行政院及所屬各機關出國報告

（出國類別：進　修）

高葡萄糖環境對視網膜血管內皮細胞及血管周邊細胞的Gap Junction之基因表現的影響—糖尿病視網膜病變的可能機轉

服務機關：台北榮民總醫

出國人：職　　稱：主治醫師

姓　　名：李安斐

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高葡萄糖環境對視網膜血管內皮細胞及血管周邊細胞的Gap Junction之基因表現的影響--糖尿病視網膜病變的可能機轉

主辦機關:

行政院輔導會臺北榮民總醫院

聯絡人／電話:

／

出國人員:

李安斐　行政院輔導會臺北榮民總醫院　眼科部　主治醫師

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關鍵詞: 高葡萄糖環境對視網膜血管內皮細胞及血管周邊細胞的Gap Junction之基因表現的影響--糖尿病視網膜病變的可能機轉

內容摘要: 職於出國期間學習利用Antisense 核苷酸來改變基因活性, 藉以探討糖尿病視網膜病變之機轉. 發現Antisense 核苷酸來抑制Gap junction Connexin 43之表現會導致細胞自殺(apoptosis)的機率. 因此在糖尿病的視網膜上,其血管周邊細胞(pericyte)及內皮細胞(endothelial cells)的Connexin 43 活性下降,有可能導致細胞環境恆定性的破壞, 影響兩種細胞間訊息的傳達. 因爲血管周邊細胞對內皮細胞的生長影響力甚大, 在兩種細胞間訊息的傳達不良的情況下, 內皮細胞及血管周邊細胞的生長可能異常, 使得視網膜微血管產生pecicyte消失及無細胞血管(ghost vessel) 產生. Antisense 核苷酸來改變基因活性也有治療疾病的潛力. 利用Antisense 核苷酸來抑制血管基底膜成分-Fibronectin, 可以導致血管通透性的改變, 可以抑制糖尿病視網膜病變中過多的血管通透性. 對於青光眼也可能有治療作用. 減少隅角小樑細胞的Fibronectin生成, 可能可以減少、房水排出的阻力,增加房水分泌來降低眼壓. 利用分子生物學的方法,特別在眼部局部而非全身的情況下, 較易得到治療效果而能減少全身性的副作用例如利用病毒載體或質體便可能有不表現或病毒繁殖過多的危險. 因此Antisense 核苷酸易施用於前房或玻璃體內,是未來疾病治療的明日之星

本文電子檔已上傳至出國報告資訊網

摘要:

職於出國期間學習利用 Antisense 核苷酸來改變基因活性，藉以探討糖尿病視網膜病變之機轉. 發現 Antisense 核苷酸來抑制 Gap junction Connexin 43 之表現會導致細胞自殺(apoptosis)的機率. 因此在糖尿病的視網膜上,其血管周邊細胞(pericyte)及內皮細胞(endothelial cells)的 Connexin 43 活性下降,有可能導致細胞環境恆定性的破壞，影響兩種細胞間訊息的傳達. 因爲血管周邊細胞對內皮細胞的生長影響力甚大，在兩種細胞間訊息的傳達不良的情況下,內皮細胞及血管周邊細胞的生長可能異常，使得視網膜微血管產生 pecicyte 消失及無細胞血管(ghost vessel) 產生.

Antisense 核苷酸來改變基因活性也有治療疾病的潛力. 利用 Antisense 核苷酸來抑制血管基底膜成分-Fibronectin，可以導致血管通透性的改變，可以抑制糖尿病視網膜病變中過多的血管通透性. 對於青光眼也可能有治療作用. 減少隅角小樑細胞的 Fibronectin 生成,可能可以減少、房水排出的阻力,增加房水分泌來降低眼壓. 利用分子生物學的方法,特別在眼部局部而非全身的情況下，較易得到治療效果而能減少全身性的副作用例如利用病毒載體或質體便可能有不表現或病毒繁殖過多的危險.

因此 Antisense 核苷酸易施用於前房或玻璃體內,是未來疾病

治療的明日之星.

目的:

糖尿病視網膜病變之早期血管病變爲視網膜血管內皮細胞及血管周邊細胞(pericyte)死亡,血管通透性增加進而造成血管阻塞,組織缺氧,以至於產生新生血管,造成眼內出血, 視網膜剝離等失明情況. 但是血管內皮細胞及血管周邊細胞死亡的原因仍不清楚.有研究顯示細胞自殺(apoptosis)可能爲其原因.

