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The Influence of the Combined Presence of Diabetes Mellitus and Hypercholesterolaemia on the Function and Morphology of Experimental Vein Grafts*

Mark G. Davies[1], Helge Dalen[2], Jay H. Kim[1], Lizzie Barber[1], Einar Svendsen[2] and Per-Otto Hagen[1,3]

Vascular Biology and Atherosclerosis Research Laboratory, [1]Departments of Surgery and [3]Biochemistry, Duke University Medical Center, Durham, North Carolina, U.S.A. and [2]Department of Pathology, The Gade Institute, University of Bergen, Bergen, Norway

Objectives: *Diabetes and hypercholesterolaemia are known risk factors for the development of atherosclerosis and are considered to influence the development of vein graft intimal hyperplasia. This study examines the combined effect of diabetes for 12 weeks (alloxan-induced) and hypercholesterolaemia for 8 weeks (1% cholesterol diet) on the formation of intimal hyperplasia and the vasomotor function of vein grafts.*

Materials and design: *Thirty-two New Zealand White rabbits underwent a carotid vein bypass graft. Eight were controls, eight were diabetic, eight were hypercholesterolaemic and eight had both diabetes and hypercholesterolaemia. All vein grafts were harvested at 4 weeks postoperatively for morphology (n = 4) or contractility studies (n = 4).*

Results: *Compared to controls, both diabetes and hypercholesterolaemia increased intimal thickness by 20% and 63% respectively; medial thicknesses of these vein grafts were unchanged compared to control. In contrast, diabetes with hypercholesterolaemia dramatically increased intimal and medial thicknesses (1.8 fold and 1.6 fold respectively, compared to control). Smooth muscle cell contractility was enhanced in both the diabetic and hypercholesterolaemic groups. The presence of diabetes with hypercholesterolaemia did not further alter the enhanced smooth muscle cell contractile responses.*

Conclusions: *This study suggests that the combination of both the atherogenic risk factors, hypercholesterolaemia and diabetes, significantly augments the formation of intimal hyperplasia in experimental vein grafts.*

Key Words: Diabetes; Hypercholesterolaemia; Vein grafts; Endothelium; Smooth muscle cell; Function; Rabbit.

Introduction

The development of intimal hyperplasia in a vein graft can lead to a haemodynamically significant stenosis within 2 years of insertion in up to 30% of peripheral bypasses.[1–3] If left untreated, these grafts fail and after either radiological or surgical interventions, the subsequent secondary patency is low.[4–6] Intimal hyperplasia results from the migration and proliferation of medial smooth muscle cells in the intima of the grafts followed by connective tissue deposition.[7] This proliferative lesion appears to be influenced by many physical, cellular and humoral factors.[7] Moreover, the presence of the risk factors, which are better associated with the development of spontaneous atherosclerosis (hyperlipidaemia, diabetes and hypertension), has been shown to contribute to the rapid development of intimal hyperplastic lesions in vein grafts.[8–12] Although the singular effects of each of these risk factors have been assessed in experimental and clinical studies, the impact of the presence of two risk factors on the development of intimal hyperplasia in vein grafts has not been fully examined.[9]

In clinical studies, hyperlipidaemia is significantly correlated with the development of vein graft atherosclerosis.[3,12,13] Hypercholesterolaemia increases the development of intimal hyperplasia in both experimental vein grafts and after experimental arterial angioplasty.[8,14–16] Diabetes appears to be a significant

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Please address all correspondence to: Per-Otto Hagen, PhD, Duke University Medical Center, P.O. Box 3473, Durham, NC 27710, U.S.A.

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factor in the development of restenosis following coronary angioplasty in clinical series,[17] and it has been shown to increase the formation of intimal hyperplasia in experimental vein grafts.[10] However, in contrast, the presence of mild systemic hypertension appears not to increase the development of intimal hyperplasia in experimental vein grafts.[11,18] The contributions of a mixed atherogenic environment to the early development of intimal hyperplasia in a vein graft are still unclear. However, it has recently been shown that the combination of systemic hypertension and hypercholesterolaemia does not have an additive impact on the promotion of intimal hyperplasia development.[11] This study investigates the effect of the combined presence of diabetes and hypercholesterolaemia on the development of intimal hyperplasia and on the changes induced in smooth muscle cell contractile phenotype within experimental vein grafts. When combined, hypercholesterolaemia and diabetes significantly augment the development of intimal hyperplasia and the increase in the medial changes observed do not further alter the physiological phenotype of the smooth muscle cells present.

Material and Methods

Thirty-two New Zealand White rabbits weighing an average of 2–2.5 kg underwent a right common carotid interposition vein bypass graft using the ipsilateral external jugular vein. Eight were controls, eight were diabetic (alloxan HCl induced 100 mg/kg I.V.; maintained for 12 weeks),[10] eight were hypercholesterolaemic (100 g/day; 1% cholesterol diet for 8 weeks; ICN Biomedical Inc., Cleveland, OH, U.S.A.) and eight had both diabetes and hypercholesterolaemia (alloxan induced diabetes for 12 weeks and placed on a 1% cholesterol diet for the final 8 weeks). Diabetes was present for 8 weeks prior to surgery and for 4 weeks after surgery. Hypercholesterolaemia was present for 4 weeks prior to surgery and for 4 weeks after surgery. In the combined group, diabetes was present for 8 weeks and hypercholesterolaemia for 4 weeks prior to surgery and both were maintained for 4 weeks after surgery. All vein grafts were harvested at 4 weeks postoperatively for morphology ($n = 4$) and contractility studies ($n = 4$). Total serum cholesterol concentrations were measured by the ferric chloride method.[19] Blood urea nitrogen and creatinine concentrations were determined by a standard auto-analyser (Beckman Astra, Beckman Scientific Instruments, Irvine, CA, U.S.A.). Animal care complied with the "Principles of Laboratory Animal Care" as formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" issued by the National Institutes of Health. (U.S. Department of Health and Human Services, NIH Publication No. 80–23, revised 1985).

Diabetic model

Diabetes was induced in 16 animals by alloxan (median dose 100 mg/kg; Sigma Chemical Company, St Louis, MO, U.S.A.) given intravenously via an ear vein as described previously.[10] Animals were maintained in the vivarium on normal chow with water *ad libitum* for the duration of the study. Blood glucose concentrations were determined every 14 days using a blood glucose meter (Ames Glucometer 3, Miles Inc., Elkhart, IN, U.S.A.) and an animal was considered diabetic if its random blood sugars were consistently greater than 13.3 mmol/l.

