurgery, National University of Singapore, Singapore

² Research Associate, Acute Brain Injury Research Laboratory, Department of Neurosurgery, National Neuroscience Institute, Singapore,

³ Assistant Professor, Department of Anatomy, National University of Singapore, Singapore

Address of Correspondence: Dr Ivan Ng, National Neuroscience Institute, Acute Brain Injury Research Laboratory, Section of Neurotrauma, Department of Neurosurgery, 11 Jalan Tan Tock Seng, Singapore 308433.

the protocol described by McIntosh et al.¹⁵ The rats were anaesthetised with a concoction of xylazine (10 mg/kg) and ketamine (150 mg/kg) intraperitoneally and allowed breathing spontaneously. A 5-mm craniotomy was made over the left parietal cortex midway between the lambda and bregma sutures. A Leur-Loc was tightly fitted and cemented to the craniotomy with dental acrylic. Injury was induced by trephination, with the impact of the rapid fluid bolus that strikes the intact dural surface producing a focal injury to the brain.¹⁶ The impact of the fluid bolus was measured in atmospheres.

Brain Water Content

The animals were sacrificed after 2 hours post-injury for both the study and sham-operated groups. The brains were removed immediately and weighed before being dehydrated at 90 degree Celsius for 72 hours.¹⁷ The brain samples were weighed again and the brain water content is estimated using the formula depicted below:

$$1 - (\text{weight after drying} / \text{weight before drying}) * 100\%$$

Horseradish Peroxidase

The animals were administered with 80 mg/kg (dissolved in 3 mL) of HRP Type VI (Sigma Chemical Co., St Louis, Mo) 5 minutes post-injury for the lateral fluid percussion injured group and post-surgery for the sham-operated group via the femoral artery over a 5-minute period. They were sacrificed 120 minutes post-administration of the HRP. The animals were perfused with 4% paraformaldehyde. The brains were removed and immersed in 4% paraformaldehyde for 24 hours and then transferred to 20% sucrose for another 24 hours at 4 degree Celsius. Sections of 40 mm mounted on poly-L-Lysine treated slides were then washed thrice with PBS. The slides were immersed in 3% hydrogen peroxide for 10 minutes and washed again thrice with PBS. The slides were stained with DAB for 10 minutes and washed thrice with deionised water before being mounted with coverslip.

Immunohistochemistry and Analysis

The brain samples were prepared in the same manner as above without the administration of HRP. Immunoreactive ZO-1, ZO-2, ZO-3, claudin-1 and occludin were detected by the labelled streptavidin-biotin method. Sections of 10 mm mounted on poly-L-Lysine-treated slides were extensively rinsed in PBS and endogenous peroxidases were quenched in 3% hydrogen peroxide for 5 minutes. After washing in PBS, slides were incubated with blocking reagent for 30 minutes (Dako LSAB Kit, Dako Carpinteria CA). Incubation with primary antibody was undertaken at 4°C overnight with a 1:50 dilution of rabbit anti-ZO-1 polyclonal antibody, rabbit anti-ZO-2 polyclonal antibody, rabbit anti-claudin-1 polyclonal antibody, rabbit anti-occludin polyclonal antibody (Zymed Laboratories Inc., San Francisco, CA, USA) and mouse anti-ZO-3 monoclonal antibody (Chemicon International, Temecula, CA, USA). Following three rinses with PBS, slides were incubated with linking antibody (Dako) for 10 minutes before washing with PBS, and followed by 10 minutes with streptavidin-HRP diluted as recommended by the manufacturer and incubated for 8 minutes with the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako). After incubation, samples were rinsed thrice with deionised water. Samples were counterstained with 0.5% methyl green for 1 minute. Slides were dehydrated and mounted.

Results

All animals were monitored haemodynamically post-injury and none suffered hypotension and/ or hypoxia following injury. Brain oedema was evident two hours after trauma; the brain's water content in the injury group was higher than that in the sham-operated group ($83.97 \pm 0.21\%$, $n = 10$ versus $82.11 \pm 0.6\%$, $n = 5$) ($p < 0.05$)

(Fig. 1).

Immunohistochemically, all the tight junction proteins studied (ZO-1, ZO-2, ZO-3, claudin-1 and occludin) showed punctuated expression in the paracellular pathway between endothelial cells, evidenced by the patchy chromogen staining compared with the tight junctions in the healthy rat brain sections of the sham-operated group (Fig. 2). This staining loss was seen not only at the penumbral region of the injury created, but also further afield in the ipsilateral hippocampus (Fig. 2).

