The Diabetic State Increases the Activity but Not the Number of Peritoneal Macrophages in the GK Rat Promoting the Tube Formation of Cultured Endothelial Cells in Rat Aorta

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The influence of the diabetic state on the number and activity of intraperitoneal macrophages was investigated in the GK rat. This rat is a model of non-insulin-dependent diabetes mellitus and was established from the normal Wistar rat. The cell number of a macrophages preparation in diabetic GK rats (10–15 weeks of age) did not differ from that in normal Wistar rats (9–10 weeks of age). The co-cultured macrophages in diabetic GK rats (9–12 weeks of age) significantly increased the tube formation of aortic endothelial cells (EC) compared with that in age-matched Wistar rats. The conditioned medium obtained from a culture of macrophages from GK rats also increased tube formation more than that from a culture of macrophages from Wistar rats. The effect of macrophages in the GK rat was not influenced by interferon- γ (160 and 460 pM), but macrophages in normal rats were activated by these concentrations of interferon- γ . In conclusion, the diabetic state increased the activity but not the number of macrophages in the GK rat, to stimulate tube formation of EC. The tube-forming effect of macrophages in the diabetic state may depend on released factors which differ from that of normal macrophages.

Key words peritoneal macrophage; non-insulin-dependent diabetes mellitus (NIDDM) model (GK rat); increased tube formation; vascular endothelial cell

The growth of capillary vessels is involved in the late developing proliferative lesions associated with complications of diabetes mellitus (DM) including retinopathy.¹⁾ Various factors in these proliferative lesions are suggested as being able to induce vessel growth.^{2,3)} However, the origin and activity of factors are not well understood in *in vivo* angiogenic models. Since the process of new capillary vessel formation consists of degradation of capillary basement membranes, migration, proliferation and tube formation of vascular endothelial cells (EC),⁴⁾ *in vitro* models for angiogenesis have focused on the responses of EC. We have previously reported that interferon (IFN)- γ -activated macrophages enhance tube formation of aortic EC cultured in type I collagen gel.⁵⁾ The effects caused by the activated macrophages are due to angiogenic factors released from the macrophages, but not direct adhesion of the macrophages to EC. The macrophages in the insulin-dependent DM (IDDM) model have been reported to induce autoimmune dysfunction of the islets of Langerhans.^{6,7)} However, there is no direct evidence that the macrophages in IDDM and non-IDDM (NIDDM) exhibit angiogenic activity.

The Goto-Kakizaki (GK) rat is a non-obese model of spontaneous NIDDM, produced by selective breeding of normal Wistar rats.⁸⁾ The GK rat shows mild hyperglycemia, impaired pancreatic function, insulin resistance and thickening of the glomerular capillary basement membranes.^{8,9)} The aim of the present study was to investigate the influence of the diabetic state in the GK rat on the number and activity of peritoneal macrophages involved in tube formation of cultured vascular EC during the angiogenic process.

MATERIALS AND METHODS

Animals Male diabetic GK rats (9–32 weeks of age) were bred in the Laboratory Animal Research Center in

our university under specific pathogen-free conditions. Normal male Wistar rats (9–30 weeks of age, Japan Shizuoka Laboratory Center, Hamamatsu) were used as controls. Male prediabetic (12–15 weeks of age) and diabetic KK-CA^y mice (18–44 weeks of age) were maintained in our laboratory. Blood samples were obtained from the orbital vein plexus of fed animals. The blood glucose levels of these animals were monitored by the glucose oxidase method using a glucose analyzer (Beckman Japan, Type II, Tokyo).

Preparation of Intraperitoneal Macrophages The number of intraperitoneal macrophages in GK and Wistar rats was determined as follows; Peritoneal lavage cells were collected, washed in Ca²⁺, Mg²⁺-free phosphate-buffered saline (PBS) and incubated for 3–4 h in plastic 35-mm dishes (Falcon 3001, Becton Dickinson, Oxnard, CA, U.S.A.) with RPMI 1640 (Nissui, Tokyo) containing 20% heat-inactivated fetal bovine serum (FBS, Bioproduct, Walkersville, MD, U.S.A.), 160 U/ml benzyl penicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika, Tokyo) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Adherent cells were washed gently with PBS, detached using 0.05% trypsin–0.02% EDTA in PBS and counted in a hemocytometer. The adherent cells were used as macrophage preparations. The percentage (means $\pm$ S.E.) of macrophage preparations in Wistar (9–10 weeks of age) and GK rats (10–15 weeks of age) were $44.3 \pm 5.8\%$ ($n=4$) and $42.2 \pm 9.0\%$ ($n=5$) of total lavage cells, respectively. The macrophages in peritoneal lavage solution (2.5 ml) in KK-CA^y mice were determined by non-specific esterase staining with α -naphthyl butyrate.¹⁰⁾ Their number as a percentage in the prediabetic (12–15 weeks of age) and diabetic KK-CA^y mice (18–44 weeks of age) were $50.7 \pm 0.8\%$ ($n=8$) and $48.6 \pm 1.0\%$ ($n=21$) of total lavage cells, respectively. These percentages of macrophages were similar to that in mouse peritoneal cells.¹¹⁾

