CLINICAL RESEARCH

In-vitro venous prostacyclin production, plasma 6-keto-prostaglandin $F_{1\alpha}$ concentrations, and diabetic retinopathy

T M E DAVIS, E BOWN, D R FINCH, M D MITCHELL, R C TURNER

Abstract

Previous studies have shown that vessels from diabetics produce less prostacyclin in vitro than those from normal controls. To determine whether this decreased production is related to complications elective biopsy of a superficial forearm vein was performed on 12 insulin-dependent male diabetics, six with nil or minimal and six with proliferative retinopathy, and seven male controls. Vein segments from the diabetics and controls produced similar amounts of prostacyclin in vitro (medians 0.11 and 0.19 ng/mg tissue respectively), but the segments from the diabetics with nil or minimal retinopathy produced less than those from the diabetics with proliferative retinopathy (medians 0.09 and 0.18 ng/mg respectively). Preoperative plasma immunoreactive concentrations of 6-keto-prostaglandin $F_{1\alpha}$ were not significantly different between the controls and the diabetics (medians 101 and 116 pg/ml respectively). In a separate study, however, 11 diabetics with duration of disease of over 10 years and nil or minimal retinopathy had significantly lower concentrations than a matched group of 16 with background or proliferative retinopathy (medians 79 and 121 pg/ml respectively).

These results do not support an association between reduced prostacyclin production and diabetic retinopathy.

Introduction

Prostacyclin is a prostaglandin produced by the vessel wall and other tissues. It is a powerful vasodilator and inhibitor of platelet aggregation in vitro,¹ and inhibition of platelet aggregation may be important in the prevention of atherosclerosis and arterial thrombosis.² Vascular disease is a major complication of diabetes mellitus, and several studies have been concerned with the production and metabolism of prostacyclin in diabetes.

Depressed vascular prostacyclin production in vitro was first shown in diabetic rats treated with streptozocin³ and subsequently in preliminary work with arterial segments removed at operation⁴ and specimens obtained at elective vein biopsy⁵ from diabetic patients. A recent study showed that insulin treatment of diabetic rats restores vascular production of prostacyclin to normal.⁶ Uncontrolled diabetes in man is linked with the development of both large-vessel⁷ and small-vessel^{8,9} disease, and decreased vascular output of prostacyclin might be one of the factors responsible for this association.

Some results obtained, however, have been inconsistent. Isolated, perfused hearts of acutely diabetic rats produce prostacyclin in increased amounts.¹⁰ Plasma concentrations of the stable prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$), which might reflect in-vivo production of prostacyclin, are normal in young patients with juvenile-onset diabetes of short duration and without complications,¹¹ but there is conflicting evidence as to whether diabetics with microangiopathy have lower concentrations than matched controls.^{12,13}

We measured venous prostacyclin production in vitro and plasma concentrations of 6-keto-PGF $_{1\alpha}$ in normal subjects and diabetics with and without retinopathy to determine whether prostacyclin production and metabolism are altered in diabetics with microangiopathy.

Patients and methods

We studied seven male controls (median age 45 years) and 12 male insulin-dependent diabetics (median age 34 years, median duration of disease 21 years). No subject had other systemic disease or clinical evidence of atherosclerosis as assessed from the history, physical

Diabetes Research Laboratories, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford OX2 6HE

T M E DAVIS, MB, research fellow

E BOWN, HNC, chief technician

R C TURNER, FRCP, clinical reader

John Radcliffe Hospital, Oxford OX3 7LJ

D R FINCH, FRCS, clinical lecturer, Nuffield department of surgery (now at Princess Margaret Hospital, Swindon)

M D MITCHELL, DPHIL, MRC senior fellow, Nuffield department of obstetrics and gynaecology

examination, and electrocardiogram. None of the subjects was receiving medication other than insulin. The proportion of smokers (those smoking more than one cigarette or equivalent a day) in the two groups was similar, as was the daily cigarette consumption of the smokers in each group.

The diabetics were further divided into two equal subgroups. Six had nil or minimal retinopathy (defined as no retinopathy on ophthalmoscopy and not more than 15 microaneurysms per standard 13° macular-based field on fluorescein angiography) and six had proliferative retinopathy. These two subgroups were matched for age, body weight, duration of disease, insulin treatment (units/70 kg), and number of smokers (table).