Operative procedure

Anaesthesia was induced and maintained with subcutaneously injected ketamine hydrochloride (60 mg/kg, Ketaset, Bristol Laboratories, Syracuse, NY, U.S.A.) and xylazine (6 mg/kg, Anased, Lloyd Laboratories, Shenandoah, IA, U.S.A.). Antibiotic prophylaxis with 30000 IU/kg of benzanthine and procaine penicillin (Durapen, Vedco Inc., Overland Park, KA, U.S.A.) was given intramuscularly at the time of induction. Surgery was performed using an operating microscope (JKH 1402, Edward Weck Inc., Research Triangle Park, NC, U.S.A.) under sterile conditions. After exposure through a midline longitudinal neck incision, the right external jugular vein was identified. Its branches were cauterised, at a distance from the vein to minimise injury, and it was then dissected out. Following excision, the vein was kept moist in a heparinised Ringer lactate solution (5 IU/ml, Heparin, Elkins-Sinn Inc., Cherry Hill, NJ, U.S.A.) for approximately 15 min while the right common carotid artery was identified, dissected and both proximal and distal control obtained. Heparin (200 IU/kg) was administered intravenously. A proximal longitudinal arteriotomy was made and one end of the reversed jugular vein was anastomosed to the artery in an end-to-side manner using continuous 10-O microvascular monofilament nylon suture (Ethilon, Ethicon Inc., Somerville, NJ, U.S.A.). The distal anastomosis was performed in a similar manner. Throughout the

procedure, care was taken to avoid unnecessary instrumentation of the vein graft. The right common carotid was ligated and divided between the two anastomoses with 4-O silk sutures. Haemostasis was achieved and the wound was subsequently closed in layers.

Harvest procedure

Using the same anaesthetic regimen described above, the original incision was reopened and the vein graft isolated. Vessels for isometric tension studies were sectioned *in situ* into 5 mm segments, excised and immediately mounted in organ baths containing Krebs solution (vide infra). The animal was killed with an intravenous pentobarbital overdose (100 mg/kg, pentobarbital sodium, Anthony Products, Arcadia, CA, U.S.A.). For those vessels taken for histology, the proximal common carotid artery was cannulated following systemic heparinisation (200 IU/kg intravenously). An infusion of Hanks Balanced Salt Solution (HBSS, Gibco Laboratories, Life Technologies Inc., Grand Island, NY, U.S.A.) was begun and the animal killed. The infusion of HBSS was continued for 3 min at a pressure of 80 mmHg. Thereafter, fixation was commenced with a 5 min perfusion at room temperature of a 2% glutaraldehyde solution made up in a 0.1 mol/l cacodylate buffer, pH 7.4, supplemented with 0.1 mol/l sucrose to give a vehicle osmolarity of approximately 300 mOsm. The distal artery was then ligated and the fixative was left in the graft at a pressure of 80 mmHg. Simultaneously, the exterior of the graft and the attending vessels were soaked in the same fixative. After 60 min, the specimen was removed and glutaraldehyde-fixed for another 24 h at 4 °C prior to further processing. All specimens were then processed for light microscopy. Following standard procedures, the specimens were stained with a modified Masson's trichrome and Verhoeff's elastin stain. The intima and media were delineated by identification of the demarcation between the criss-cross orientation of the intimal hyperplastic smooth muscle cells and circular smooth muscle cells of the media. The outer limit of the media was defined by the interface between the circular smooth muscle cells of the media and the connective tissue of the adventitia. The area of the lumen, intima and media of each graft were quantified by videomorphometry (Innovision 150, American Innovision Inc., San Diego, CA, U.S.A.). To determine the mean thickness of each layer, the luminal, intimal and medial radii were derived (area = πr^2) and the intimal and medial thicknesses were calculated. An intimal ratio of the areas was also calculated (intimal area/[intimal + medial areas]).

For scanning electron microscopy (SEM), mid-portion specimens of the glutaraldehyde-fixed grafts were rinsed with the same buffer solution as described above, dehydrated in ascending concentrations of ethanol, transferred to absolute acetone, critical point dried from CO_2, mounted on specimen stubs and sputter-coated with gold-palladium according to standard techniques. All specimens were examined in a Philips 500 scanning electron microscope (N.V. Philips, Eindhoven, The Netherlands) at an accelerating voltage of 12 or 20 kV. For transmission electron microscopy (TEM), representative sections from the mid-portion of the glutaraldehyde-fixed grafts were post-fixed for 1 h at room temperature in 1% osmium-tetroxide dissolved in the same cacodylate buffer as used for the glutaraldehyde solution. After dehydration in ascending concentrations of ethanol, the specimens were transferred to propylene oxide and finally embedded in Epon 812 monomer. Polymerisation was carried out overnight at 40 °C followed by 2 days at 60 °C. Ultrathin sections were cut with a diamond knife on a Reichart ultramicrotome (Reichart Optische Werke AG, Vienna, Austria), contrasted with uranyl acetate and lead citrate, and then examined in a Philips 300 transmission electron microscope (N.V. Philips, Eindhoven, The Netherlands) or a Zeiss 10A transmission electron microscope (Oberkochen, Germany) both operated at 40 or 60 kV.

Isometric tension studies

After harvesting as described above, 5 mm rings from each vein graft were suspended from two stainless steel hooks in 5 ml capacity organ baths containing oxygenated Krebs solution (122 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$, 15.4 mmol/l $NaHCO_3$, 1.2 mmol/l KH_2PO_4 and 5.5 mmol/l glucose, oxygenated with a mixture of 95% O_2 and 5% CO_2 at 37 °C). One hook was fixed to the bottom of the bath and the other was connected to a force transducer (Myograph F-60, Narco Bio-Systems, Houston, TX, U.S.A.). The isometric responses of the tissue were recorded on a multichannel polygraph (Physiograph Mk111-S, Narco Bio-Systems, Houston, TX, U.S.A.). The tissues were then placed under 0.5 g tension and allowed to equilibrate in physiologic Krebs solution for 1 h. During the equilibration period, the Krebs solution was replaced every 15 min. Following equilibration, the resting tension was adjusted in increments from 0.25–2.5 g and the

Table 1. Blood chemistry

	Control	Diabetes	Hypercholesterolaemia	Diabetes/Hypercholesterolaemia
Blood glucose	182±61	328±125*	174±54	285±37*
Blood urea nitrogen	17±4	22±2	23±1	23±1
Creatinine	1.13±0.13	1.14±0.7	1.02±0.05	1.24±0.22
Serum cholesterol	88±11	91±13	1898±249*	2806±570*

Concentrations of blood glucose, blood urea nitrogen, creatinine and cholesterol in control (n=8), hypercholesterolaemia (n=8), diabetes (n=8) and diabetes/hypercholesterolaemia (n=8) animals at harvest. Values are the mean ± S.E.M. expressed as mg/dl. There are significant differences in the blood glucose between the non-diabetic and diabetic groups. Serum cholesterol is four-fold higher in the two groups on the 1% cholesterol diet. Blood urea and creatinine are similar between the four groups.
*$p<0.05$.

contractile response to a modified oxygenated Krebs solution (60 mmol/l KCl, 66.7 mmol/l NaCl, 1.2 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$, 15.4 mmol/l $NaHCO_3$, 1.2 mmol/l KH_2PO_4 and 5.5 mmol/l glucose) was determined to establish a length–tension relationship. The tension at which a ring generated its maximal contractile response to the modified Krebs solution was considered the optimal resting tension for that particular ring, and the ring was set at this tension for subsequent studies. Norepinephrine (10^{-9}–10^{-5} mol/l) was added cumulatively in half molar increments and the isometric tension developed by the tissue was measured. After washout and re-equilibration, dose response curves were obtained for bradykinin (10^{-10}–10^{-5} mol/l), histamine (10^{-8}–10^{-3} mol/l) and serotonin (10^{-9}–10^{-5} mol/l). For all experiments, the same agonist was added to each chamber in the same sequence to control for possible previous exposure-induced changes in subsequent responsiveness. These preparations retain reproducible contractile properties for up to 12 h. All compounds were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were dissolved in distilled water with the exception of norepinephrine which was dissolved in 10^{-3} mol/l HCl.