Gap junction 爲細胞間小分子交通的管道,由 Connexin 蛋白所組成.各種離子,ATP, 及其他重要訊號傳導因子可經由它往來於細胞間,是維持細胞環境恆定性的重要管道.先前之研究發現在高葡萄糖環境中血管內皮細胞之 gap junction 活性降低. 由於血管內皮細胞及血管周邊細胞緊密接觸,其間有 gap junction 存在,因此我們研究血管周邊細胞的 Gap junction

之基因表現是否受高葡萄糖環境抑制.

其次,爲了解降低的 gap junction 活性是否與細胞死亡增加有關,我們於血管內皮細胞上利用 antisense 核苷酸抑制 Connexin 43 蛋白,來分析細胞自殺死亡是否增加.期以增加對糖尿病視網膜病變機轉之了解.

方法(I)

1. 從牛眼視網膜分離血管周邊細胞,予以培養七天.實驗組之培養液加葡萄糖至 30 mM
2. 從細胞分離所有蛋白,以西方點墨法分析定量 connexin 43
3. 從細胞分離所有 RNA,以 RT-PCR 半定量分析 connexin 43 mRNA 的量
4. 細胞培養於載玻片上,利用螢光抗體染色法偵測 Cx43 蛋白之分布
5. 共同培養血管周邊細胞及血管內皮細胞利用螢光抗體染色法偵測兩種細胞間 Cx43 蛋白之分布.

(II)

1. 培養血管內皮細胞
2. 將 antisense Cx43 核苷酸植入血管內皮細胞, 24 小時後取出所有細胞分離所有蛋白,以西方點墨法分析定量 connexin43
3. 將 antisense Cx43 核苷酸植入血管內皮細胞, 24 小時後取出所有細胞分離小分子 DNA,作 DNA 階梯電泳
4. 平行實驗用螢光 TUNEL 染色法偵測正在進行 apoptosis 的細胞

結果:

(I) 血管周邊細胞培養於高葡萄糖環境中, 呈現 CX43

降低的特性. Cx43 蛋白的量及 mRNA 均較控制組低.

螢光抗體染色法偵測 Cx43 蛋白之分布, 不論是血管周邊細胞單獨培養或是與血管內皮細胞共同培養,螢光點(即 CX43 蛋白之所在)之數量皆減少.

(II) 血管內皮細胞以 antisense Cx43 核苷酸處理後其 Cx43 蛋白量降低,表示 antisense Cx43 核苷酸確有抑制 Cx43 的效果. 同時,DNA 電泳的結果與 TUNEL 染色之結果一致. 即降低 Cx43 的基因表現將導致細胞自殺(apoptosis)的機率.

討論:

在血管周邊細胞培養於高葡萄糖環境中,其 gap junction 活性下降,顯示於高葡萄糖環境中微血管環境恆定性遭到破壞. 由於糖尿病最主要的症候就是高血糖,因此在糖尿病視網膜的微血管中微血管環境恆定性可能遭到破壞. 至少血管周邊細胞間及血管周邊細胞與內皮細胞間之 gap junction 活性降低.此項變化有可能導致血管周邊細胞與內皮細胞生長速度的變化,而在臨床上產生血管病變. Gap junction 活性的異常有可能是視網膜血管細胞在糖尿病

的情況下所產生的最早的細胞變異之一,而演變成整個視網膜功能異常. 在了解視網膜血管細胞在糖尿病的情況下產生變異的分子機制後, 才能更進一步發展出預防或治療的對策.

附錄:

1. 美國視覺及光學會 2002 年發表之論文摘要

   High glucose inhibits Connexin 43 expression and GJIC activity in retinal pericyte.

2. 美國糖尿病學會 2002 年發表之論文摘要

   High glucose-induced Connexin43 downregulation and apoptosis in rat microvascular endothelial cells

3. 將於美國視覺及光學會 2003 年發表之論文摘要

   High glucose-induced fibronectin overexpression inhibits trabecular meshwork cell permeability

附錄 1-1

SUBMISSION STEPS

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Summary:
Control/Tracking Number: 02-A-2189-ARVO
Activity: Abstract
Current Date/Time: 12/7/2001 2:47:37 PM

High Glucose Inhibits Connexin 43 Expression and GJIC Activity in Retinal Pericytes.

