Perspectives in Diabetes

Pathobiology of Endothelial and Other Vascular Cells in Diabetes Mellitus

Call for Data

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Because the pathogenetic understanding of diabetic vascular complications remains fragmentary, and even the best available interventions may prove insufficient to arrest the progression of certain lesions, new avenues of investigation should be pursued. One of these should be the early in vivo investigation of the cells that endure the pathological process (pericytes and endothelial and mesangial cells), preferably in humans. The abnormal vascular architecture (i.e., capillary acellularity, microaneurysms, thickened basement membranes, and mesangial expansion) and the hemostatic and hemodynamic alterations observed in diabetes point to an adaptive/maladaptive replicative and biosynthetic program triggered by the metabolic perturbation, but positive documentation of cellular changes in vivo remains grossly insufficient. Critical review of current knowledge of microangiopathy permits elaboration of specific questions that, with the tools provided by the new molecular technology, may be posed about vascular cells in situ. Knowing whether and how the cell types involved in the vascular complications of diabetes modify their differentiated functions may offer novel targets for intervention and, most important, should provide a much needed “sounding board” against which to test the viability and refine the focus of pathogenetic hypotheses. Diabetes 40:653–59, 1991

Investigations proceed from the initial availability of scattered information, through a phase of extensive data gathering and formulation of hypotheses, into the phase of causal links and reconciliation of theretofore seemingly disjointed findings, and on to verifiable knowledge. Most would concede that the investigation of the pathogenesis of the vascular complications of diabetes has not yet yielded definitive knowledge, and many would agree that it is currently in a phase of accelerated data gathering and formulation of hypotheses. Claims that the investigation has entered the phase of causal links and unifying concepts must wrestle with several instances of discordant experimental evidence in identical or compatible models (1–8) and with the fact that leading contenders for the role of pivotal mechanism—increased polyol-pathway and nonenzymatic glycosylation—do not appear to engage in much cross talk (1,9).

How then do we channel current information into the type of knowledge that would support the advocacy of specific preventive or interventive measures? One answer, of course, is that this is being done with the Diabetes Control and Complications Trial (10). We contend, however, that because certain aspects of diabetic vascular complications are already known to defy any intervention that the Trial might recommend (4,11), the pathogenetic investigation must continue and find new avenues. We see as especially promising the early in vivo investigation of the cells that endure the pathological process, preferably in humans. Knowing how the cell types involved in the vascular lesions of diabetes adapt or maladapt in vivo to the abnormal metabolic environment—by changing their turnover, their differentiated biosynthetic profile and functions, and potentially their information molecules—would provide a much needed “sounding board” against which to verify the relevance, interactions, and limitations of specific mechanistic constructs.

Although this may appear a formidable task, we face this juncture relatively well equipped. Insofar as many studies have identified the aspects of the natural history, histological lesions, and functional abnormalities of diabetic angiopathy that need explanation. In addition, in vitro studies have begun to uncover how the cell types chiefly involved in microangiopathy can react to selected metabolic perturbations. In this article, we address how critical review of the above studies, combined with growing knowledge of the biology of vascular cells, permits elaboration of an initial set of questions to be asked of cells exposed to the diabetic...
environment and how the new molecular technology may provide experimental approaches.

CELLULAR REPLICATION
Three diabetic vascular lesions propose the question of whether and how the diabetic milieu affects cellular replication: acellular capillaries, microaneurysms, and mesangial expansion.

Acellular capillaries. Extensively described in the retina (12,13), acellular capillaries have also been observed in skeletal muscle (14,15) and renal glomeruli (15) of long-term diabetic patients. We can infer from data on decreased microvascular density that acellular capillaries may also occur in the conjunctiva of diabetic patients (16) and the brain of rats with experimental diabetes (17). Acellular capillaries have a central role in the evolution of retinopathy because they are nonperfused and, hence, herald tissue ischemia and neovascular proliferation.

It has never been clear whether capillary occlusion (e.g., by microthrombotic events) is the cause or the consequence of the disappearance of microvascular cells in diabetic vessels. There are observations compatible with the former possibility (4), but two features of the accelerated cell disappearance suggest cell suffering/impaired cell renewal as primary events. First, the cell type more conspicuously affected in diabetic retinal microvessels is the pericyte, a cell that has no direct contact with intraluminal processes. Pericytes are cells of mesodermal origin that, encased in the basement membrane, envelop microvessels and are intimately associated with the endothelial cells lining the vessels (18). The normal retinal microvasculature has a uniquely high ratio of pericytes to endothelial cells (1:1), and the ratio declines early in the course of diabetes due to the demise of pericytes (12,19). The second feature pointing to impaired cell renewal as a primary consequence of diabetes is that retinal capillaries become acellular and nonperfused even after correction of the abnormal metabolic milieu (4), when imbalances in the clotting process or platelet abnormalities are no longer an issue (7,20).