Data and statistical analysis

The isometric responses of the rings were converted to percent maximal response and were plotted against the negative logarithm of the agonist dose to produce dose response curves. The EC_{50} value (the concentration for the half maximal response) for contraction or relaxation of each ring was calculated by logistic analysis[20] and is expressed as the pD_2 (defined as $-\log_{10}[EC_{50}]$). The actual maximal contractile response to each agonist is expressed in milligrams of force generated and is then standardized to the contractile ratio (contractile ratio = actual maximal contraction to an agonist/actual maximal contraction to 60 mmol/l KCl). Data are expressed as the mean ± standard error of the mean (S.E.M.) with n equaling the number of animals studied. Statistical differences between groups were tested by multifactorial analysis of variance (MANOVA) using a Duncan *post-hoc* test, when appropriate, and a p-value less than 0.05 was regarded as significant.

Results

General

All animals survived to harvest and all grafts were patent. The serum cholesterol concentration of the cholesterol-fed animals were significantly greater than the control or diabetic animals (Table 1). Likewise, blood sugars of the non-cholesterol and cholesterol fed diabetic animals were significantly greater than the non-cholesterol and cholesterol fed, non-diabetic animals (Table 1). Serum biochemistry was equivalent in the four groups of animals (Table 1). The recorded mean arterial blood pressures were not different between the four groups of animals at harvest.

Morphology

At harvest, there were areas of visible intramural accumulations of cholesterol in the middle and distal segments of the grafts from the hypercholesterolaemic and hypercholesterolaemia/diabetic animals only. All vein grafts developed intimal hyperplasia which was characterised, on light microscopy, by a random criss-cross orientation of the smooth muscle cells in the intima with slender smooth muscle cells in the media which were arranged in a regular circular pattern. There were varying degrees of connective tissue interspersed between the smooth muscle cells. The light microscopic appearance of the diabetic vein

Table 2. Dimensional analysis

	Control	Diabetes	Hypercholesterolaemia	Diabetes/Hypercholesteroaemia
Intimal area (mm^2)	0.87±0.12	1.74±0.34*	2.53±0.29**	2.72±0.80**
Medial area (mm^2)	1.03±0.12	1.45±0.34	1.41±0.15*	3.07±1.45**†
Intimal ratio	0.45±0.04	0.55±0.03*	0.64±0.01*	0.53±0.04*†

Values are the mean ± S.E.M. Intimal ratio = [intimal area]/[intimal + medial areas].
*$p<0.05$, **$p<0.01$ compared to control vein graft; †$p<0.05$ for the effect of diabetes on hypercholesterolaemic vein grafts.

grafts were similar to the control vein grafts. In the vein grafts from animals fed with a high cholesterol diet, there were areas in the intimal hyperplasia of lipid accumulation, and many foam cell formation and lipid containing smooth muscle cells were observed; these were better defined in electron microscopy.

The dimensional analysis of the control and diabetic vein grafts are shown in Table 2. There was a two-fold increase in the area of intimal hyperplasia in the diabetic vein grafts compared to the control vein grafts with the intimal ratio showing a 22% increase (Table 2). There was no significant difference in the medial areas between the control and diabetic vein grafts. The presence of hypercholesterolaemia increased the intimal area by nearly three-fold while the medial area increased by 37% compared to control group (Table 2). Hypercholesterolaemia significantly increased the intimal ratio (42%) of the vein grafts. The intimal area of the combined disease group was similar to the hypercholesterolaemic group (Table 2). However, in contrast to the hypercholesterolaemic group, there was a two-fold increase in medial areas of the vein grafts. This medial area was three-fold greater than the control vein grafts (Table 2). With the combined increase in intimal and medial areas, the intimal ratio was reduced compared to the hypercholesterolaemia but remained 17% greater than the control group.

Compared to control vein grafts, the presence of diabetes resulted in a 20% increase in intimal thickness without a change in medial thickness (Figs. 1 and 2), while the presence of hypercholesterolaemia produced a 63% increase in intimal thickness without a change in medial thickness (Figs. 1 and 2). In the presence of both diabetes and hypercholesterolaemia, the thickness of the intima and the media of the vein grafts was significantly increased (177% and 158%, respectively) compared to the control group or compared to the vein grafts from either the diabetic or the cholesterol-fed animals (Figs. 1 and 2).

In SEM, the vein grafts from the control group were completely covered by intact sharply outlined endothelial cells with occasional junctional stomata (Fig. 3). The vascular surface of the vein grafts from the diabetic group differed considerably from that of the control group. Most notable was the absence of the surface microextensions, the wavy cell borders with increased numbers of stomata, and occasional clefts in the intercellular junctions of the endothelium (Fig. 4). In the vein grafts from both groups of cholesterol-fed animals, the endothelial cells had faint and indistinct

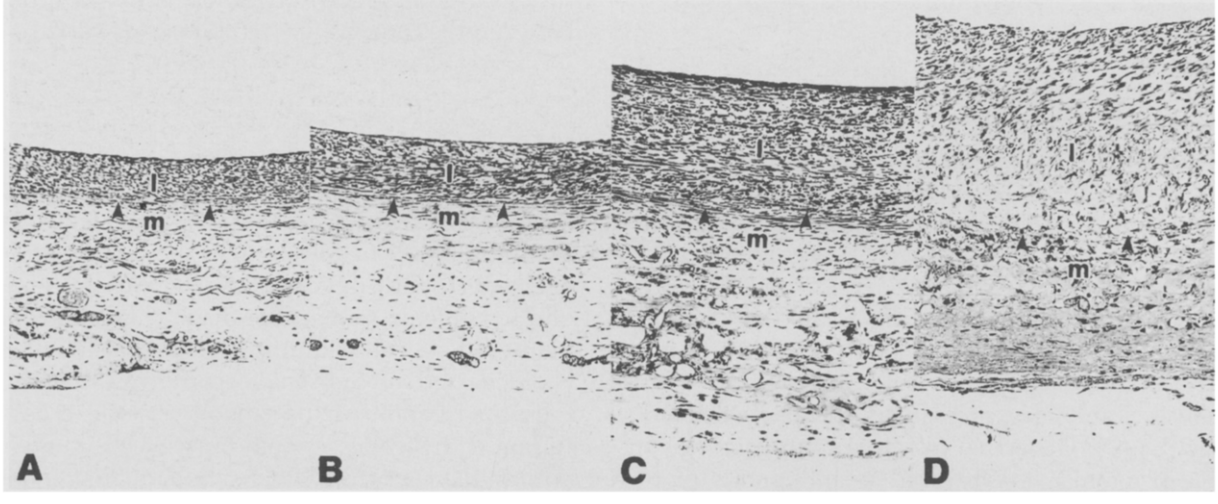

Fig. 1. A composite photomicrograph of a cross-section of the wall of a control vein graft (**A**), diabetic vein graft (**B**), hypercholesterolaemic vein graft (**C**) and hypercholesterolaemia with diabetes vein graft (**D**). I = intimal hyperplasia. m = media. The arrows indicate the demarcation between the intimal hyperplasia and the media. Magnification × 100 (reproduced here at 80%).

cellular outlines. There were more frequent stomata on the surface with fingerlike extensions and occasional breaks at the endothelial cell borders (Figs. 5 and 6). The peculiar shallow surface depressions of the endothelial cells are shrinking artefacts due to dehydration corresponding to the sites of the cytoplasmic large vacuoles seen in transmission electron microscopy (Figs. 9 and 10). The fractures and perforations are, on the other hand, a result of the impact of the electron beam suggesting a fragile/friable endothelium.