Details of controls and patients studied. Values are medians (and ranges)

	Controls (n = 7)	Diabetics	
		Nil or minimal retinopathy (n = 6)	Proliferative retinopathy (n = 6)
Age (years)	45 (25-56)	33 (23-50)	39 (33-47)
Body weight (kg)	76 (54-86)	73 (69-79)	78 (66-83)
Duration of diabetes (years)		14 (11-33)	22 (15-30)
Insulin treatment (units/70 kg)		58 (41-106)	65 (36-72)
No of smokers	3	4	3
Plasma glucose (mmol/l)	4.7 (4.1-5.4)	12.7 (5.2-20.7)	13.2 (6.3-25.3)
Glycosylated haemoglobin (%)	6.9 (6.1-8.0)	12.7 (8.4-18.2)	12.7 (9.1-14.0)

Conversion: SI to traditional units—Plasma glucose: 1 mmol/l $\approx$ 18 mg/100 ml.

Before the study ethical permission was obtained from the Oxfordshire Area Health Authority to perform elective biopsy of a superficial forearm vein on all subjects. Informed consent was obtained in all cases before operation.

The biopsies were performed between 10 am and midday. Subjects had not been required to fast but had taken no aspirin-containing medications in the previous week. Thirty minutes before operation blood was taken from the right antecubital vein for determination of plasma 6-keto-PGF_{1 α} concentration by radioimmunoassay^{11 14} (the first sample), plasma glucose concentration (glucose oxidase, Boehringer kit), and percentage glycosylated haemoglobin.¹⁵

A length of vein, unsuitable for venepuncture or as the site of an intravenous infusion, was selected from the superficial venous network on the extensor aspect of the proximal left forearm. The overlying skin was anaesthetised by an injection of 2 ml of 1% Xylocaine without adrenaline and the vein exposed through a 3 cm incision performed under strict aseptic conditions. A 2 cm length of vein was excised rapidly from between secured catgut ties and immersed in ice-cold oxygenated Krebs Henseleit buffer containing 11.0 mmol glucose/l (200 mg/100 ml) for transport to the laboratory. The skin was closed with interrupted 5/0 sutures.

Within two minutes the length of vein was removed from the buffer, placed on a watch glass, and quickly debrided to its adventitial layer with minimal stretch and distortion so that endothelial damage was minimised.¹⁶ It was weighed and cut into 1-2 mm rings with a razor blade. The rings were immediately placed in Krebs Henseleit buffer (four to five minutes after excision) at 0.1 mg tissue/ μ l buffer and incubated at 22°C.

Aliquots of 5 μ l supernatant were taken at three, six, and 10 minutes' incubation. The supernatant prostacyclin concentration (expressed in ng/mg wet weight of tissue) was determined using the bioassay technique described by Harrison *et al.*³ This technique is based on prostacyclin inhibition of platelet aggregation induced by adenosine diphosphate. The maximal slope of the aggregometer tracing for each submaximal dose of synthetic prostacyclin was measured, and a standard curve was constructed using a spline-function curve-smoothing technique,¹⁷ which defined the curve without assuming its mathematical form and permitted interpolation.

All statistical analysis was by means of non-parametric tests.¹⁸

Results

Every vein-segment supernatant inhibited adenosine-diphosphate-induced platelet aggregation, which was verified as being due to prostacyclin by its abolition after both 15 seconds' boiling¹ and addition of specific antiserum.³

The supernatant prostacyclin concentrations at three, six, and 10 minutes were not significantly different (medians 0.15, 0.15, and 0.19 ng/mg respectively; Friedman χ^2 test, $p > 0.5$), and therefore the median of the three concentrations determined for each subject was used as a measure of venous prostacyclin production in the first three minutes of incubation.

Although there was a large range of tissue wet weights (from 5 to 57 mg), these were not related to the median supernatant prostacyclin concentrations expressed in ng/mg wet weight of tissue ($r_s = 0.05$, $n = 19$). Thus using different-sized veins, incubated in proportional volumes of buffer, did not bias results.

FIG 1—Prostacyclin production by vein segments in vitro, showing group median values.

FIG 2—Prostacyclin production in vitro related to pre-operative plasma 6-keto-PGF_{1 α} concentrations.

The vein segments from the controls produced more prostacyclin than those from the diabetics, but the difference was not significant (Mann-Whitney U test, $p = 0.30$, two-tailed). The segments from the diabetics with nil or minimal retinopathy, however, produced significantly less prostacyclin in vitro than those from the diabetics with proliferative retinopathy ($p < 0.02$; fig 1). When only the diabetics were considered prostacyclin production did not correlate significantly with either the preoperative plasma glucose concentration ($r_s = 0.05$, $n = 12$) or percentage glycosylated haemoglobin ($r_s = -0.19$, $n = 12$).