A. Li[1], T. Sato[1], S. W. Hassan[1], R. Haimovici[2], S. Roy[1].
[1]*Ophthalmology, Boston University School of Medicine, Boston, MA;*
[2]*Gundersen Eye Center, Boston University Medical Ctr, Boston, MA;*

Purpose: To determine whether gap junction protein, connexin-43 (Cx43), plays a role in the maintenance of vascular homeostasis in diabetic retinopathy. This study examined whether high glucose condition alters the expression of Cx43 and gap junction intercellular communication (GJIC) activity in retinal pericytes. **Methods:** Western blot analysis was performed to determine Cx43 protein level in human and bovine retinal pericytes grown for 8 days in normal (5mM) or high (30mM) glucose medium. In parallel experiments performed with human retinal pericytes, GJIC activity was assessed using the scrape load dye transfer technique. **Results:** Western blot analysis showed Cx43 expression was reduced in bovine retinal pericytes (53.8±16.9% of control, p=0.01, n=4) and in human retinal pericytes (49.0±23% of control, p=0.02, n=4) grown in high glucose medium compared to cells grown in normal medium. The ability of the cells to transfer Lucifer yellow through gap junctions was also reduced in high glucose condition (49±25% of control, p=0.04, n=3). **Conclusion:** High glucose condition reduces Cx43 expression in retinal pericytes and inhibits GJIC activity. Maintenance of retinal vascular homeostasis through gap junctions may be disturbed by high glucose condition and result in pericyte dysfunction in diabetic

retinopathy.

A. Li, None; **T. Sato**, None; **S.W. Hassan**, None; **R. Haimovici**, None; **S. Roy**, None.

Other Data Collected:

Reviewing Code (Complete): 154 diabetic retinopathy: basic mechanisms - RC
Keyword (Complete): 388 diabetic retinopathy ; 340 cell-cell communication ; 614 vascular cells
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March 13, 2002

Sayon Roy
Ophthalmology
Boston University School of Medicine
715 Albany Street
Boston, MA 02118

Dear Dr. Roy:

I am pleased to inform you that your abstract, High Glucose-Induced Downregulation of Connexin-43 Expression and Apoptotic Endothelial Cell Loss in Diabetic Retinopathy, has been selected for Poster Presentation at the American Diabetes Association's 62nd Scientific Sessions June 14-18, 2002 in San Francisco, California. It will also be printed in the Scientific Sessions Abstract Book, the June supplement to *Diabetes*.

Your poster has been assigned presentation number 820 in the category, Complications, Ocular, and it will be displayed for one day (Satuday, Sunday or Monday) in the General Poster Session in the Exhibit Hall. Please refer to the attached schedule for your assigned day. **New this year**, there will be a Poster Session Reception each evening. Please plan to be at your poster on your assigned day during both the two-hour mid-day time period and the Poster Session Reception.

Since you will not receive this notification until after the pre-registration deadline, ADA will honor the pre-registration fee of $260 for Association members or $405 for non-members. You can register by contacting our registration and housing company at 866-268-0195 (U.S./Canada) or 972-349-5433 (International) or online at www.diabetes.org/am02. In order to receive the early discounted rate, you must mention you are an abstract author to have your name verified in the author database. Hotels are filling up quickly, so please register for the meeting and make your hotel reservations as soon as possible.

Enclosed are Poster Presentation Instructions and a Travel Grant Application. In the event you are unable to attend the meeting, it is your responsibility to assign a co-author to present your abstract. If you have questions, please contact the Kelly Kidwell at 703-299-5503 or abstracts@diabetes.org.

On behalf of the Scientific Sessions Planning Committee, I would like to thank you for your contribution to this year's meeting and your support of the American Diabetes Association.

Sincerely,

Linda Cann

Linda Cann, MSEd
Director, Professional Education

Enclosure

Title: High Glucose-Induced Connexin 43 Downregulation and Apoptosis in Rat Microvascular Endothelial Cells

Sayon Roy* and An-Fei Li. 1 Boston, MA, United States.