In transmission electron microscopy, common morphological features of all the vein grafts were the numerous layers of smooth muscle cells (SMCs) representing the development of intimal hyperplasia. The SMCs were embedded in a matrix containing poorly developed and disorganised connective elements. In the control vein grafts, the vascular surface was covered by a monolayer of intact endothelial cells. The SMCs of the subendothelium were predominantly of a contractile phenotype. Only a few synthetic phenotypes were observed (Fig. 7). Compared with the control vein grafts, the endothelial cells of the diabetic vein grafts appeared relatively thicker than those in the control vein grafts and contained greater amounts of ribosomes. However, unlike the grafts from either the control or cholesterol-fed animals, the

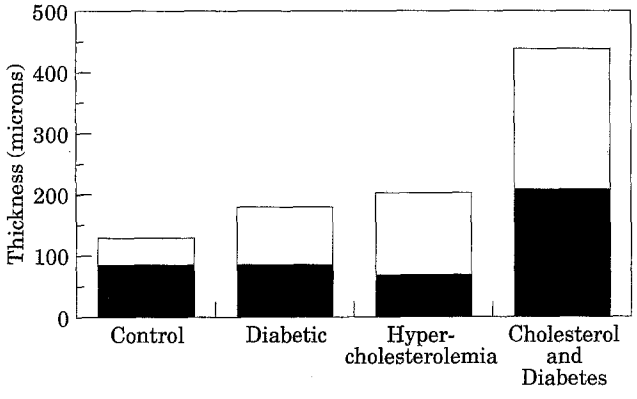

Fig. 2. The intimal and medial thicknesses of the graft wall of the control, diabetic, hypercholesterolaemic and hypercholesterolaemia with diabetes groups. Values are the mean thickness in μm. (□) intima; (■) media.

Fig. 3. Scanning electron microscopy of the vascular surfaces of a control vein graft. The vascular surface of the control vein graft is covered with bulging, oval-shaped endothelial cells (EC) that have distinct cell borders (arrows). The surface of each cell is covered with abundant minor surface extensions (white dots). Magnification $\times$2200 (reproduced here at 50%).

Fig. 4. Scanning electron microscopy of the vascular surfaces of a diabetic vein graft. The surface ultrastructure of the endothelium from the diabetic vein graft is dramatically different from that seen in the control vein graft. The bulging endothelial cells (EC) are almost devoid of surface processes (white dots). Stomata (S) can be identified in the cell borders (arrowed) which, in these grafts, are characterised by their wavy pattern (arrows). Magnification $\times$2200 (reproduced here at 50%).

Fig. 5. Scanning electron microscopy of the vascular surface of a hypercholesterolaemic vein graft. The hypercholesterolaemic vein graft has flat irregularly outlined endothelial cells (EC) with a scanty number of surface processes (white dots). Long finger-like structures (arrowed) often extend from the slightly wavy cell borders. Note the peculiar round and shallow surface depressions (*). The few surface fractures (F) and minor surface perforations (arrowheads) represent artifacts caused by the impact of the electron beam and suggesting a fragile/friable endothelium. Magnification $\times$ 2200 (reproduced here at 50%).

endothelial cells of the diabetic vein grafts revealed predominantly narrow intracellular contact points and occasional gaps (stomata) between adjacent endothelial cells. In the subendothelium, the vast majority of the SMCs were characterised as synthetic phenotypes (Fig. 8). However, unlike the grafts from the control and cholesterol fed animals, the endothelial cells of the diabetic vein grafts had narrow intracellular contact points and occasional gaps between adjacent endothelial cells (Fig. 8). In the subendothelium, the vast majority of the SMCs were characterised by synthetic phenotypes (Fig. 8). The luminal surface of the vein grafts from the cholesterol fed animals was covered by a continuous layer of relatively thick endothelial cells. Beneath this monolayer, there were multiple layers of smooth muscle cells (Figs. 9 and 10). Many of the endothelial cells and smooth muscle cells contained numerous cytoplasmic vacuoles and enhanced numbers of ribosomes. Interspersed between the smooth muscle cells, there were vacuole-filled macrophages (foam cells). Macrophages with little or no lipid accumulation could be identified deeper in the wall (Figs. 9 and 10). The number of foam cells and macrophages present varied from region to region in the graft wall in both cholesterol fed groups. In general, the numbers of foam cells in the vessel wall and the number of macrophages at the adventitial

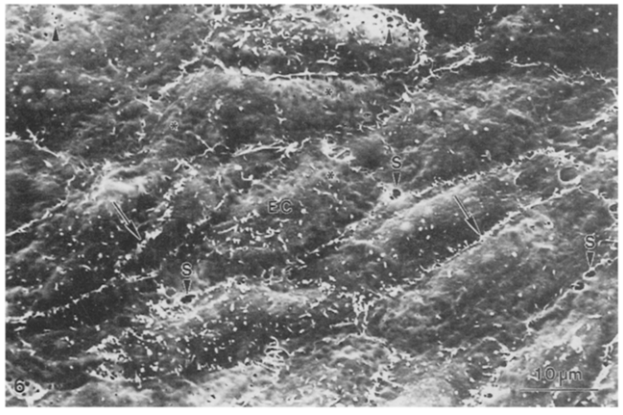

Fig. 6. Scanning electron microscopy of the vascular surface of a hypercholesterolaemic and diabetic vein graft. Apart from minor differences in cellular outlines and curvature, the surface of the endothelial cells (EC) lining the hypercholesterolaemia with diabetes vein grafts is almost identical with that of the hypercholesterolaemic vein grafts. Finger-like surface processes at the cell borders (arrowed), stomata (S) minor surface perforations (arrowhead) and shallow depressions (*) are readily identified. Magnification ×2200 (reproduced here at 50%).