Preoperative plasma 6-keto-PGF_{1 α} concentrations were not significantly different between diabetics and controls (Mann-Whitney U test, $p = 0.83$, two-tailed) nor between the two subgroups of diabetics ($p = 0.16$). Prostacyclin production did not correlate significantly with plasma 6-keto-PGF_{1 α} concentrations ($r_s = 0.19$, $n = 17$; fig 2).

Figure 3 shows the pooled results of two previous studies from our laboratories,^{11 12} in which plasma 6-keto-PGF_{1 α} concentrations were determined by radioimmunoassay in normal subjects and diabetics. Samples were assayed blind and in random order. The interassay coefficient of variation for all assays was 16%.¹² The plasma 6-keto-PGF_{1 α} concentrations in the diabetics overall were not significantly different from those in the controls (Mann-Whitney U test, $p = 0.28$, two-tailed). The diabetics with nil or minimal retinopathy and longer

duration of disease, however, had significantly lower concentrations than the two other groups of diabetics studied—namely, a matched group with background or proliferative retinopathy and similar duration of diabetes ($p < 0.02$) and diabetics with nil or minimal retinopathy and duration of disease less than 10 years ($p < 0.02$). Only two values found in the latter two groups were outside the range found in the controls.

FIG 3—Plasma 6-keto-PGF_{1α} concentrations in normal controls and diabetics with differing severities of retinopathy and duration of disease, showing group median values.

All the diabetics who underwent elective vein biopsy in the present study had also been included in a previous study in which plasma 6-keto-PGF_{1α} concentrations were determined.¹² Thus the plasma concentration of this prostacyclin metabolite had been measured at least twice in these patients, and in some of the controls. Among 15 such paired samples, taken from 21 to 122 days apart, the median difference in plasma 6-keto-PGF_{1α} concentration was 53 pg/ml (range 3–142 pg/ml). This is more than twice our interassay coefficient of variation.

Discussion

The concentrations of prostacyclin present in the vein-ring supernatants were constant from three to 10 minutes' incubation time. This finding is consistent with findings of Moncada *et al.*,¹⁹ who showed that production of prostacyclin occurred in the first three minutes of incubation of arterial and venous rings, and Jorgensen *et al.*,²⁰ who found that prostacyclin added to washed platelets suspended in saline abolished thrombin-induced platelet aggregation for 12–15 minutes. The median of the three measurements of supernatant prostacyclin for each subject was considered to be the most reliable estimate of production during the first three minutes of incubation.

Although the vessels from the diabetics did not produce significantly less prostacyclin than those from the controls, the trend was in that direction, as previously reported.^{4,5} Vessels from the diabetics with nil or minimal retinopathy produced significantly less prostacyclin than those from the matched diabetics with proliferative retinopathy, which seems not to support the suggestion that reduced prostacyclin production has a role in the development of microangiopathy. Alternatively, a

vascular "self-protection" mechanism may operate analogous to that found in spontaneously hypertensive rats²¹: as vascular disease progresses so the viable endothelium is stimulated to produce increased amounts of prostacyclin.

The prostacyclin production of vessels from diabetics bore no relation to glucose control as assessed by either plasma glucose concentration or percentage glycosylated haemoglobin. Normalisation of vascular prostacyclin output by insulin treatment of diabetic rats⁶ was associated with a fall in plasma glucose concentration from a high mean value of about 17 mmol/l (300 mg/100 ml) to values below those in control animals.

The pattern of prostacyclin production in the three matched groups is reflected by the plasma 6-keto-PGF_{1α} concentrations in larger numbers of similarly matched subjects. Although the correlation between these two measures was not significant in the present study, there is great day-to-day variability in 6-keto-PGF_{1α} concentrations within subjects, which cannot be accounted for by intra-assay and interassay variation alone. An association between in-vitro prostacyclin production and plasma 6-keto-PGF_{1α} concentrations might be disguised by this unless larger numbers of subjects were studied.

Although circulating prostacyclin may have no effect on platelet aggregation in vivo,²² local release within both small and large vessels might still be an important factor in preventing vascular disease. In-vitro studies of prostacyclin production by small vessel segments might therefore be informative. We wished to estimate prostacyclin production promptly with the tissue in a state as near as possible to that in situ. The lack of sustained prostacyclin release by incubated vessel segments, however, means that our results, and those of similar in-vitro studies,^{3–6} might represent simply the amount of immediately available tissue prostacyclin. Prolonged incubation with added substrate may enable the activities of the enzymes necessary for prostacyclin production (cyclo-oxygenase and prostacyclin synthetase) to be studied, but whether this would give a better indication of in-vivo production is uncertain.