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Tube Formation by Cultured Aortic Endothelial Cells
Primary cultured EC from the thoracic aorta of Wistar rats (9 weeks of age) were prepared as reported.^{5,12} EC were cloned from 100–200 primary cells in Dulbecco's modified Eagle medium (DMEM, Nissui) supplemented with 10% FBS, benzyl penicillin potassium and streptomycin sulfate on 0.03% type I collagen (1-AC, Koken, Tokyo)-coated 16-mm dishes (Corning, Corning, NY, U.S.A.). The EC (16–30th passages) were cultured 2–6 weeks after confluence in 10% FBS-DMEM without antibiotics under 5% CO₂ and 95% air. The post confluent cells were washed with PBS and 0.02% EDTA in PBS, detached by 0.25% trypsin–0.02% EDTA in PBS and harvested in 10% FBS-DMEM without antibiotics. The EC $[(3.3 \pm 0.1) \times 10^4/16\text{-mm dish}]$ were cultured in 10% FBS-DMEM at 37°C in an atmosphere of 5% CO₂ and 95% air for 20–24 h on a 0.15% type I collagen gel in a 16-mm dish, and then overlaid with the same concentration of collagen gel. The peritoneal lavage cells $[(6.4 \pm 0.03) \times 10^4 \text{ cells}/16\text{-mm dish}]$ from GK and Wistar rats (9–12 weeks of age) were plated on the overlaid collagen gel for 3 h with 2% FBS-DMEM, washed with PBS, and co-cultured for day 0 (4–5 h), 2, 4 and 6 in 2% FBS-DMEM with or without recombinant murine IFN- γ (4.6×10^6 units/mg protein, Genentech, San Francisco, CA). In some experiments, the lavage cells $[(6.0 \pm 0.1) \times 10^5 \text{ cells}/16\text{-mm dish}]$ of both rats were plated for 3 h, washed, and cultured in 2% FBS-DMEM with or without IFN- γ for 4 d. Medium conditioned during culture of macrophages was centrifuged and filtered through a 0.3 μm Millipore filter (Nihon Millipore Kogyo, Yonezawa) and stored at –30°C. The conditioned medium (CM, 50 μl) was incubated in the EC-cultured medium (0.45 ml) for 4 d. The medium was changed every other day.

Measurement of Tube Formation Tubes that developed from EC were photographed with a Leitz Diavert camera equipped with a Wild Photoautomat MPS45 (Leitz, Germany). Four fields selected randomly in each 16-mm dish were photographed at $\times 36$. Typical photographs of such tubes were presented in a previous paper.⁵ These experiments were repeated at least 3 times. The lengths of all tubes longer than 94 μm were measured and summed with Graphic Software MEAS I (Graphtec Corp., Tokyo) as an index of tube formation.⁵

Statistical Analysis Data were expressed as means $\pm$ S.E. and tested by one-way analysis of variance. The significance of any differences was assessed by Scheffé's or Tukey's test at $p=0.05$ or 0.01 .

RESULTS

Comparison of the Number of Peritoneal Macrophage Preparations in GK and Wistar Rats The number of cells in peritoneal macrophage preparations in the diabetic GK rat was compared with that in the normal Wistar rat. The number of macrophages in GK rats (10–15 weeks of age) did not differ from those in normal Wistar rats at 9–10 weeks of age, but the blood glucose levels in the GK rats were significantly higher than in Wistar rats (Table 1). At 31–32 weeks of age, the number of macrophages in GK

Table 1. Number of Cells in Intraperitoneal Macrophage Preparations in Diabetic GK and Normal Wistar Rats