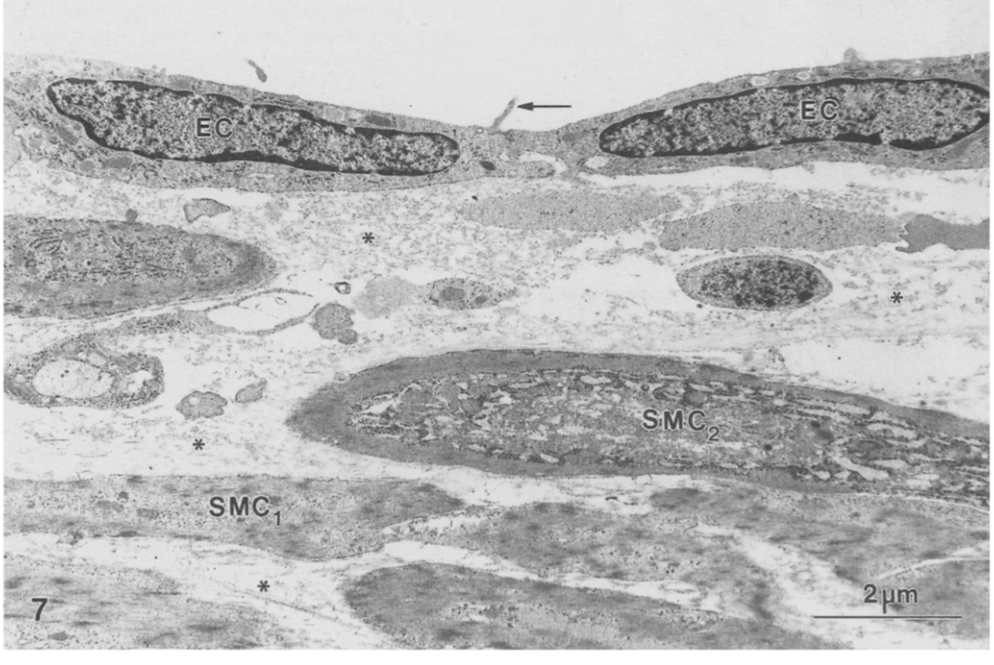

Fig. 7. Transmission electron micrograph of the cross-section of walls from a control vein graft. A common finding of the subendothelium in all groups is the intimal hyperplasia composed of numerous layers of smooth muscle cells (SMC) embedded in a matrix of poorly developed and organised connective tissue (*). The control vein graft shows well defined endothelial cell (EC) with convex surfaces. A slender surface extension (arrowed) is seen adjacent to the intercellular junction. The majority of the intimal smooth muscle cells (SMC_1) were largely filled with myofilaments (contractile phenotypes). However, scattered smooth muscle cells (SMC_2) containing abundant free and membrane bound ribosomes (synthetic phenotypes) were identified. Note the poorly developed and disorganised connective tissue. Magnification ×2200 (reproduced here at 80%).

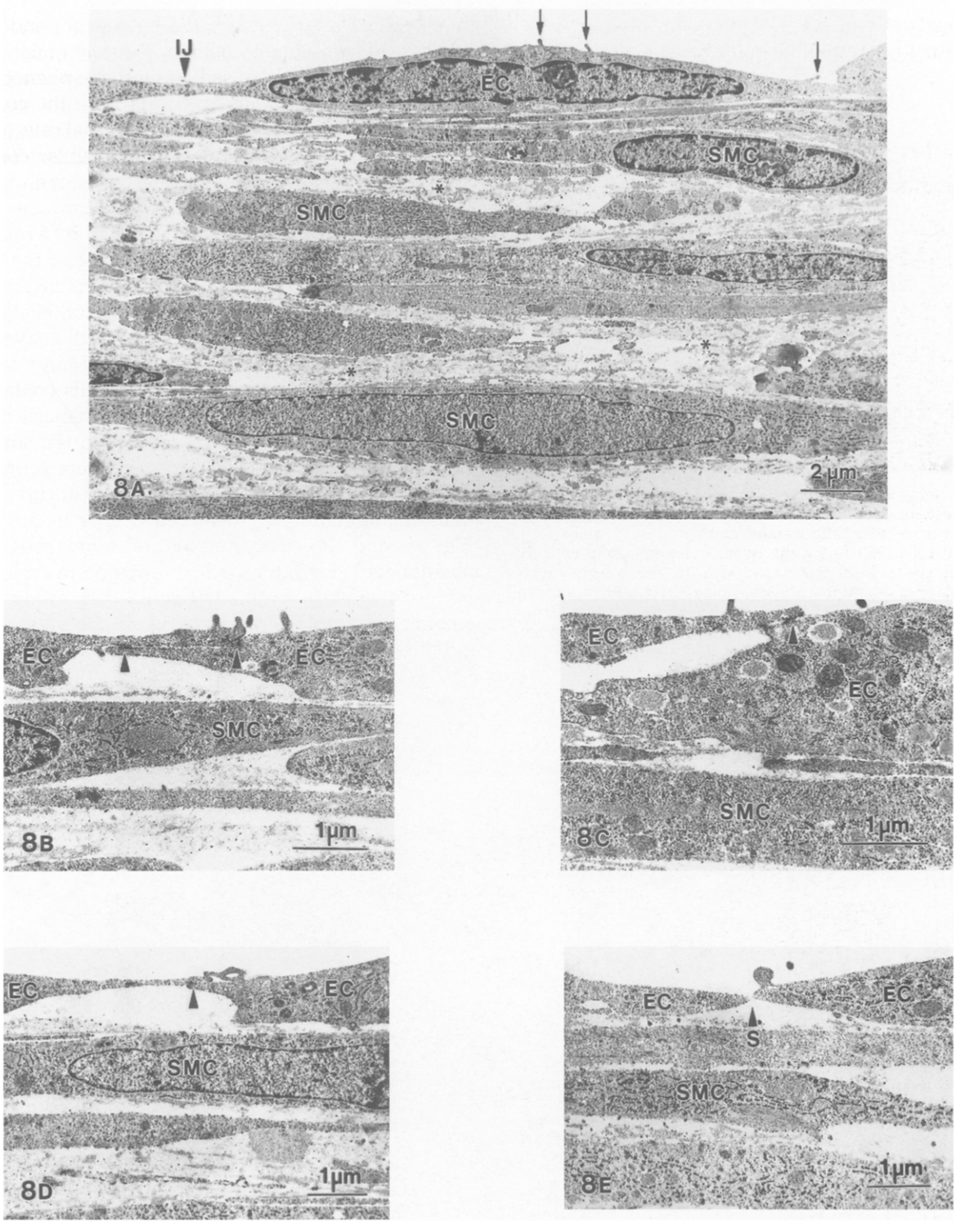

Fig. 8. Transmission electron micrographs of the cross-section of walls from a diabetic vein graft. A common finding of the subendothelium in all groups is the intimal hyperplasia composed of numerous layers of smooth muscle cells (SMC) embedded in a matrix of poorly developed and organised connective tissue (*). The ultrastructural pattern of the diabetic vein graft differ from the control vein grafts. Most notable is the abundance of smooth muscle cells (SMC) of the synthetic phenotype, endothelial cells (EC) with an enhanced amount of free and membrane bound ribosomes in their cytoplasm and the peculiar morphology of the intercellular junctions (IJ). Commonly, such junctions are composed of long and thin cellular processes overlapping each other (8B and 8C) or making end-to-end contacts (8D). The adjacent cell membranes are interconnected by desmosomes (arrowheads). Intercellular stomata (S) are occasionally identified (8E). Magnification ×2200 (reproduced here at 80%).

surface in the cholesterol-fed diabetic animals (Figs. 9 and 10) were less than in the cholesterol-fed.