Light microscopy also demonstrated extravasation of HRP into the interstitial space 2 hours following lateral fluid percussion injury. At lower magnification, we observed localised HRP extravasation in the cortex and hippocampal region in the ipsilateral hemisphere of injured rat brain. At higher magnification, the HRP is phagocytosed by the microglial cells (Fig. 3).

Discussion

Increased intracranial pressure (ICP) is a frequent accompaniment following TBI and intractable intracranial hypertension has been identified as a significant cause of mortality in the neurocritical care setting. The primary injury and subsequent secondary insults contribute to neuronal cell demise via multiple pathways, such as excitotoxicity, apoptosis and lipid peroxidation. One of the presumed injurious events that occur is a breakdown of the BBB with extravasation of fluid into the extracellular compartment, with its attendant deleterious effects on cell membrane function and metabolism.^{18,19} Nevertheless, to date there are no reports on the characterisation of the biological derangement of the BBB with regards to the effect of TBI on the protein structural integrity that maintains the BBB.

The structural basis of the BBB has been the subject of intense study and its elaborate network of complex tight junctions has been investigated morphologically by freeze-fracturing and ultrathin sectioning and physiologically by measurements of paracellular permeability and transepithelial/transendothelial electrical resistance.^{20,21} The molecular organisation of the BBB tight junctions has been elucidated and consists of occludin,^{2,22} claudin-1,³ ZO-1,⁵ ZO-2,⁷ and ZO-3.⁸ While occludin is a transmembrane protein of approximately 65 kDa, ZO-1 (220 kDa), ZO-2 and ZO-3 are submembrane proteins and claudin-1 is a major cell adhesion molecule of the tight junction.

In our study, the BBB appeared from a morphological perspective to be altered by TBI significantly at 2 hours post-injury. We employed 2 methods to prove that punctuated expression of the tight junction proteins was the cause of BBB disruption, namely, brain water content and HRP assessment. At 2 hours, the ipsilateral hippocampus showed patchy loss of occludin, claudin-1, ZO-1, ZO-2 to ZO-3 protein expression. This loss of immuno-expression correlated with the increase in brain water content and HRP extravasation, suggesting that there was developing vasogenic oedema from tight junction disruption. To date this is the first publication that documents the patchy disruption of the BBB following TBI. What is important was, in addition to the localised areas of BBB disruption at the site of the contusion was the evidence further afield (hippocampus). The interrupted protein expression may be caused by endothelial cell injury from the effects of mediators, such as bradykinin, arachidonic acid, histamine, free radicals and polyamines.^{23,24}

Modulation of protein expression has been employed as a therapeutic strategy in a wide variety of degenerative and psychiatric disorders to good effect. Tight junction proteins, in the same manner, bear promise as a target for pharmacologic intervention. Also, this new finding has important implications as the understanding of the

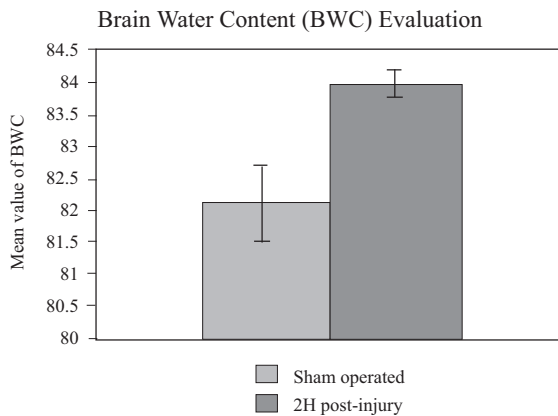


Fig. 1. Brain water content (BWC) were evaluated using the wet/dry weight technique. Results show that brain odema was evident 2 hours after trauma, the brain's water content in the injury group ($83.97 \pm 0.21\%$, $n = 10$) was higher than that in the sham-operated group ($82.11 \pm 0.60\%$, $n = 5$) ($P < 0.05$).