Age (weeks)	Blood glucose level (mg/dl)	Body weight (g)	Cell number of macrophage preparations (× 10 ⁶)	
			(/rat)	(/100 g body weight)
GK				
10—15	224 ± 9 ^{b)}	313.4 ± 8.8	8.76 ± 0.28	2.80 ± 0.05
20	225 ± 13	373.7 ± 4.7	9.26 ± 0.76	2.48 ± 0.19
31—32	257 ± 13 ^{b)}	371.1 ± 8.6 ^{b)}	6.44 ± 0.39 ^{a)}	1.74 ± 0.09
Wistar				
9—10	103 ± 6	325.4 ± 12.2	9.48 ± 0.34	2.91 ± 0.08
30	81 ± 7	599.6 ± 19.2	11.74 ± 1.34	1.96 ± 0.21

Values represent the means $\pm$ S.E. of 3–4 rats. a) $p < 0.05$, b) $p < 0.01$: significantly different from the corresponding values in Wistar rats.

Table 2. Number of Cells in Intraperitoneal Macrophage Preparations in Prediabetic and Diabetic KK-CA^y Mice

Age (weeks)	Blood glucose level (mg/dl)	Body weight (g)	Cell number of macrophage preparations ($\times 10^6/\text{ml}$)
15	132 $\pm$ 8	30.3 $\pm$ 1.4	2.30 $\pm$ 0.11
18	203 $\pm$ 16	37.8 $\pm$ 1.2	2.30 $\pm$ 0.05
24	467 $\pm$ 27	39.0 $\pm$ 1.8	2.03 $\pm$ 0.05
33	487 $\pm$ 45	36.5 $\pm$ 3.8	2.09 $\pm$ 0.08
44	439 $\pm$ 28	37.5 $\pm$ 1.5	2.69 $\pm$ 0.09

Non-specific esterase-positive cells were measured as macrophages in peritoneal lavage solution. Values represent the means $\pm$ S.E. of 4–8 mice.

rats was significantly lower than in Wistar rats at 30 weeks of age. The body weights of GK rats were also significantly less than those of Wistar rats. However, the number of macrophages per 100 g GK rat at these ages did not differ from the number per 100 g Wistar rat at a corresponding age. These results demonstrated that the number of macrophages was not influenced by the diabetic state in the GK rat at 10–15 weeks of age.

The number of peritoneal macrophages in diabetic KK-CA^y mice (18–44 weeks of age), another diabetic model of NIDDM,¹³ was compared with that in prediabetic mice (15 weeks of age). The blood glucose levels of KK-CA^y mice increased from 18 weeks of age, but the number of macrophages did not differ from that in prediabetic mice (Table 2). These results also demonstrated that the high blood glucose levels did not lead to an increase in the number of peritoneal macrophages in these diabetic models.

Activity of Peritoneal Macrophage Preparations in the GK Rat with Respect to Tube Formation of Aortic Endothelial Cells The effect of co-cultured peritoneal macrophage preparations isolated from the lavage cells $[(6.4 \pm 0.03) \times 10^4 \text{ cells}/16\text{-mm dish}]$ in GK rats (9–12 weeks of age) was compared with that in age-matched Wistar rats in terms of tube formation of EC. The co-cultured macrophage preparations in GK rats significantly increased tube formation from day 4 to 6 in culture to a greater extent than that in age-matched Wistar rats (Fig. 1). These results demonstrate that the peritoneal

Fig. 1. Time-Dependent Effects of Co-cultured Peritoneal Macrophage Preparations in GK Rats on Tube Formation from Aortic EC

The EC were co-cultured in type I collagen gel for 0 (4–5 h), 2, 4 and 6 d with macrophage preparations in diabetic GK (●) and normal Wistar (○) rats at 9–12 weeks of age. Differences in the total tubular length from 0.99 ± 0.33 ($n=6$; day 0), 3.03 ± 0.06 ($n=10$; day 2), 3.05 ± 0.07 ($n=25$; day 4) and 3.08 ± 0.07 mm/mm² dish ($n=16$; day 6) with 2% FBS alone are expressed as the means $\pm$ S.E. of the average of 4 analyses ($n=5-7$). a) $p < 0.01$: significantly different from macrophages in the normal Wistar rat on the same day.

Fig. 2. Effects on Tube Formation of Co-cultured IFN- γ -Treated Macrophage Preparations and CM Derived from IFN- γ -Treated Macrophage Preparations in GK Rats

Left: The EC in gel were co-cultured with macrophage preparations in GK (●) and Wistar rats at 9–12 weeks of age (○) with 0, 46, 160 and 460 pM IFN- γ for 4 d. Right: the CM (10-fold dilution) derived from the macrophage preparations in GK (●) and Wistar rats at 9–12 weeks of age (○), precultured with or without IFN- γ for 4 d, were exposed to EC culture for 4 d. Differences in total tubular length from the value with 2% FBS alone (data shown in the legend to Fig. 1) are expressed as the means $\pm$ S.E. of the average of 4 analyses ($n=3-6$). a) $p < 0.01$: significantly different from macrophages in the normal Wistar rat. b) $p < 0.01$: significantly different from that without IFN- γ .

macrophages in diabetic GK rats enhance tube formation of EC during the angiogenic process.