Compromised cell renewal can be entertained if the cell types in question are capable of replication, and this is clearly the case for endothelial cells. Endothelial cell turnover has been documented in all vascular beds examined (21,22); if extremely slow in retinal microvessels under unperfused conditions (22), it can be accelerated by ischemia (23) or physical-chemical injury (24). Pericyte turnover has been scantily investigated and in the retina has been found to be extremely slow (22,25) or undetectable (26,27). Five studies have addressed whether diabetes alters the replicative activity of microvascular cells and have yielded conflicting data. In streptozocin-induced diabetic rats, thymidine incorporation was found to be increased in retinal endothelial cells (25) but substantially decreased in cardiac capillaries (28); in the retina of obese hyperglycemic mice, no differences were found (27), and in diabetic mice, both a decrease (29) and an increase (26) in retinal endothelial cell labeling were reported. The most conclusive statement that can be made about these observations is that they were obtained in poor animal models of diabetic retinopathy and with a detection method (in vivo [1H]thymidine incorporation) plagued with possible interpretative pitfalls (25,26) and not very sensitive because it detects cycling cells solely through the narrow window of S phase (30). Moreover, the method cannot be applied to human studies.

An effort to define the replicative capabilities of human microvascular cells and the early effects of diabetes therein would help sort many hypotheses about the pathogenesis of acellular capillaries. If pericytes rarely replicate in vivo and/or diabetes does not affect or actually enhances pericyte and endothelial cell replication, the accelerated disappearance of these cells in diabetes would have to be studied as resulting primarily from events that compromise their survival and eventually exhaust a limited replicative potential. That the replicative potential of retinal endothelial cells is normally limited is suggested by their disappearance from the capillaries of the peripheral retina in older individuals (31). If pericyte and endothelial cell replication is decreased in diabetes, a complex but well-defined cellular function would become a relevant target for studies. These studies could incorporate and develop the observation that, in vivo, a high glucose level is a perturbation sufficient to hamper both pericyte (32) and endothelial cell (33) replication.

Microaneurysms. In the diabetic microcirculation, vascular acellularity commonly coexists with areas of endothelial clustering, the best defined of which are microaneurysms. These saccular expansions of the capillary wall, although not uniquely occurring in diabetes (13), are most common in the retina of diabetic patients (12,13) and have also been observed in diabetic kidney (34) and heart (35). Many authors refer to the cellularity of retinal microaneurysms as "endothelial proliferation" (3,12) and interpret such proliferation as an aborted attempt at neovascularization (36) or as a reparative process triggered by the selective loss of pericytes and subsequent weakening of the vessel wall (12). Ashton (13) noted long ago "a most misleading phenomenon . . . that endothelial cells can readily become detached from their basement membrane and slide along the capillary lumen, so that they aggregate in clusters and closely resemble proliferations . . .", but no experimental evidence exists to date supporting either interpretation of endothelial cell clustering in diabetic microvessels.

One reason to be especially intrigued by the question of whether microaneurysms represent groups of neoproliferated endothelial cells is that microaneurysms are found more abundantly in areas where pericytes appear to be selectively lost (12), and pericytes have been proposed to be negative regulators of endothelial cell proliferation (18,37,38). Recent in vitro studies show that pericytes inhibit endothelial cell migration (37) and proliferation (38) and that both functions are mediated by transforming growth factor-β in its activated form, which in turn is only generated when endothelial cells and pericytes are in contact or close proximity (37,38). This exciting model of cell-cell interaction demands to be tested in vivo.

Mesangial expansion. Mesangial cells are pericytes' first cousins insofar as both cell types are derived from smooth muscle cells and adapted to unique topography and functions. Akin to pericytes, mesangial cells are monocucleated cells embedded in extracellular matrix and in contact, through their processes, with the basement membrane and endothelial lining of the glomerular capillaries (39). The
pathobiology of these cells is central to various glomerular diseases, because whether inflammation or metabolic perturbations are the triggering event, mesangial expansion becomes a feature of the disease (40) and is in diabetes a likely mechanism for loss of kidney function (41).