Physiological responses

Compared to control vein grafts, the presence of hypercholesterolaemia enhanced while diabetes did not change smooth muscle cell contractility in vein grafts. The combined presence of hypercholesterolaemia and diabetes maintained the overall enhanced vasoreactivity of the vein grafts induced by hypercholesterolaemia (Tables 3 and 4). Vein grafts from the control and diabetic groups responded in a concentra-

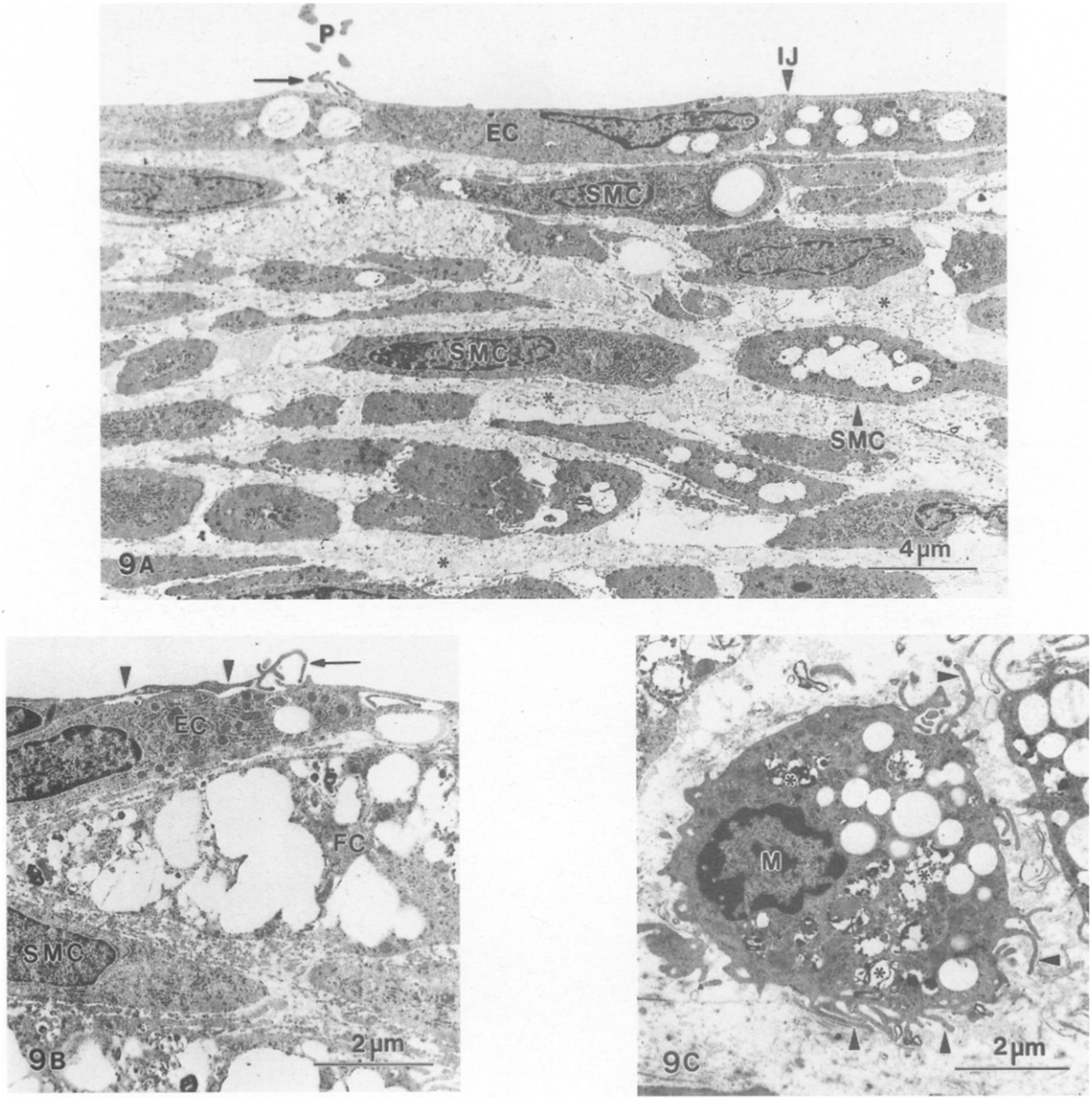

Fig. 9. Transmission electron micrographs of the cross-section of walls from a hypercholesterolaemic vein graft. A common finding of the subendothelium in all groups is the intimal hyperplasia composed of numerous layers of smooth muscle cells (SMC) embedded in a matrix of poorly developed and organised connective tissue (*). A characteristic ultrastructural feature of the hypercholesterolaemic vein grafts are the large vacuoles residing in the cytoplasm of both the endothelial (EC) and smooth muscle cells (SMC). In addition, the subendothelium contains numerous vacuole-filled cells: foam cells (FC) and macrophages (M). The latter cells are characterised by the long and slender surface processes and the intracellular vacuoles with electron dense debris (*). The profile of the intercellular junctions varies from a transverse (IJ; 9A) to a diagonal orientation (arrowheads). Note the long, slender surface processes (arrows) at the cell borders. The majority of the smooth muscle cells are characterised as synthetic phenotypes. P = blood platelets. Magnification ×2200 (reproduced here at 80%).

tion dependent manner to norepinephrine, serotonin, bradykinin and histamine without significant changes in their respective sensitivities (Table 3). The actual maximal contractions to norepinephrine, serotonin, histamine and bradykinin were increased in the diabetic vessels compared to controls. In addition, in the diabetic vein grafts, there was an increase in the maximal contractions generated in response to KCl by

