Angiogenic sprouting from the aortic vascular wall is impaired in the BB rat model of autoimmune diabetes

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Abstract

**Background.** Diabetes is associated with impaired neovascularization leading to reduced revascularization of ischemic tissue and impaired wound healing. Neovascularization is a complex process, involving resident endothelial cells as well as circulating endothelial progenitor cells. Endothelial progenitor cells in diabetes were previously shown to be numerically reduced and functionally impaired. We hypothesize that diabetes also has a long-term effect on angiogenic cells residing in the vessel wall. To test this hypothesis, angiogenic sprout formation from *ex vivo* cultured aortic rings isolated from diabetic and non-diabetic BioBreeding (BB) rats was assessed.

**Methods.** Diabetes prone BB (BBDP) rats spontaneously develop autoimmune diabetes and were suboptimally treated with insulin by subcutaneous implantation of slow-release insulin-pellets. Neonatally thymectomized BBDP rats, pre-diabetic BBDP rats and diabetes resistant BBDR rats served as non-diabetic controls. After follow-up thoracic aortas were harvested and cultured *in vitro* in Matrigel to induce sprout formation. Sprout length was quantified after 4, 7, 10 and 14 days of culture. The total number of sprout-derived cells was measured and *in vitro* proliferative capacity of sprout cells was quantified. Finally, expression of Flk-1, CD31 and smooth muscle α-actin on sprout cells was determined.

**Results.** Mean blood glucose levels in diabetics was significantly elevated compared with non-diabetics. Both long-term and short-term diabetes significantly reduced sprout formation (p<0.05 vs. non-diabetics). Reduced sprout length in diabetics was reflected by significantly reduced numbers of sprout cells that could be isolated (p<0.05 vs. non-diabetics). Isolated sprout cells from diabetics revealed significantly reduced proliferative capacity after *in vitro* culture (p<0.05 vs. non-diabetics). Immunofluorescent staining indicated an endothelial phenotype of both freshly isolated and *in vitro* cultured sprout cells as indicated by CD31 and Flk-1 expression and absence of smooth muscle α-actin expression.

**Conclusions.** Diabetes in BB rats impairs angiogenic sprouting from cells residing in the vascular wall, independent of effects on circulating cells or circulating angiogenic/anti-angiogenic factors. The angiogenic impairment of diabetic sprout cells is, to some extent, imprinted upon the cells.
Introduction

Diabetes mellitus is associated with impaired neovascularization leading to reduced revascularization of ischemic tissue, impaired wound healing, embryonic vasculopathy, and organ transplant rejection in diabetic patients. On the other hand, pathologically enhanced neovascularisation is also observed in diabetes, contributing to diabetic retinopathy, diabetic nephropathy, and possibly atherosclerotic plaque destabilization. Neovascularization is a complex process, involving growth factors, cytokines, and both resident endothelial cells as well as circulating cells. Circulating factors in the diabetic milieu directly influence neovascularization. Hyperglycemia decreases endothelial cell proliferation in vitro and myocardial interstitial fluid from dogs with experimental diabetes impairs angiogenic tube formation by cultured endothelial cells. The diabetic milieu also influences circulating cells. Monocytes from diabetic patients respond poorly to angiogenic chemotactic factor VEGF-A and circulating angiogenic endothelial progenitor cells were previously shown to be numerically reduced and functionally impaired in diabetes.

It is currently unclear what the effect of diabetes is on angiogenic cells residing in the vessel wall, which are potent contributors to neovascularization. We hypothesized that diabetes would attenuate their angiogenic capacity and therefore assessed angiogenic sprout formation from ex vivo cultured aorta rings isolated from diabetic and non-diabetic diabetes-prone BioBreeding (BBDP) rats. We furthermore determined the phenotype of the outgrowing sprout cells. This sprouting-assay provides a tool to specifically study the long-term effect of diabetes on the angiogenic capacity of cells residing in the vessel wall, thereby excluding the direct influence of both circulating factors and circulating cells.

Material and Methods

Diabetes Prone BioBreeding BBDP rats

Both male and female BBDP/Wor rats were used. BBDP rats spontaneously develop autoimmune diabetes due to the absence of regulatory T cells and are used to model human type 1 diabetes. Age-matched neonatally thymectomized BBDP rats and pre-diabetic BBDP rats served as non-diabetic controls. All animal use was in accordance with the guidelines of the "Principles of laboratory animal care" and the Animal Ethics Committee of the University Medical Center Groningen. In this study, two experimental groups were included. Group 1 (long-term group) had a long diabetic course (~45 wks diabetes) and consisted of 15 diabetic BBDP rats and 8 age-matched neonatally thymectomized non-diabetic BBDP rats. Group 2 (short-term group) had a relatively short diabetes duration and consisted of 8 diabetic BBDP rats (~6 wks diabetes; ~17 wks of age) and 7 pre-diabetic BBDP rats (8 wks of age). Diabetic rats were suboptimally treated with insulin by subcutaneous implantation of slow-release Linplant insulin-pellets (LinShin, Scarborough, Canada).
Aortic ring sprouting assay