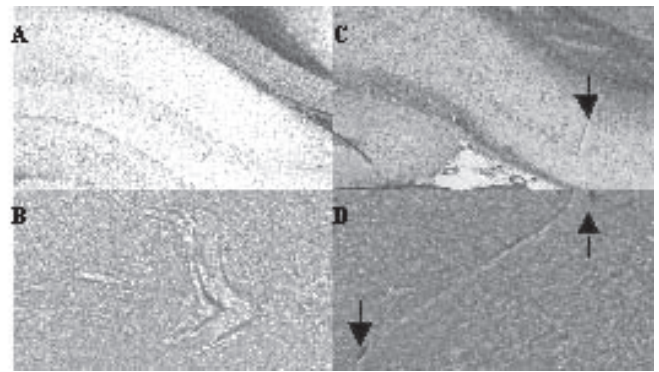


Fig. 3. Light microscopy showing extravasation of horseradish peroxidase (HRP) into the extracellular compartment 2 hours following lateral fluid percussion injury. HRP were infused 5 minutes post-surgery (for the sham-operated) and post-injury (for the injury group) via the femoral artery, and the rats were sacrificed 2 hours later. A-B show lower (50X) and higher (400X) magnification of the healthy rat brain section from the sham-operated group with no evidence of HRP extravasation. C — At lower magnification (50X), observations of localised HRP extravasation were found in the cortex and hippocampal region in the ipsilateral hemisphere of injured rat brain. D — At higher magnification of 400X, it can be seen that the HRP were phagocytosed by the microglial cells. The HRP were stained with DAB chromogen.

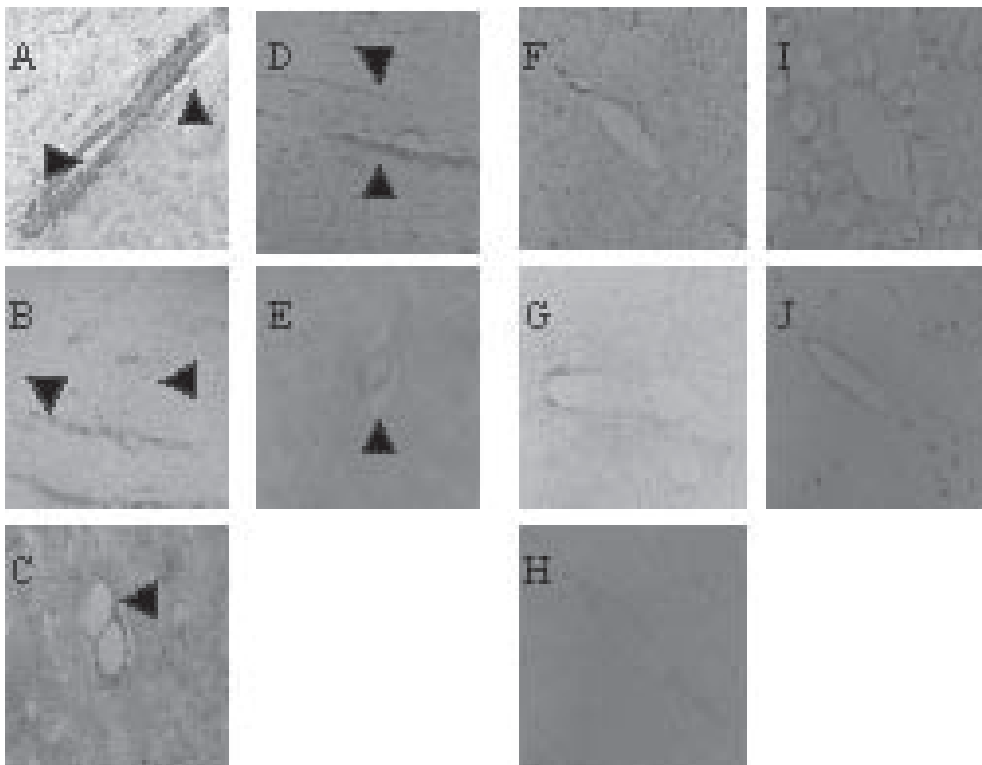


Fig. 2. Immunohistochemistry analysis of changes in the tight junction proteins morphology, the structural basis of the BBB, after lateral fluid percussion injury in rats. A-E (arrows) show the clear outline of tight junction proteins drawing individual endothelial cell's boundary with immunoreactive ZO-1, ZO-2, ZO-3, Claudin-1 and Occludin antibodies respectively. The lining of the endothelial cells of the blood vessel wall in the hippocampal region of the sham-operated rat brain were stained with DAB chromogen and observed at 400X magnification. F-J show the punctuated pattern of the outline that encircle each individual endothelial cells in the brain sections of rats 2 hours following lateral fluid percussion injury. Note especially the tight junction proteins in the paracellular pathway between 2 individual endothelial cells are compromised, evident with the absence of chromogen staining which were normally found in the healthy rat brain sections of the sham-operated group.

basic molecular pathology of the BBB disruption may lead to new avenues of delivering drugs into the CNS. For instance, vascular endothelial growth factor (VEGF) has been shown to reduce occludin expression. It is conceivable that blocking VEGF expression, which presumably is produced as a result of hypoxia, can lead to the preservation of the BBB in pathological states.²⁵

Conclusion

Following TBI, there is a punctuated expression of tight junction proteins that constitute the BBB. This appears to be associated with the development of vasogenic oedema that can contribute to ICP. Understanding the molecular mechanism of BBB breakdown may facilitate novel therapeutic intervention to preserve this barrier and prevent cerebral oedema.