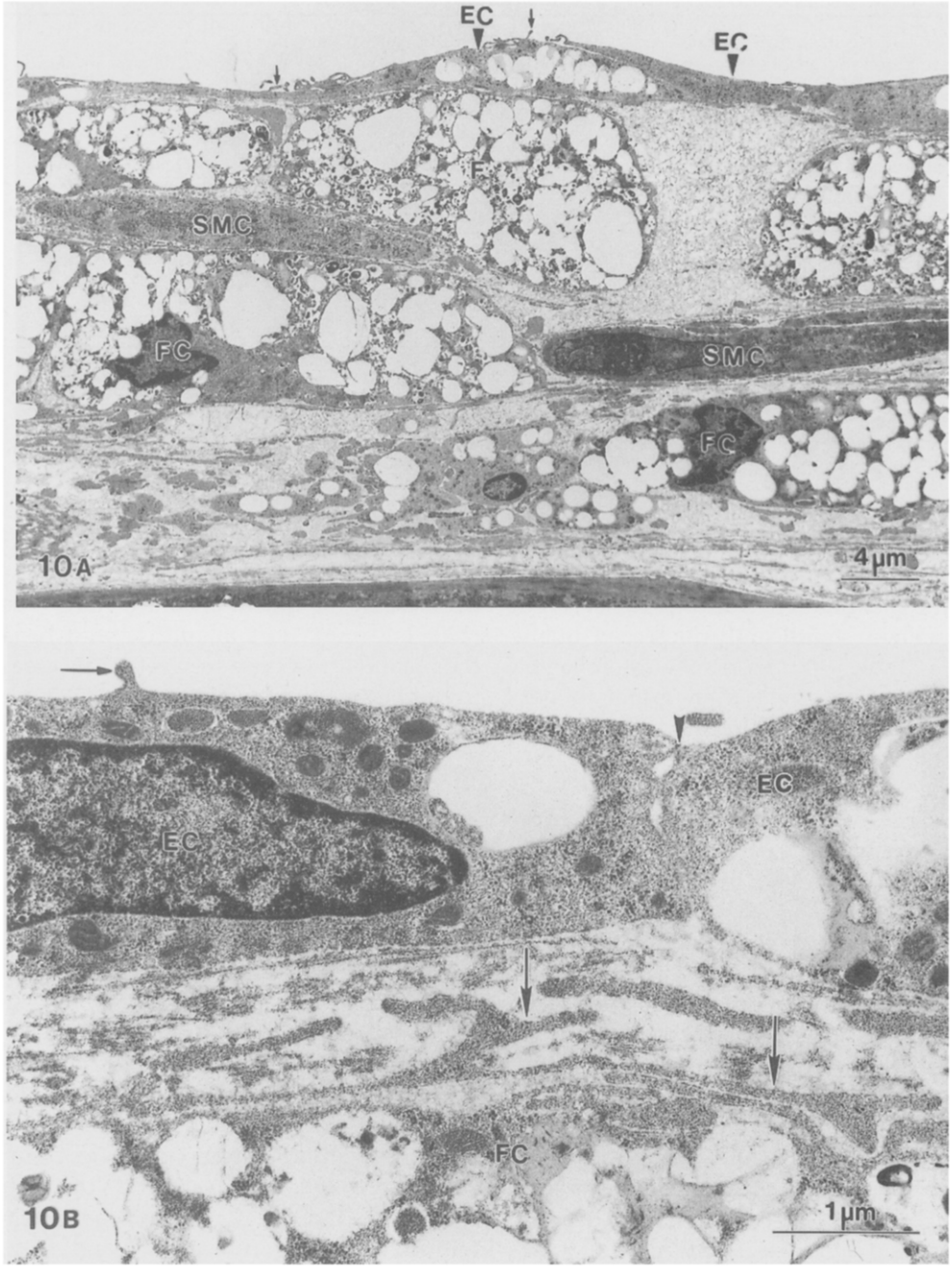

Fig. 10. Transmission electron micrographs of the cross-section of the walls of a hypercholesterolaemic and diabetic vein graft. A common finding of the subendothelium in all groups is the intimal hyperplasia composed of numerous layers of smooth muscle cells (SMC) embedded in a matrix of poorly developed and organised connective tissue. The hypercholesterolaemia with diabetes vein graft shows an ultrastructural pattern very similar to the hypercholesterolaemic vein graft. However, the smooth muscle cells (SMC) appear less vacuolated. Furthermore, foam cells are less numerous and accumulate predominantly in the upper part of the subendothelium. No characteristic macrophages were identified, although the long and slender cytoplasmic processes (large arrows) seen in 10B strongly indicates transformation of a macrophage into a foam cell. Minor surface processes on the endothelial cells are indicated by small arrows. Magnification ×2200 (reproduced here at 80%).

Table 3. Sensitivity in control and diseased vessels

	Control	Diabetes	Hypercholesterolaemia	Diabetes/Hypercholesteroaemia
Norepinephrine	6.09±0.09	6.15±0.13	6.40±0.09*	6.43±0.22
Serotonin	6.20±0.15	6.21±0.10	6.89±0.06**	6.21±0.11†
Bradykinin	5.60±0.15	6.49±0.33	6.30±0.10*	6.17±0.09
Histamine	5.13±0.10	4.88±0.15	5.47±0.18*	5.07±0.11

The EC_{50} values to the agonists in control and diseased vessels. Values are the mean ± S.E.M. of the pD_2.
* $p<0.05$, ** $p<0.01$ compared to control vein graft; † $p<0.05$ for the effect of diabetes on hypercholesterolaemic vein grafts.

Table 4. Maximal responses in control and diseased vessels

	Control	Diabetes	Hypercholesterolaemia	Diabetes/Hypercholesteroaemia
Norepinephrine	1.62±0.37	1.47±0.39	2.13±0.19*	1.18±0.14†
Serotonin	0.41±0.27	1.04±0.30*	4.19±0.03**	2.25±0.32*††
Bradykinin	0.38±0.21	0.42±0.11	2.40±0.13**	1.29±0.29**††
Histamine	1.69±0.15	2.37±0.35*	0.60±0.03*	0.68±0.14*

The maximal responses to the agonists in control and diseased vessels. The KC1 responses are 300±33 mg, 519±138 mg, 216±30 mg and 280±40 mg for the control, diabetes, hypercholesterolaemia and diabetes/hypercholesterolaemia groups respectively. Values are the mean ± S.E.M. of the contractile ratio.
* $p<0.05$, ** $p<0.01$ compared to control vein graft; † $p<0.05$, †† $p<0.01$ for the effect of diabetes on hypercholesterolaemic vein grafts.

the vein grafts from diabetic group (519 ± 138 mg) compared to controls (300 ± 33 mg). However, when the contractile responses are standardised to this KCl response, only the maximal contractions to serotonin and histamine remain significantly increased in the diabetic group (Table 4). Hypercholesterolaemia increased the sensitivity of the vein grafts to norepinephrine, serotonin, bradykinin and histamine (Table 3). The maximal actual contractile forces generated in response to potassium chloride (216 ± 30 mg) and histamine were reduced, those to norepinephrine were unchanged, while those to bradykinin were increased by the presence of hypercholesterolaemia (Table 4).

There were significant statistical interactions between diabetes and hypercholesterolaemia when the combined presence of hypercholesterolaemia and diabetes in vein grafts was tested in the MANOVA model ($p < 0.05$). Subsequent analyses, therefore, examined two subsets: the impact of hypercholesterolaemia on diabetic vein grafts and the impact of diabetes on hypercholesterolaemic vein grafts. The presence of diabetes in hypercholesterolaemic vein grafts significantly decreased the sensitivity of the serotonin responses (Table 3). There were no significant changes in the sensitivity of the responses elicited by norepinephrine, bradykinin and histamine (Table 3). Furthermore, there were no significant changes in the actual maximal responses induced by the potassium chloride, norepinephrine, serotonin, bradykinin or histamine (Table 4). The presence of high cholesterol concentrations in the diabetic vein grafts produced no significant changes in the sensitivity of the responses to any of the contractile agonists tested (Table 3). However, there was a significant decrease in the maximal contractions induced by KCl (280 ±40 mg), norepinephrine and histamine while the maximal response to serotonin increased and that to bradykinin remained unchanged (Table 4).