Aortas were harvested from the diabetic and non-diabetic BBDP rats under sterile conditions and flushed with saline to remove residual blood. Then ~1mm (group 1) and 0.65mm (group 2) thick aortic rings were transversally cut manually or using an automatic tissue chopper (McIlwain Tissue Chopper, The Mickle Laboratory Engineering Co. LDT., Gomshall, Surrey, England), respectively. Aortic rings were centrally positioned in the wells of 96-well tissue culture plates containing cold liquefied BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Alphen aan den Rijn, The Netherlands), and overlaid with EC medium (RPMI1640 medium containing 2 mM L-glutamine, 50 µg/ml endothelial cell growth factor (ECGF), 5 units/ml heparin, 100 U/ml penicillin, 100 µg/ml streptomycin and 20% fetal calf serum). Cultures were performed in duplicate (group 1) or triplicate (group 2). Plates were then placed at 37°C 5% CO₂, allowing the matrix solution to solidify. Sprout length in the long-term diabetic group (group 1) was measured after 10 days of culture. In the short-term diabetic group (group 2), sprout length was measured after 4, 7, 10 and 14 days of culture in order to evaluate kinetics of sprout formation, with having medium replenished at the moment of measurements. Sprout length was quantified (expressed in arbitrary units) as mean maximal sprout length from the perimeter of the aortic ring to the most distal tip of the angiogenic sprout in four quadrants of each aortic ring under an inverted microscope at 40-fold magnification using a microscopic grid.

Sprout cell isolation

To evaluate the absolute number of sprout cells, sprouts growing in Matrigel™ from the short-term diabetics and pre-diabetics (group 2) were isolated and counted. Medium and aorta rings were removed from the Matrigel™ cultures after which remaining sprouts and Matrigel™ were incubated with dispase (BD, Alphen aan den Rijn, The Netherlands, 2.5 caseinolytic units/well) at 37°C, 5% CO₂ for 2 hours to ensure complete dissociation. Cultures were performed in triplicate and after enzymatic digestion the total solution obtained from triplicate cultures was diluted in 10 ml PBS. Absolute numbers and viability of isolated sprout cells were determined using a haemocytometer and Trypan Blue exclusion. Viability was consistently >98% (not shown). Cell counting was performed after 4, 7, 10 and 14 days of culture in Matrigel™. Cells isolated after 10 days of culture in Matrigel™ were either replated in fresh EC medium for continued cell culture (Evaluation of sprout cell proliferative capacity: described below) or resuspended in PBS + 2.5% BSA for cytospots. Cytospots were prepared by spinning a total of 5x10⁴ isolated cells onto glass-slides (5 min., 550 rpm) using a Shandon Cytospin 4 (Thermo Fisher Scientific).

Evaluation of sprout cell proliferative capacity

To determine the proliferative status of sprout cells, cytospots (5x10⁴ cells/spot) of cells isolated after 10 days of culture in Matrigel™ were immunostained for Ki67, a marker which is expressed during all active phases of cell replication but not in resting cells. Cytospots were acetone-fixed for 12 min and dried for 1 hr at room temperature (RT). Blockade of endogenous peroxidase (0.03% H₂O₂ in PBS for 10 min) was followed by
incubation (1 hr, RT) with α-Ki67 monoclonal antibody (DakoCytomation Denmark A/S, Glostrup, Denmark). Subsequently, cytopsots were incubated (30 min, RT) with horseradish peroxidase-conjugated rabbit-α-mouse antibody (DakoCytomation Denmark A/S, Glostrup, Denmark) followed by visualisation with diaminobenzidine (DAB, Sigma-Aldrich, Zwijndrecht, The Netherlands). Cells were counterstained with hematoxylin and coverslipped in Depex mounting medium. All Ki67+ cells per cytopspot (5x10^4 cells) were counted.

To further evaluate the proliferative capacity of isolated sprout cells after replating, 2x10^4 cells/well were seeded in EC medium in 6-well tissue culture plates that were precoated (1 hr) with 1 ml/well Matrigel” (1 mg/ml). Cells were cultured for 96 hrs (4 days) after which they were trypsinized and counted using a haemocytometer and Trypan Blue exclusion. In some experiments cells were cultured in Matrigel”-coated 8-wells chamberslides (Lab-Tek”, Nunc, VWR International B.V., Amsterdam, The Netherlands) for phenotypic analysis.