Abstract Body:
Loss of retinal vascular endothelial cells is a characteristic and early lesion of diabetic retinopathy. Breakdown of homeostasis is associated with apoptotic cell death, a mechanism by which endothelial cells are lost in diabetes. Recent studies indicate that high glucose reduces the expression of connexin 43 (Cx43), a gap junction protein, and disrupts vascular homeostasis in microvascular endothelial cells. Because Cx43 plays a critical role in maintenance of vascular homeostasis, we determined whether Cx43 expression and gap junction intercellular communication (GJIC) activity are altered in microvascular endothelial cells grown in high glucose or exposed to H2O2, an inducer of apoptosis. Rat microvascular endothelial cells (RMEC) were grown in normal (5mM), or high (30mM) glucose medium for seven days or in normal medium for six days and then exposed to 0.05mM H2O2 for 16 hours. Western blot analysis showed that Cx43 protein level was reduced in RMECs grown in high glucose medium compared to cells grown in normal medium (58% ± 30% of control, p=0.020), and in parallel experiments, RMECs grown in normal medium and then exposed to H2O2 also showed reduced Cx43 protein level (67 ± 14% of control, p=0.002). Scrape load dye transfer (SLDT) assay performed in cells grown in high glucose medium or cells exposed to H2O2 showed reduced GJIC activity compared to cells grown in normal medium (67 ± 11% of control, p=0.0002), and (64 ± 16% of control, p=0.001), respectively. Quantification of cell viability and apoptotic index based on acridine orange and ethidium bromide uptake showed higher number of cells with fragmented nuclei and apoptotic bodies in cultures grown in high glucose medium or exposed to H2O2 compared to cells grown in normal

medium (2.2-fold and 3.2-fold of control, respectively). Findings from this study indicate that high glucose-induced downregulation of Cx43 expression and inhibition of GJIC activity may disrupt cellular homeostasis and result in apoptosis of microvascular endothelial cells. Breakdown of homeostatic balance may play an early role in initiating apoptosis in high glucose cells.

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Association for Research in Vision and Ophthalmology, May 4 - 9, 2003, Ft. Lauderdale, Florida

Control/Tracking Number : 03-A-711-ARVO **Activity** :Abstract **Current Date/Time** : 12/6/2002 4:01:06 PM **High Glucose-Induced Fibronectin Overexpression Inhibits Trabecular Meshwork Cell Permeability** *A.-F.Li, A.Chen, S.Roy.* Ophthalmology, Boston University School of Medicine, Boston, MA. **Purpose**: To study whether high glucose-induced fibronectin (FN) overexpression plays a role in the cellular permeability of human trabecular meshwork (HTM) cells. **Methods**: HTM cells were grown in normal (5mM) or high glucose (30mM) medium for 10 days. In addition, two separate groups of HTM cells grown in high glucose medium for 7 days were transfected with FN antisense phosphorothioate oligonucleotides targeted against the translation initiation site of the FN transcript to modulate high glucose-induced fibronectin (FN) overexpression, or random phosphorothioate oligonucleotides. Cells treated with antisense or random oligos were analyzed 72 h after transfection. FN protein expression was assessed using Western blot analysis and immunofluorescense microscopy. In parallel, HTM cells were grown on polyester membrane inserts of 24 transwell plates in normal or high glucose medium. When cells reached confluency, in vitro permeability assay was performed by adding fluorescein (0.5 mg/ml) to the upper chamber and monitoring its passage into the lower chamber of the transwells. Presence of fluorescein in the lower chamber was determined by spectrophotometric readings performed at 60 minute time point. **Results**: Western blot analysis and immunofluorescence microscopy showed significant increase in FN expression in HTM cells grown in high glucose medium compared to cells grown in normal medium (127±14% of control, p=0.018; 118±11% of control, p=0.009, respectively). Permeability of fluorescein molecules in HTM cell monolayer decreased in cells grown in high glucose medium (87±9% of control, p=0.004). HTM cells transfected with antisense phosphorothioate oligonucleotide against FN showed significant reduction in FN expression to near normal level (94±15% of control, p=0.009) and an increase in permeability (98±7% of control, p=0.01); whereas, random oligonucleotide had no effect on FN expression or in vitro permeability. **Conclusion**: Excessive FN synthesis by trabecular meshwork cells may contribute to blockage in the outflow facility and the development of primary open angle glaucoma. **Commercial Relationship**: A. Li, None; **A. Chen**, None; **S. Roy**, None.

Reviewing Codes (Complete): 174 glaucoma: trabecular meshwork - GL **Keyword, Presentation and Grant ID (Complete)**:
Grant Identification : ADA
(1) : 602 trabecular meshwork
(3) : 504 outflow: trabecular meshwork
(2) : 403 extracellular matrix
Presentation Preference : Poster Only

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