國科會研究成果報告

計畫名稱:

The study on high-glucose induced apoptosis of human pericyte

高葡萄糖引致之人類周皮細胞凋亡之研究

計畫編號: **NSC88-2314-B-002-309**

執行期限: **87/8/1 - 88/7/31**

主持人:廖朝崧

執行機構:台大醫學院

報告日期:**88/10/31**

中文摘要

為了探討糖尿病性視網膜病變之可能機轉，我們研究高葡萄糖濃度對人類周皮細胞凋亡之影響。人類周皮細胞取自於人類大網的微細血管，以 collagenase 消化而得。周皮細胞以 M199 培養液加入 20%胎牛血清培養。試驗高葡萄糖時，葡萄糖濃度為 5.5mmol/l (1mg/ml)及 28mmol/l (5mg/ml)。此外，insulin (100μu/ml)之影響亦加以研究。細胞凋亡之測定利用顯微鏡觀察以及利用 Cell Death Detection ELISA PLUS Kit (Boehringer Mannheim)。

結果發現在正常葡萄糖濃度下(1mg/ml)周皮細胞仍有相當之細胞凋亡情形，培養 24 小時比 4 小時細胞凋亡量有意義之增加，但繼續培養至 72 小時，則凋亡並未繼續增加。高葡萄糖處理比正常葡萄糖處理在 4 小時即可見明顯之細胞凋亡情形。此一差異持續至 72 小時。很有趣的發現是，Insulin 之處理並未影響高葡萄糖導致的細胞凋亡，雖然數值上呈現減少細胞凋亡之趨勢。

總結之，取自人類大網微細血管之周皮細胞雖然會受高葡萄糖之作用而增加其生長，但其細胞凋亡亦增加，且在 4 小時之作用時已呈明顯表現，並持續至 72 小時。而 Insulin 並不能有效減少細胞凋亡之發生，雖然呈現有此一趨勢。這些發現或可能有助於糖尿病性視網膜病變的機轉及治療之研究。

Abstract

In order to investigate the possible mechanisms of diabetic retinopathy we studied the effects of high glucose on apoptosis of human pericyte. Pericyte was obtained from microvessel of human omentum by collagenase digestion. Pericyte was cultured in Medium 199 supplemented with 20% fetal bovine serum. To study the effects of high glucose, the concentration of glucose was adjusted to 5.5 mmol/l (1 mg/ml) or 28 mmol/l (5 mg/ml). The effects of insulin (100 μU/ml) were also studied. Apoptosis was detected by microscopy observation and mainly by using Cell Death Detection ELISA PLUS Kit (Boehringer Mannheim).

The results showed that even under culture with physiological glucose concentration, there was significant apoptosis. The apoptosis increased as incubation for 24 hours. But, as the incubation time extended further to 72 hours, the apoptosis showed no further increase. High glucose treatment increased apoptosis significantly at as early as 4 hours and persisted even up to 72 hours of incubation. It was interesting to note that insulin treatment demonstrated no definite salutary effects on preventing apoptosis induced by high glucose, although there appeared such a tendency.

It is concluded that pericyte derived from human omental microvasculature, although stimulated to grow at high glucose concentration, showed significant increase in apoptosis as early as 4 hours and persisted up to 72 hours. Insulin treatment was not effective in decreasing the apoptosis induced by high glucose concentration, although there was such a tendency. These findings may have some implications for the mechanisms and management of diabetic retinopathy.

Keywords: pericyte, apoptosis, diabetes mellitus, high glucose, retinopathy

BACKGROUND

Microcirculation plays important roles in physiological and pathological conditions. Microcirculation is constituted of metarteriole and capillary (1). Two types of cells, the endothelial cell and the pericyte, compose the microvasculature which is devoid of smooth muscle cells (2). Of these cells the pericyte is embedded in basement membrane on the outer surface of capillary while the endothelial cells are lining the inner side of the vessels.

Pericyte was first described by Rouget in 1873. Although known for more than a century, studies on pericyte are much limited, as comparing with that on endothelial cells. The origin of pericyte is not definitely determined yet. The function of pericyte is still not totally settled. Suggested functions for pericyte include: transformation into smooth muscle cell, dynamic control of microcirculation, phagocytosis and the regulation of neovascularization (3).