**Phenotypic analysis of sprout cells**

To demonstrate an EC-phenotype of isolated sprout cells, cytopsots were stained using the following antibodies: α-CD31 (clone TLD-3A12, mlG1, BD Pharmingen), α-Flk-1 (clone A-3, mlgG1, Santa Cruz Biotechnology), mAb HIS43” (own produced hybridoma tissue culture supernatant, mlgG1) and α-alpha-SMA (vascular smooth muscle cells clone 1A4, mlgG2a, DakoCytomation A/S). After aceton fixation (12 min, -20°C) cytopsots were incubated with primary monoclonal antibodies (1 hr, RT). Cytopsots were then incubated (30 min, RT) with TRITC-conjugated goat-α-mouse IgG1 and FITC-conjugated goat-α-mouse IgG2a antibodies (both from Southern Biotechnology Associates, Birmingham, Alabama, USA) diluted in PBS + 3% normal rat serum. After nuclear staining with DAPI cytopsots were embedded in Citifluor. Cytopsots were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems Nederland B.V., Rijswijk, The Netherlands).

**Statistical analysis**

Data are expressed as mean±SEM and were analyzed using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, USA) using a two-tailed Students’ t-test. A p-value of <0.05 was considered statistically significant.

**Results**

**Diabetes onset and glycaemic control**

Non-thymectomized BBDP rats developed diabetes with a median onset of diabetes at the age 84 days (group 1; long-term) and 75 days (group 2; short-term), respectively. Age-matched control thymectomized BBDP rats did not become diabetic. In group 1 mean follow-up time was 324 and 386 days for the diabetic and thymectomized non-diabetic BBDP rats, respectively. Mean blood glucose level after long-term diabetes was 15.1±0.3 mmol/L versus 5.3±0.1 mmol/L in thymectomized non-diabetic controls (Figure 1A, p<0.0001). In group 2 mean follow-up time was 120 and 56 days for the short-term diabetic
and pre-diabetic BBDP rats, respectively. Mean blood glucose level after diabetes onset was 20.8±1.0 mmol/L versus 7.4±0.3 mmol/L in pre-diabetic controls.

Figure 1. Long-term diabetes (~45 wks) impairs aortic sprouting. (A) Mean blood glucose levels in group 1: long-term diabetics (DM, n=15) and age-matched thymectomized non-diabetic controls (no DM, n=8). (**p<0.0001) (B) Quantification of the relative sprout length from aortic rings revealed significantly reduced (*p<0.05) sprout formation in vitro after exposure to hyperglycemic conditions in diabetic BBDP rats in vivo compared to age-matched thymectomized non-diabetic control BBDP rats.

Angiogenic sprouting from aorta rings is impaired in diabetic animals

In both diabetic and non-diabetic BBDP rats we observed sprouts starting to emerge from the vascular wall and growing outward after 4 days of culture in Matrigel™. All rings generated sprouts of comparable morphology although sprout length and sprout density was reduced in the diabetic rats. In group 1 (long-term group) maximal sprout length in aortic rings measured at day 10 was significantly lower in diabetic rats than those from thymectomized non-diabetic controls (0.94±0.06 vs. 0.77±0.03, p<0.05, Figure 1B). In order to determine the kinetics of sprout development, sprout length was determined in the short-term diabetics and pre-diabetic BBDP rats at 4, 7, 10 and 14 days after culture in Matrigel™. Figure 2A shows representative photomicrographs depicting reduced sprout formation in rings from diabetic rats (right panel) compared with rings from pre-diabetic rats (left panel). Quantitative analysis revealed reduced sprout length in diabetic rats at 4, 7 and 10 days after culture in Matrigel™ reaching the level of statistical significance at day 4 (** p<0.01) and day 10 (* p<0.05) compared with pre-diabetic rats (Figure 2B). After 14 days of culture in Matrigel™ sprouts had reached the border of the well, and thereby maximal sprout length, in rings from both diabetic and pre-diabetic rats.

Our microscopic analysis of developing sprouts suggested reduced sprout density in rings obtained from diabetic rats. Reduced sprout length and sprout density is anticipated to result in the presence of decreased numbers of sprout cells in diabetic rats compared with pre-diabetic rats. To test this assumption we determined the absolute number of cells that could be isolated from sprouts that had grown out of aortic rings from short-term diabetic and pre-diabetic BBDP rats. Cells were isolated by enzymatic digestion using dispase followed by counting using a haemacytometer. As shown in Figure 2C absolute numbers of cells isolated from sprouts from diabetic rats were significantly reduced (at days
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4, 7 and 14) compared with the numbers of cells isolated from sprouts from pre-diabetic rats. (* p<0.05, ** p<0.01, *** p<0.0001)

Aortic sprout cells from diabetic rats have a lower proliferative status

A possible explanation for reduced sprout length and density in diabetic rats is a reduced proliferative capacity of sprout cells. To test this possibility we determined the proliferative status of sprout cells by performing an immunostaining for the proliferation marker Ki67 on cytosots of cells isolated from sprouts that were cultured for 10 days in Matrigel™. Figure 3A shows the nuclear staining for Ki67 in dividing cells. Compared with pre-diabetic controls, diabetic rats had significantly (*** p<0.01) less Ki67-positive cells per 5x10⁴ spotted cells (Figure 3B) suggesting a lower proliferation status of sprout cells derived from diabetic rats.