Although mesangial expansion is primarily attributable to accumulation of extracellular matrix, definition of whether mesangial cell hyperplasia occurs in any particular disease entity would greatly assist in reconstructing the signaling cascade. In vivo, most mesangial cells are quiescent but capable of replication (42), and in vitro, they proliferate in response to growth factors, cytokines, matrix components, and vasoactive substances (39). Considering that many of these mitogenic substances are increased in the plasma of diabetic patients (43,44) and are also synthesized by glomerular mesangial and endothelial cells (39,40), thus potentially regulating growth through autocrine or paracrine effects, and that increased levels of kidney insulinlike growth factor I are found during renal hypertrophy in experimental diabetes (45), it is reasonable to question whether there is any evidence of mesangial cell proliferation in the human diabetic kidney.

To this effect, Østerby (46) warned against the difficulties of correctly interpreting light-microscopy studies often performed without quantitative techniques. Using quantitative electron microscopy, Østerby failed to detect hyperplasia of mesangial cells in kidney biopsies obtained from insulin-independent diabetic patients up to 5 yr after onset of the disease, despite evidence of increased capillary and mesangial basement membrane material (46). This is not in contrast with the finding of an increased number of total nuclei per open glomerulus at advanced stages of human diabetic nephropathy (47), because at these stages, the enlarged glomeruli no longer reflect a primary effect of diabetes but rather a compensatory response to the widespread occlusive pathology.

Østerby's demonstration that the number of mesangial cell nuclei is not increased in the kidneys of short-term diabetic patients does not, however, exclude the possibility that a constant cell number may be maintained through accelerated cell death and replication, a state that may have substantial consequences from the biosynthetic standpoint. This possibility is purely speculative but is entertained because of the untoward effects of diabetes on the survival of other cells (pericytes) with origin and functions similar to mesangial cells. Clearly, only studies of cellular turnover in vivo can address these issues and help reconstruct how intrinsic cellular characteristics, topography, and cell-cell interactions may modulate any effect of the diabetic milieu on cellular replication.

CELLS AND EXTRACELLULAR MATRIX

Thickening of basement membranes is a widespread phenomenon in diabetes affecting mostly but not only the microvasculature (48); accumulation of mesangial matrix is the central lesion of diabetic nephropathy (41); the development of both abnormalities requires the presence of the metabolic perturbations of diabetes (49). It may thus be argued that a preeminent feature of diabetes is disturbed homeostasis of the extracellular matrix caused by some elements of the abnormal milieu.

Three interrelated aspects of the disturbed homeostasis require clarification: whether the primary abnormality originates in the cells or from the matrix, whether increased synthesis occurs for both the mesangial matrix and basement membrane proper, and which step in the synthetic process is upregulated by the diabetic milieu. It has been postulated that nonenzymatic glycosylation of basement membrane components and subsequent chemical rearrangements to form advanced glycosylation end products (AGEs) may lead to accumulation of extracellular matrix because of decreased turnover of the modified molecules, cross-links with permeated plasma proteins, and proliferation of matrix and cells in response to AGE-induced secretion of growth factors (9). Increased nonenzymatic glycosylation of basement membrane (type IV) collagen does indeed occur in human diabetes (50), but decreased solubility has been documented only for glycosylated interstitial (type I) collagen (51), and decreased susceptibility to proteolytic degradation has been shown only for basement membranes glycosylated in vitro in the presence of 1 M glucose (52). The potential complex consequences of AGE formation and deposition in tissues may be difficult to verify as long as their nature remains elusive (6). The rapid accumulation of glomerular matrix after onset of both human and experimental diabetes (53) is incompatible with any etiology other than increased synthesis, which may thus represent the primary abnormality. Increased synthesis of glomerular matrix components is amply demonstrated in experimental diabetes (54–59), but whether expansion of mesangial matrix and thickening of glomerular basement membranes share in the same pathogenesis remains unclear.

Regarding basement membranes proper, a primary effect of diabetes on their synthesis is strongly suggested by in vitro studies but insufficiently verified in vivo. In line with the demonstration that elevated blood levels of aldohexose (as achieved in experimental galactosemia) are capable of inducing basement membrane thickening (4), high concentrations of glucose have been shown to increase synthesis of basement membrane components by pericytes (32), endothelial cells (60,61), and glomerular epithelial cells (62) within a few days of exposure in vitro. In endothelial cells, the upregulated expression of collagen IV and fibronectin is independent of glucose-induced modification of the deposited matrix (63). In vivo, retinas of rats with 12–48 wk of experimental diabetes show increased activity of enzymes of collagen synthesis (64) but unchanged levels of collagen IV and laminin mRNAs (65). The in vivo observations may be looked at as contradictory or combined to suggest that diabetes increases synthesis of basement membrane components through effects exerted at the translational or posttranslational level. To draw such a conclusion and extrapolate it to events in human diabetes would be premature, however, for the following reasons: 1) both the protein and the mRNA data apply to whole retinas rather than to isolated microvessels, leaving open the possibility of confounding contributions from sources other than microvascular cells; 2) the sensitivity, and thus the quantitative power, of filterhybridization techniques as used by Poulsom et al. (65) in the study of rare retinal microvascular transcripts can justifiably be questioned (66); 3) in the same animal model, the elevated kidney mRNA levels (57,59) point to a pre-
translational effect of diabetes on synthesis of matrix components; and 4) in human endothelial cells in vitro, the overexpression of basement membrane components induced by high glucose levels is mediated at the level of transcription without evidence of translational regulation (63).