The studies on pericyte are hindered by the special location of pericyte in the microvascular wall which makes it difficult for isolation and culture. The lack of specific markers for its identification is also contributory. In the reported studies, pericyte is most frequently isolated and cultured from bovine retina (4). To our knowledge, pericyte isolated and cultured from human tissue has never been reported. In past 2 years we successfully developed a method to isolate and grow human pericyte from omentum and have studied the effects of high glucose and insulin and some cardiovascular drugs on the growth and collagen synthesis of human pericyte (with grants from NSC). We have identified the pericyte by excluding it from endothelial cell and smooth muscle cell by microscopic morphology and by immunofluorescence markers.

In diabetes mellitus, acellular capillaries and microaneurysm are important complications involving microvasculature. It has long been noted that in diabetic patients pericytes are selectively lost, especially in the retinal capillaries (5). The thickening of basement membrane of vascular bed is also a common finding in diabetes mellitus (6). The mechanisms for these changes are not clear.

The reduction of pericytes may play important pathogenetic roles for the proliferative changes of the retina vessels in diabetic retinopathy. Recently it has been reported that apoptosis can be induced by high glucose in human umbilical vein endothelial cells (7). Yet, in another report, apoptosis was demonstrated for cultured bovine retinal pericytes but not for endothelial cells when ambient glucose concentration is decreased abruptly (8). In this report we used the pericytes we cultured to study the high glucose induced apoptosis on these cells.

METHODS

A. Isolation and culture of human pericyte

The method was a modification of that of Wagner (9). Omentum obtained from patients undergoing abdominal operation was rinsed in chilled normal saline. Pieces of fat pad devoid of visible blood vessels, about 0.5 cm in diameter, were obtained by cutting with scissors. About 5 grams of fat tissue were used in each experiment. The fat tissue was first treated with 5 ml of 0.25% trypsin-EDTA (Gibco). The digestion proceeded for 30' at 37° C.

After digestion, the fat tissue was washed with 40 ml normal saline and then centrifuged at 3000 rpm for 10'. The fat tissue was then digested with 5 ml type II collagenase (0.75%, Worthington) at 37° C until almost digested, usually about 15'. The digestant was washed with 40 ml normal saline and the sediment was filtered through a stainless steel sieve (200 mm holes). The retained materials were then washed down and cultured in a 3.5-cm Petri dish. Culture medium was composed of Medium 199 supplemented with 20% fetal bovine serum, 20 mM HEPES, 5 U/ml heparin, 100 IU/ml penicillin and 0.1 mg/ml streptomycin (10).

The culture dish was observed every day. Contaminating fibroblast and omental mesothelial cells (11), smooth muscle cells and endothelial cells were carefully picked out with a sterile needle. At confluence, the cells were trypsinized and subcultured at 1:3 ratio. The pericytes of 3rd to 7th passage were used for experiments.

B. Identification of the pericyte

As previously reported (3,4), there are no specific markers for pericyte identification. Pericytes were identified by the characteristic morphology of large cells with irregular shape containing phase-dense fibers. They could be excluded from endothelial cells by applying immunofluorescence staining with antibody against von Willebrand factor for endothelial cells (12). Pericyte could be stained with antiactin antibodies.

C. Study procedure

I. Treatment of pericytes with high glucose concentration

1) Increased glucose concentration

The pericyte were cultured in M 199 medium supplemented with 20% FBS until near confluence. In this medium the glucose concentration is 1.0 mg/ml, a physiological concentration. For testing the effects of high glucose

concentration, the glucose concentration was increased to 28 mmol/l (5.0 mg/ml). This treatment was extended for up to 72 hr. At 4 hr, 24 hr and 72 hr, the cells were checked for apoptosis (see below).

2) Fluctuation of glucose concentration

As demonstrated by Li et al., fluctuation of glucose may play important role in inducing apoptosis (8). Yet, in their study only abrupt glucose reduction was investigated. In our experiments we proposed to treat the cells with repeated fluctuation of glucose concentrations between normal and high glucose. Yet, due to inadequate pericytes for experiments this procedure was not done.