Discussion

After insertion into the arterial circulation, vein grafts consistently develop intimal hyperplasia which histologically, is a combination of increased numbers of smooth muscle cells and connective tissue synthesised by the smooth muscle cells.[21] The processes leading to the development of vein graft intimal hyperplasia begin after implantation, with smooth muscle cell migration from the media and proliferation within the intima.[7,9,22] In time, this proliferative phase is followed by a more chronic phase of connective tissue deposition. Over one quarter of peripheral vein bypass grafts develop significant stenotic lesions within one year of implantation and histologically, the majority of these lesions can be defined as intimal hyperplasia.[3,9] Thus, studies designed to delineate those factors which may accelerate the development of intimal hyperplasia and means to control its formation are of considerable interest. Based on the similarities in their respective pathologies, intimal hyperplasia and spontaneous atherosclerosis have been grouped

within the same spectrum of vascular disease termed "accelerated atherosclerosis".[23]

The serum cholesterol concentrations are high in this study and exceed those usually measured in hypercholesterolaemic patients. The markedly elevated levels are due to the cholesterol metabolism of the rabbit and the diet used. These higher serum cholesterol concentrations induce rapid atheroma development in the rabbit and have been used as a model for human atheroma. Thus, although the serum cholesterol concentrations on a 1% diet are obviously not clinically applicable, the resulting atheromatous lesions can be. The intimal hyperplasia of human vein grafts retrieved one month after aorto-coronary bypass appear to consist mainly of proliferating smooth muscle cells with only scattered macrophages in the subendothelium.[24,25] The intimal hyperplastic lesions of vein grafts from the cholesterol fed animals in this study are composed predominantly of lipid-laden smooth muscle cells between which are macrophages in various stages of foam cell formation. The intimal hyperplasia develops more rapidly in these vein grafts compared to controls.[8] Functionally, hypercholesterolaemia induces supersensitivity of the vein grafts to all the contractile agonists. There are suggestions that the probable mechanism for this increased contractility are altered receptor-independent calcium mediated responses.[26] Low levels of low density lipoproteins have been documented to alter calcium metabolism of vascular smooth muscle cells.[27] Specifically, the augmented serotonin response in hypercholesterolaemia has been suggested to be due to the direct interaction of oxidised low density lipoproteins with smooth muscle cells, an increase in serotonin activated Ca^{2+} influx and/or an increase in smooth muscle cell—cell to cell coupling.[28] Such functional changes may be considered markers for the subsequent development of atherosclerosis.[29,30] Clinically, the presence of diabetes, usually of the non-insulin dependent variant, does not significantly affect long-term vein graft patency.[31,32] The endothelial linings of the vein grafts from the diabetic group showed ultrastructural changes compared to those of the control or the hypercholesterolaemic vein grafts, giving an impression of poor endothelial integrity. The alterations in endothelial cell morphology may be associated with the increase in intimal hyperplasia observed. Although there were significant morphological changes in the endothelium of these vein grafts, the presence of diabetes did not change the sensitivity of the vein grafts to any of the contractile agonists compared to controls. Morphologically, the smooth muscle cells in the intimal hyperplasia were similar to the control vein grafts. These findings suggest that, from both an ultrastructural and physiological perspective, the smooth muscle cell phenotype has not been further modulated by diabetes, even though the volume of smooth muscle cells present has obviously increased. This is, in contrast, to results in the hypercholesterolaemic group, where an increased intimal thickness was associated with significantly altered smooth muscle cell morphology and contractility.

In the group of animals with the combination of hypercholesterolaemia and diabetes, there were further morphological changes compared to either the control, hypercholesterolaemic or diabetic groups. Both the intimal and medial layers showed substantive proliferative responses. Although diabetes induces an intimal response, it does not change the physiological phenotypes present. Therefore, the dramatic response observed in both layers probably reflects the physiological changes primarily induced in the smooth muscle cells by hypercholesterolaemia. This study demonstrates that when cholesterol-modulated smooth muscle cells are additionally stimulated by the high serum glucose concentrations *in vivo*, there is a resultant intimal and a medial growth response. This does not happen when hypertension is combined with hypercholesterolaemia.[11] Ultrastructurally, the vein grafts from the combination of hypercholesterolaemia and diabetes had less numerous foam cells than the hypercholesterolaemic group and these cells were predominantly located in the subendothelium. There were fewer foam cells deep in the wall and the numbers of macrophages at the adventitial surface were also decreased. Other studies on the combination of hypercholesterolaemia and diabetes in the rabbit have suggested that the degree of net lipid transfer into the arterial wall and the mural cells is less than in hypercholesterolaemia alone.[33–35] This is due to an accumulation of large triglyceride-rich lipoproteins that do not permeate the vessel wall. These observations could explain the apparent decrease in intramural lipid accumulation in this study.

The increased intimal and medial thicknesses in the presence of hyperlipidaemia and diabetes are consistent with the present understanding of atherogenesis, that the presence of both conditions results in accelerated atheosclerotic lesion formation. However, the mechanisms, whereby, such an exaggerated response is mediated are unclear. One explanation is that hyperglycaemia is critical to the diabetes-induced vascular proliferative responses.[35,36] It has been shown that serum from diabetic humans can stimulate the growth of cultured fibroblasts, smooth muscle cells and endothelial cells.[37] Furthermore, hyperglycaemia induces non-enzymatic glycosylation of key cellular

proteins to produce acute glycosylation endproducts (AGE).[35,36] AGE are capable of modulating cellular responses to growth factors and cytokines and may be responsible for the altered morphology and vascular responses observed in diabetes. These AGEs react with specific AGE receptors on both endothelial and smooth muscle cells, induce cytokine production in macrophages, increase vascular permeability and inactivate nitric oxide.[35,36] Moreover, AGE receptors have been documented on macrophages. Stimulation of these macrophage AGE receptors increases macrophage monokine synthesis and secretion. These monokines (cachectin, TNF and IL-1) increase cell proliferation and matrix synthesis directly, and indirectly induce growth factor synthesis and release from endothelial cells.[35,36] The presence of diabetes, and in particular, high glucose concentrations increase lipoprotein oxidation. Oxidized LDLs are significant stimuli of smooth muscle cell proliferation and endothelial cell dysfunction.[38] These effects, in combination with the effects of high lipid concentrations on macrophages and vessel endothelial and smooth muscle cells, could explain the considerable increased intimal and medial hyerplasia development seen in the vein grafts of the combined hypercholesterolaemia and diabetes animals.

The contractility of the smooth muscle cells in the vein grafts from the combined hypercholesterolaemia with diabetes animals appears to reflect predominantly the presence of hypercholesterolaemia. The presence of diabetes in the hypercholesterolaemic vein grafts did not further augment the contractile responses elicited, whereas the addition of a cholesterol diet to diabetic animals induced sensitivities closer to those observed in the cholesterol fed group. There was a notable exception, in that the response to serotonin decreased. The precise reason is unclear. However, from previous studies, the responses to serotonin in vein grafts appear to correlate with the intimal ratio which reflects both intimal and medial dimensions rather than intimal thickness.[8,39] The vein grafts in the combined hypercholesterolaemia with diabetes group had intimal ratios much lower than those from the hypercholesterolaemia group alone, which can be accounted for by the significant medial response observed in the combined hypercholesterolaemia with diabetes group.

In conclusion, the combination of both the atherogenic risk factors, hypercholesterolaemia and diabetes, significantly augments the formation of intimal hyperplasia in experimental vein grafts, without further aggravating the alterations in smooth muscle cell physiological phenotype induced by the systemic vascular diseases.

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