An additional issue potentially critical to reconstruction of the pathophysiology of abnormal basement membranes in diabetes is whether the decreased amount of heparan sulfate proteoglycans documented in the glomerular matrix (67) extends to basement membranes in other vascular districts and whether it reflects decreased synthesis, as postulated in the Steno hypothesis of microvascular complications (68) but not unanimously observed (58).

Resolution of the above uncertainties through attribution of specific biosynthetic activities to discrete cell types, possibly at early stages of diabetes, would greatly enhance our converson on several provocative issues. Although in diabetes the degree of mesangial expansion and glomerular basement membrane thickening behave in parallel (49), experimental galactosemia, which leads to basement membrane thickening in both the retina and the glomerulus, does not cause mesangial expansion (4). In experimental and human diabetes, return to normoglycemia reverses (41) or arrests (69) mesangial expansion but does not appear to slow the rate of glomerular basement membrane thickening (41,69) or progression of retinopathy (4,11). Unless the turnover of the mesangial matrix is substantially faster than that of basement membranes, it is reasonable to question whether the behavior of basement membrane–producing cells in certain tissues is peculiar. In diabetic animals, up-regulated synthesis of matrix components outlasting the metabolic abnormalities by several weeks suggests that some cellular changes induced by diabetes may have a prolonged half-life (54,55,59). Phenotypic changes persisting beyond removal of the inducing perturbation and propagated through several cell divisions have been observed in smooth muscle cells explanted from hypertensive pulmonary arteries (70), in hepatocytes exposed to estrogens (71), and in human endothelial cells cultured in high glucose concentrations (59). Attribution of persistently altered biosynthetic phenotypes to discrete cell types of known turnover would provide new starting points for the study of reversibility of lesions.

Finally, the pathogenetic perspective on matrix accumulation in diabetes must take into account that increased matrix synthesis occurs in response to experimental cellular injuries of diverse nature (72), that aging is characterized by widespread vascular basement membrane thickening (15), and that other glomerulopathies involve accumulation of mesangial and basement membrane material (40). It has recently been shown that elevated local expression of transforming growth factor-β accounts for the accumulation of extracellular matrix in experimental glomerulonephritis (73). Comparison of cellular events in these different physiopathological entities could be a step toward definition of similarities and differences.

CELLS, HEMOSTASIS, AND HEMODYNAMICS
The increased synthesis of extracellular matrix by vascular cells in diabetes may be viewed as a facet of a program of altered biosynthetic activity whereby cells attempt to adapt to a subtly noxious environment. The altered program may encompass other products, and certain characteristics of diabetic angiopathy suggest modified production of molecules involved in hemostasis and regulation of vascular tone. Because these are complex functions modulated by multiple influences, events occurring at the level of vascular cells may not be primary or sole determinants. Nevertheless, identification of any such event would represent progress in knowledge, if not a potential target for novel interventions.

Hemostasis. Hypercoagulability (20), decreased fibrinolysis (74), and platelet hypersensitivity (7) have been described in diabetes. The contribution of a disturbed vascular endothelium to each and all of these abnormalities can be readily hypothesized within many constructs on the basis of the extraordinary repertory of thrombotic/antithrombotic substances that endothelial cells can elaborate (75); in vivo verification could begin at the level of documented abnormalities of specific products.

Several studies have reported increased circulating levels of von Willebrand factor in diabetic patients (76), and whether this reflects increased endothelial synthesis/release, decreased efficiency of binding/storage in the extracellular matrix, or decreased clearance remains undetermined. In vitro exposure of human endothelial cells to high glucose levels does not increase the von Willebrand factor mRNA levels (unpublished observations), although it does increase the intracellular quantity of the factor but not its release (76). It would be informative to verify in vivo these results and the observations that human endothelial cells exposed to high glucose levels express tissue plasminogen activator (tPA; unpublished observations) and PA inhibitor 1 (63) and exhibit increased synthesis and activity of tissue factor in response to thrombin perturbation (77). These findings may be relevant to the elevated levels of PA and PA inhibitory activity that accompany depressed fibrinolysis in diabetic patients (74) and to the increased thrombin activity observed in the diabetic state (20). In addition, because endothelial cell surface heparan sulfate proteoglycans exhibit anticoagulant properties, clarification of whether expression of these molecules is decreased in diabetic vascular endothelium in vivo would help interpret not only abnormal matrix characteristics but also variations in the blood compatibility of the vascular lining.