3) Effects of insulin on apoptosis induced by high glucose

In the experiments mentioned above, the effects of insulin were tested by adding insulin to the culture medium at concentration of 100 mU/ml. At predetermined time points (4 h, 24 h, 72 h), apoptosis of the treated pericytes was checked.

4) Effects of cycloheximide on apoptosis of pericytes in high glucose concentration

Cycloheximide is an antibiotic which inhibits protein synthesis and nuclear division of karyotic cells. In Li's study, cycloheximide exerted a protective effect for apoptosis of pericytes (8) while in the study of Higuchi et al. (13), cycloheximide enhances apoptosis. To test the effects of cycloheximide on apoptosis of pericytes due to high glucose, cycloheximide (10 mg/ml) was proposed to be added to the culture medium containing either normal (5.5 mmol/l) or high glucose (28 mmol/l) and apoptosis was later checked. Yet, due to inadequate pericytes for experiments this procedure was not done.

II. The detection of apoptosis

1) Microscopic observation

The cultures were observed for the presence of typical changes of apoptosis - shrinkage of the cells with condensed nuclei. The percentage of apoptotic cells to total cells were calculated for 5 randomly selected fields (5).

2) Using Cell Death Detection ELISA PLUS Kit (Boehringer Mannheim)

(1) Pericytes were cultured in 6-well plates Cells culture in 6-well culture plate, 20 K cells/250 ml/well.
(2) At near-confluence, the cells were treated, in duplicate, with either normal (5.5 mmol/l) or high glucose (28 mmol/l) with or without insulin added.
(3) At 4h, 24 h and day 72 h, tests for apoptosis were carried out.
(4) In measuring apoptosis, the medium was first discarded. The cells were lysed by 250 ml lysis buffer by incubate for 30' at room temperature.

(5) After centrifugation at 200 g for 10', 20 μl of the supernatant (cytoplasmic fraction) with 80 μl of immunoreagent (containing anti-histone-biotin and anti-DNA-POD) were added to streptavidin-coated wells, and were shaked (500 rpm) for 2 h at room temperature.
(6) At complete reaction, substrate solution was applied, incubated for 10-20', measurement at 450 nm spectrophotometer.

RESULTS

1. Results of culturing pericytes

This year, the culture of pericytes was not smooth. It took a long time for the new assistant to be familiar with the procedures of experiments. Therefore, we did not get so frequent success in obtaining enough pericytes to complete all the experiments we intended to do.

All cells obtained showed typical appearance for pericytes. They were large flat cells with multiple processes. They overlapped each other frequently. They were easily differentiated from endothelial cells, smooth muscle cells and mesothelial cells by morphology.

2. Effects of high glucose on pericyte apoptosis

As shown in Table 1, when glucose concentration was normal, there was mild apoptosis when the treatment duration was 4 hours. At 24 hours of treatment with glucose of 5.5 mmol/l, apoptosis was significantly increased ($p<0.05$). This change was not evident at 72 hours of treatment. When glucose concentration increased from 5.5 mmol/l to 28 mmol/l, apoptosis was much increased at 4 hours ($p<0.005$) and at 72 hours ($p<0.01$), but only with tendency of increase ($p<0.1$) at 24 hours.

3. The effects of insulin on apoptosis induced by high glucose

The increased apoptosis induced by high glucose was decreased partially after adding insulin to the high glucose condition at 4 hours ($p<0.05$) and at 72 hours ($p<0.05$) but not at 24 hours . In comparing apoptosis in high glucose treatment with and without addition of insulin, there showed no difference at all 3 time points of 4 hour, 24 hour and 72 hour. The decreased of apoptosis induced by insulin addition seemed more prominent at 4 hours.

DISCUSSION

The prevalence of diabetes mellitus has been reported increasing in Taiwan. Diabetes mellitus is important for its serious complications which include coronary artery disease, neuropathy, nephropathy and proliferative retinopathy (14). Diabetes mellitus is the leading cause of blindness (15). Pericyte has been found to be markedly decreased in the retina of patients with diabetes mellitus (5). In diabetes, thickening of basement membrane of vascular bed is also a common finding (6). In this report we studied the effects of high glucose, with or without addition of insulin, on apoptosis of pericytes.