A direct association between reduced prostacyclin production and diabetic retinopathy is not supported by the results of these two studies. Nevertheless, investigations into the metabolism of prostacyclin by diabetics and the magnitude and duration of its effect on diabetic platelets are necessary to determine whether this prostaglandin has a role in the development of microangiopathy.

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Impaired monocyte function in liver cirrhosis

AVI HASSNER, YEHUDIT KLETTER, DORIT SHLAG, MEDINA YEDVAB, MOSHE ARONSON, SHLOMO SHIBOLET

Abstract

Monocyte function in patients with cirrhosis of the liver was measured by phagocytosis and killing of *Candida pseudotropicalis* and *C. albicans*. Both variables were significantly decreased in the patients compared with controls. Control monocytes exposed for two hours to patients' serum showed a significant decrease in intracellular killing compared with control monocytes incubated in autologous serum. This suggests the presence of an inhibiting factor in the patients' serum. This inhibitory factor passed through a dialysis membrane that permitted the passage of molecules of less than 12 000 daltons. Treating monocytes from patients with trypsin significantly increased phagocytosis, indicating that the possible inhibitory factor was attached to the monocyte surface. Metabolism of monocytes during phagocytosis as determined by chemoluminescence was significantly lower in monocytes from patients compared with controls, indicating metabolic impairment.

Monocytes are a component of the monocyte-macrophage system, which includes Kupffer's cells. Impairment of the function of these cells, which have a pivotal role in clearing portal blood, might well be extremely important in the development of chronic liver disease.

Introduction

Various defects have been described in the immune defence system of patients with liver cirrhosis that might explain the increased susceptibility of these patients to infection. Impaired delayed hypersensitivity reactions,¹ impaired humoral function,² and impaired production of complement by the diseased liver³ have been described. In a review on this subject, however, Sherlock⁴ stated that there were no data showing decreased function of Kupffer's cells in liver cirrhosis but as a result of collateral circulation these cells were bypassed. While Kupffer's cells and monocytes are considered to be components of the monocyte-macrophage system,⁴ evaluation of monocyte function in patients with cirrhosis of the liver might contribute to our understanding of the factors leading to the increased tendency for infection. We therefore investigated phagocytosis and intracellular killing by blood monocytes from patients with liver cirrhosis.

Subjects and methods

We studied 40 patients (17 women, 23 men) who had liver cirrhosis that had been proved on biopsy. The mean age was 41 (range 25-67) years. Twenty-eight patients had alcoholic cirrhosis, eight post-necrotic cirrhosis, and the others cryptogenic or an undefined type of liver cirrhosis. None had signs of infection during the study, and none received antibiotics, steroids, or other immunosuppressive drugs. We also studied 32 subjects (12 women, 20 men) who were healthy or had angina and served as control group. None of them had diabetes mellitus or received drugs during the study.

Mononuclear cells from heparinised venous blood were isolated by buoyant centrifugation in Ficoll-Paque (Pharmacia).⁵ *Candida pseudotropicalis* and *C. albicans* were grown and phagocytosis and intracellular killing measured according to the method of Territo and Cline.⁶ Serum from patients was dialysed for 24 hours at 4°C against physiological saline through dialysis tubing with a 12 000 dalton cut-off (A H Thomas Co, Philadelphia, USA). Monocytes from healthy donors were incubated for 120 minutes at 4°C in autologous serum, untreated patients' serum, and patients' serum after dialysis. After incubation the cells were washed twice in Hanks's Balanced Salt Solution and heparin, and phagocytosis and intracellular killing in autologous serum were measured. Monocytes from patients were incubated for 30 minutes at 37°C in 0.25% trypsin, washed twice, and their function subsequently evaluated.

Chemoluminescence was measured in monocytes from the controls and patients 30 minutes after incubation with *C. albicans* in autologous

Department of Medicine D, Ichilov Municipal Hospital of Tel-Aviv, and Department of Histology and Cell Biology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

AVI HASSNER, MD, house physician and instructor in internal medicine
YEHUDIT KLETTER, PHD, research fellow, department of histology and cell biology

DORIT SHLAG, MD, house physician

MEDINA YEDVAB, MD, deputy head of department of internal medicine and lecturer

MOSHE ARONSON, PHD, associate professor, department of histology and cell biology

SHLOMO SHIBOLET, MD, head of department of internal medicine and associate professor