Acknowledgement

This study was sponsored in part by the National Medical Research Council Grant NMRNC/05772/001.

REFERENCES

- Kniesel U, Wolburg H. Tight junctions of the blood-brain barrier. *Cell Mol Neurobiol* 2000; 20:57-76.
- Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, et al. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 1993; 123: 1777-88.
- Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J Cell Biol* 1998; 143:391-401.
- Tsukita S, Furuse M. Overcoming barriers in the study of tight junction functions: from occludin to claudin. *Genes Cells* 1998; 3:569-73.
- Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 1986; 103:755-66.
- Gumbiner B, Lowenkopf T, Apatira D. Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc Natl Acad Sci USA* 1991; 88:3460-4.
- Jesaitis LA, Goodenough DA. Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the *Drosophila* discs-large tumor suppressor protein. *J Cell Biol* 1994; 124:949-61.
- Haskins J, Gu L, Wittchen E, Hibbard J, Stevenson BR. ZO-3. A novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. *J Cell Biol* 1998; 141:199-208.
- Baethmann A, Lehr D, Wirth A. Prospective analysis of patient management in severe head injury. *Acta Neurochir Suppl (Wien)* 1998; 71:107-10.
- Durward QJ, Del Maestro RF, Amacher AL, Farrar JK. The influence of systemic arterial pressure and intracranial pressure on the development of cerebral vasogenic edema. *J Neurosurg* 1983; 59:803-9.
- Schulz J, Plesnila N, Eriskat J, Stoffel M, Pruneau D, Baethmann A. LF16-0687: a novel non-peptide bradykinin B2 receptor antagonist reduces vasogenic brain edema from a focal lesion in rats. *Acta Neurochir Suppl* 2000; 76:137-9.
- Barzo P, Marmarou A, Fatouros P, Corwin F, Dunbar J. Magnetic resonance imaging-monitored acute blood-brain barrier changes in experimental traumatic brain injury. *J Neurosurg* 1996; 85:1113-21.
- Beaumont A, Marmarou A, Hayasaka K, Barzo P, Fatouros P, Corwin F, et al. The permissive nature of blood brain barrier (BBB) opening in edema formation following traumatic brain injury. *Acta Neurochir Suppl* 2000; 76:125-9.
- Ikeda Y, Wang M, Nakazawa S. Simple quantitative evaluation of blood-brain barrier disruption in vasogenic brain edema. *Acta Neurochir Suppl* 1994; 60: 119-20.
- McIntosh TK, Vink R, Noble LJ, et al. Traumatic brain injury in the rat: characterization of lateral fluid percussion injury model. *Neuroscience* 1989; 28:233-44.
- Lighthall JW, Dixon CE, Anderson TE. Experimental models of brain injury. *J Neurotrauma* 1989; 6:83-97.
- Vaz R, Sarmento A, Borges N, Cruz C, Azevedo T. Experimental traumatic cerebral contusion: morphological study of brain microvessels and characterization of the oedema. *Acta Neurochir (Wien)* 1998; 140:76-81.
- Bralet J, Schreiber L, Bouvier C. Effect of acidosis and anoxia on iron delocalization from brain homogenates. *Biochem Pharmacol* 1992; 43:979-83.
- Hekmatpanah J, Helmapanah CR. Microvascular alterations following cerebral contusion in rats. Light, scanning, and electron microscope study. *J Neurosurg* 1985; 62:888-97.
- Brightman MW, Reese TS. Junctions between intimately apposed cell membranes in the vertebrate brain. *J Cell Biol* 1969; 40:648-77.
- Rascher G, Wolburg H. The tight junctions of the leptomeningeal blood-cerebrospinal fluid barrier during development. *J Hirnforsch* 1997; 38: 525-40.
- Ando-Akatsuka Y, Saitou M, Hirase T, Kishi M, Sakakibara A, Itoh M, et al. Interspecies diversity of the occludin sequence: cDNA cloning of human, mouse, dog, and rat-kangaroo homologues. *J Cell Biol* 1996; 133:43-7.
- Koenig H, Trout JJ, Goldstone AD, et al. Capillary NMDA receptors regulate blood brain barrier function and breakdown. *Brain Res* 1992; 588:297-303.
- Wahl M, Unterberg A, Baethmann A, et al. Mediators of blood brain barrier dysfunction and formation of vasogenic edema. *J Cereb Blood Flow Metab* 1998; 8:621-34.
- Wang W, Dentler WL, Borchardt RT. VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. *Am J Physiol Heart Circ Physiol* 2001; 280:H434-40.