We found that apoptosis occurred even at physiological glucose concentration. The apoptosis increased as incubation extended to 24 hours but no further increase as observed up to 72 hours (Table 1). The high glucose would increase apoptosis as early as 4 hours (Table 1, Fig. 1). This effect persisted up to 72 hours with persistent increase in apoptosis (Table 1, Fig. 1).

It is a pity that we can not study on the fluctuation of glucose levels on apoptosis due to inadequate apoptosis available for use. From the results showing acute but persistent effects of high glucose, we suspect that fluctuation of glucose would exert more effects to induce more apoptosis. If this is the case, then strict sugar control would expect to render salutary effects for preventing or delaying retinal changes in chronic diabetic patients.

Although insulin supplement seemed to exert no significant effect on the apoptosis induced by high glucose ($p>0.05$), the data showed a tendency to decrease it (Table 1). This finding is out of our expectation. This observation may be due to inadequate number of experiments or due to higher variations among these experiments. From this results it is proposed that insulin may be not effective on the point of blindness prevention for diabetic patients. As mentioned, diabetic retinopathy is still neither preventable nor curable (15).

As previously observed, high glucose exerted a stimulating effect on the growth of pericyte, at the concentrations of 2, 4, and 8 mg/ml, up to 4 days of treatment (unpublished data). This observation is not conflicting to the findings from the present study in which pericyte growth was not measured but apoptosis was the parameter of measurement. Insulin was found to enhance the growth of pericyte in our previous study, especially at higher glucose concentrations. Yet, in the present study, insulin seemed not significantly reduced apoptosis induced by high glucose. These findings may be used to explain the fact that insulin does not improve retinopathy in chronic diabetics.

REFERENCES

1. Milnor WR. Cardiovascular Physiology. New York, Oxford University Press, 327-332, 1990.
2. Ross MH, Romrell LJ, Kaye GI. Histology. A Text and Atlas. 3rd Ed., Baltimore, Williams and Wilkins, 309-313, 1995.
3. Sims DE. The pericyte - A review. Tissue Cell 1986;18:153-74.
4. D'Amore PA. Culture and study of pericytes. In Piper HM (ed.): Cell Culture Techniques in Heart and Vessel Research. 1990:299-314, Springer-Verlag, Berlin.
5. Cogan DG, Toussaint D, Kuwabara T. Retinal vascular patterns. IV. Diabetic retinopathy. Arch Ophthalmol 1961;66:366-78.
6. Osterby R. Basement membrane morphology in diabetes mellitus. In: Diabetes Mellitus. Theory and Practice. Rifkin H, Porte D Jr, eds. New York, Elsvier, 220-33, 1990.
7. Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J, Gessel A, Waldhausl W. High-glucose-triggered apoptosis in cultured endothelial cells. Diabetes 1995;44:1323-7.
8. Li W, Liu X, Yanoff M, Ye X. Cultured retinal capillary pericytes die by apoptosis after an abrupt fluctuation from high to low glucose levels: a comparative study with retinal capillary endothelial cells. Diabetologia 1996; 39:537-47.
9. Wagner RC, Mathews MA. The isolation and culture of capillary endothelium from epididymal fat. Microvasc Res 1975;10:286-97.
10. Smirnov VN, Orekhov AN: Smooth muscle cell from adult human aorta. In Piper HM (ed.): Cell Culture Techniques in Heart and Vessel Research. Berlin, Springer-Verlag, 1990:271-289.
11. van Hinsbergh VWM, Kooistra T, Scheffer MA, an Bockel JH, van Muijen GNP. Characterization and fibrinolytic properties of human omental tissue mesothelial cells. Comparison with endothelial cells. Blood 1990;75:1490-7.
12. Jaffe EA, Nachman RL, Becker CG, et al. Culture of human endothelial cells derived from umbilical veins. J Clin Invest 1973;52:1745-56.
13. Higuchi M, Singh S, Aggarwal BB. Characterization of the apoptotic effects of human tumor necrosis factor: development of highly rapid and specific bioassay for human tumor necrosis factor and lymphotoxin using human target cells. J Immunolog Methods 1995;178:173-81.
14. Krolewski AS, Warram JH. Epidemiology of late complications of diabetes. In Kahn CR, Weir GC (eds.): Joslin's Diabetes Mellitus, 13rd Ed. Philadelphia, Lea & Febiger, 1994:605-619.
15. Aiello LM, Cavallerano JD. Occular complications of diabetes mellitus. In Kahn CR, Weir GC (eds.): Joslin's Diabetes Mellitus, 13rd Ed. Philadelphia, Lea & Febiger, 1994:771.