Effects of Interferon- γ on Macrophage Preparations in the GK Rat with Respect to Tube Formation We have previously reported that IFN- γ releases some factors from macrophages in normal Wistar rats to enhance tube formation.⁵⁾ To confirm that the tube-forming effect of co-cultured macrophages in GK rats is due to factors released from macrophages, the effects on tube formation

of CM obtained from the macrophage preparations isolated from lavage cells [$(6.0 \pm 0.1) \times 10^5$ cells/16-mm dish] in GK rats (9–12 weeks of age) were compared with those in age-matched Wistar rats. The CM (10-fold dilution) obtained from diabetic macrophage preparations cultured for 4 d significantly increased tube formation to a greater extent than that from normal macrophages (Fig. 2, right). These results support the hypothesis that macrophages in the diabetic GK rat enhance tube formation by releasing some angiogenic factors.

IFN- γ (160 and 460 pM) did not affect the tube-forming activity of co-cultured macrophages in GK rats as far as tube formation was concerned (Fig. 2, left). The tube-forming effects of CM derived from IFN- γ (160 and 460 pM)-treated diabetic macrophages were also similar to those from untreated diabetic macrophages (Fig. 2, right). These results demonstrate that treated IFN- γ does not influence the promoting effect of macrophages in the diabetic GK rat as far as tube formation is concerned.

DISCUSSION

One of the primary causal factors for the development of diabetic microangiopathy, including retinopathy, is a prolonged hyperglycemic state.^{14,15)} The present study demonstrates that higher levels of blood glucose in the diabetic GK rats activate intraperitoneal macrophages to increase tube formation of aortic EC during the angiogenic process. The tube-forming effect of diabetic macrophages is due to abnormal release of some angiogenic factors, but not to an increase in the number of macrophages. This result is supported by an evidence that the number of peritoneal macrophages in diabetic KK-CA^y mice, another model of NIDDM,¹³⁾ is not increased in the diabetic state.

The mechanism of diabetes-induced angiogenesis in microangiopathy is unclear. In the present assay, aortic EC were used for the assay of tube formation in the angiogenic process, because both EC derived from capillary and arterial vessels form similar capillary-like tubes in collagen gel.^{16,17)} Chronic hyperglycemia is suggested to lead to the accumulation of non-enzymatically advanced glycosylation endproducts (AGEs) on proteins.¹⁸⁾ AGEs bind to their receptors on monocytes and macrophages to release a number of cytokines.¹⁸⁾ These findings suggest two possible mechanisms of macrophage activation. One may be a direct activation of hyperglycemia and/or AGEs on macrophages. The other may be their modification of the genetic information of the diabetic macrophages, although the major histocompatibility complex antigens were not determined in the GK rat. The elevated glucose-induced activation of macrophages may be one of the causes of microangiopathy.

Vlassara *et al.* have suggested that IFN- γ produced by AGE-activated T cells may enhance AGE-induced activity of monocytes.¹⁸⁾ Our results demonstrate that IFN- γ (160 and 460 pM) does not influence the tube-forming activity of macrophages in the diabetic GK rat. Two possibilities could account for the apparently deficient action of IFN- γ on macrophages in the GK rat: firstly, the action of IFN- γ may be down-regulated by some factors produced by macrophages in the diabetic state;

secondly, since IFN- γ production may be at a maximum by inflammatory cells in the diabetic GK rat *in vivo* and by diabetic macrophages *in vitro*, IFN- γ treatment will not affect the activity of diabetic macrophages. The sensitivity of treated IFN- γ on macrophages in the diabetic GK rat is clearly different from that in normal macrophages.⁵⁾ These results suggest that the diabetic state of the GK rat in the NIDDM model modifies the activity of macrophages in terms of tube formation in angiogenesis.

In conclusion, the diabetic state in the GK rat increases the activity but not the number of peritoneal macrophages to promote tube formation of aortic EC in the angiogenic process. The tube-forming effect of macrophages in the diabetic state may depend on the release of angiogenic factors, and differs from that of normal macrophages.

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