Figure 2. Kinetics of in vitro sprout-formation in aortic rings from short-term (~6 wks) diabetic (DM) and pre-diabetic (no DM) BBDBP rats. (A) Representative photomicrographs of aorta rings from pre-diabetic (no DM; left panel) and diabetic (DM; right panel) BBDBP rats after 4 days of culture in Matrigel™. (B) Quantification of sprout length from aortic rings revealed reduced sprout formation in vitro after exposure to hyperglycemic conditions in diabetic BBDBP rats in vivo (compared to pre-diabetic BBDBP rats) after 4, 7, and 10 days of culture in Matrigel™. (* p<0.05, ** p<0.01) (C) Quantification of the absolute numbers of sprout cells revealed significantly reduced numbers in diabetic rats (DM) compared with pre-diabetic (no DM) control rats after 4, 7 and 14 days of culture. (* p<0.05,** p<0.01, *** p<0.0001)
Aortic sprout cells from diabetic rats maintain their low proliferation status in vitro

To study whether sprout cells derived from diabetic rats maintain their reduced proliferative capacity under normoglycemic conditions in vitro, a fixed number of $2 \times 10^4$ cells was seeded and cultured in EC medium in 6-well plates. After 4 days of culture seeded cells had differentiated into elongated cells that expressed Flk-1 (VEGFR2) suggesting an endothelial phenotype. Occasionally SMA+ myofibroblasts were detected in these cultures (Figure 3C). After 4 days of culture, cells were trypsinized and counted. As shown in Figure 3D, in vitro culture of sprout cells obtained from diabetic rats resulted in significantly ($p<0.05$) reduced cell numbers compared with pre-diabetic controls. These data indicate that sprout cells isolated from aortic rings obtained from diabetic rats have reduced proliferative capacity which persists under normoglycemic conditions in vitro.

Figure 3. Sprout cells from short-term diabetic BBDP rats have a decreased proliferation status. (A) Representative photomicrograph of cytospots of sprout cells isolated after 10 days of culture in Matrigel™ and stained with the proliferation marker Ki67. Arrows indicate proliferating Ki67+ cells. (B) Quantification of Ki67+ cells on cytospots ($5 \times 10^4$ cells/spot) shows significantly reduced numbers of positive cells in diabetic rats (DM, n=7) compared with pre-diabetic rats (no DM, n=7). (** $p<0.01$) (C) Virtually all in vitro expanded isolated sprout cells express Flk-1 (VEGFR2) (red) whereas occasionally smooth muscle α-actin (α-SMA)-positive myofibroblasts were detected (green) (magnification x400). (D) In vitro culture of isolated sprout cells for 4 days in EC medium shows significantly reduced expansion of cells derived from short-term diabetic rats (DM, n=8) compared with pre-diabetic control rats (no DM, n=7).
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Aortic sprout cells express endothelial markers

To demonstrate that the sprout cells are predominantly endothelial cells, additional immunofluorescent stainings were performed on cytospots of sprout cells isolated after 10 days of culture on Matrigel™ using antibodies against the EC-markers CD31, Flk-1 (VEGFR2) and the antigen recognized by mAb HIS43. As shown in Figure 4, most sprout cells stained positive for CD31 and Flk-1 and to a somewhat lesser extent HIS43. Occasionally a SMA⁺ myofibroblast was detected. Similar results were obtained on isolated sprout cells that were cultured for another 4 days (not shown). Together, these data indicate that the vast majority of the sprout cells are endothelial cells.

Discussion

Understanding how diabetes affects neovascularisation is important for the development of pro- or anti-angiogenic therapeutic strategies. Our data from isolated vessels show that the effects of diabetes on neovascularisation include impaired angiogenic sprouting from cells residing in the vascular wall, independent of effects on circulating cells or circulating angiogenic/anti-angiogenic factors. As the vascular rings and isolated sprout cells were cultured ex vivo under normoglycemic conditions, the angiogenic impairment of diabetic
sprouting cells is at least to some extent imprinted upon the cells. The model of aortic ring sprouting provides a method to specifically evaluate the effect of potential pro- or anti-angiogenic interventions at the level of the resident cells in the vascular wall. To what extent the impairment observed in this study ex vivo is physiologically relevant in vivo remains to be established.

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