Table 1 Apoptosis of pericytes after treatment with high glucose

	Treatment Time (hours)		
	4 h	24 h	72 h
Glu-1	0.53±0.15	0.74±0.21	0.68±0.19
Glu-5	0.89±0.25	1.04±0.38	1.05±0.3
Glu-5-I	0.73±0.20	0.86±0.29	0.96±0.24

* Glu-1 = glucose 1 mg/ml (5.5 mmol/l); Glu-5 = glucose 5 mg/ml (28 mmol/l); Glu-5-I = glucose 5 mg/ml with insulin (100 μU/ml)
# OD for all data from a total of 9 duplicated experiments (mean±SD)

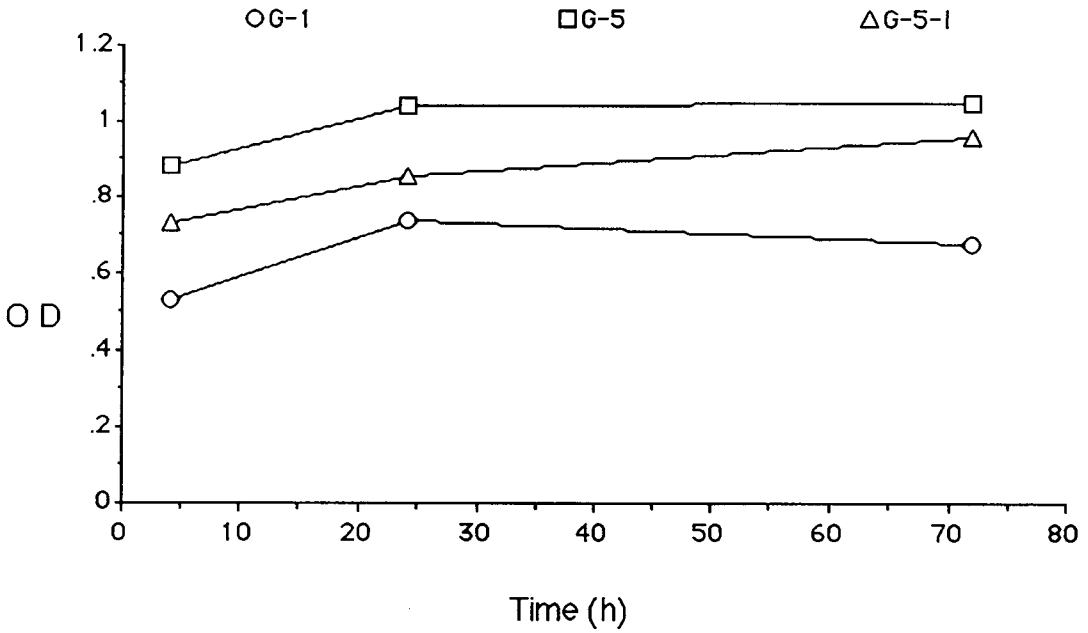

Fig. 1 Apoptosis of pericytes after treatment with high glucose
Glucose concentrations: G-1 = 1 mg/ml, G-5 = 5 mg/ml.
G-5-I = 5 mg/ml glucose with insulin (100 μU/ml)
Statistic data:
1) For G-1, 4 h vs. 24 h, $p<0.05$;
2) For G-5-I, 4h vs. 72 h, $p<0.05$;
3) For G-1 vs. G-5, at 4 h, $p<0.005$; at 24 h, $p<0.1$;
at 72 h, $p<0.01$;
4) For G-1 vs. G-5-I, at 4 h, $p<0.05$; at 72 h, $p<0.05$.