Hemodynamics. Alterations in microvascular hemodynamics resulting in increased capillary pressure and consequent increased permeation of plasma proteins have been proposed as early major events in diabetic microangiopathy (78). However, the complexity of hemodynamic changes in the kidney (79), the uncertainty about their directions in the retina (80,81), and the diversity of proposed pathogenetic mechanisms (78,79) have precluded a cohesive reconstruction of the nature and consequences of these alterations.

Several abnormalities relative to vasoactive substances produced by vascular endothelium have been described in diabetes (82). Vascular segments from diabetic patients or animals show compromised endothelium-dependent relaxation (82) indicative of impaired production/release/effects of nitric oxide, which, in turn, is possibly caused by enhanced production of vasoconstrictor prostanoids (83). The latter can be mimicked by incubation of vascular segments with...
high glucose levels (83), which, in addition, inhibit prosta-
cyclin production by vascular endothelium (84). Diabetic
patients exhibit increased circulating levels of the powerful
vasoconstrictor endothelin (44) and of angiotensin-convert-
ing enzyme (85). High glucose concentrations in vitro appear
to decrease retinal (86) but increase aortic (87) endothelial
cell secretion of endothelin. The effect of these abnormalities
would be expected to be one of generalized vasoconstriction
and, potentially, increased permeability due to the effects of
angiotensin II on vascular endothelium (88). The beneficial
effects of angiotensin-converting enzyme inhibitors on al-
buminuria in normotensive diabetic humans (89) would be
compatible with the expectation, but not so the increased
renal plasma flow and glomerular hyperfiltration of early di-
abetes (79), because both endothelin and angiotensin II induce
hypoperfusion and hypofiltration (90,91).

Because of the juxtaposition of endothelial and mesangial
cells that synthesize vasoactive substances to effector cells
(i.e., pericytes, smooth muscle, and mesangial cells) en-
dowed with specific receptors (39,88,92), autocrine and par-
acrine regulation of vascular tone is probably more important
than regulation from blood-borne substances. Hence, ex-
perimental evidence of whether and how diabetes affects
the biosynthesis of vasoactive molecules in specific districts
would provide critical input to the pathogenetic discourse
on hemodynamic abnormalities.

NOVEL INVESTIGATIVE APPROACHES
The growing knowledge about cell-cycle regulation, the clon-
ing and characterization of genes specifically expressed in
different phases of the cell cycle and genes coding for prod-
ucts of vascular cells, and the perfecting of sensitive tech-
niques to localize gene products (mRNA or protein) to
discrete cell types offer exciting new tools for investigating
the issues of interest in the most relevant targets, human
tissue.

We recently documented that intact RNA can be recovered
from microvessels isolated from human diabetic and non-
diabetic eyes up to 36 h postmortem and that specific tran-
scripts can be accurately measured in such material (66).
Informative tissue from kidneys or peripheral vascular beds
might additionally be accessible through biopsies, and for
in situ studies, fixed tissue can be used.

In situ investigation of the products of genes expressed
at growth arrest (93,94), during emergence of cells from
quiescence (95,96), and throughout later phases of the cell
cycle (35,97) can be expected to provide definition of the
replicative activity of discrete cell populations with a height-
ened degree of sensitivity (inclusion of all stages of active
cycling) and precision (positive identification of quiescent
cells). Indeed, comparing in vivo thymidine labeling with
detection of cyclin/proliferating cell nuclear antigen (PCNA),
Gordon et al. (30) found a fivefold greater sensitivity of
our method in detecting replicating cells in rat tissues. They
have successfully applied the method based on cyclin/
PCNA expression to the detection and characterization of
proliferating cells in human coronary arteries and athero-
sclerotic lesions (30). Regarding matrix proteins, factors
involved in hemostasis, and vasoactive peptides—all prod-
ucts present in the circulation—mRNA studies offer the
possibility of beginning to investigate the topography of syn-
thesis.

It is likely that the most sensitive detection techniques will
need to be used. In RNA extracted from whole tissue, filter
hybridization may only visualize abundant transcripts; to
study transcripts other than actin in human retinal micro-
vessels, we had to resort to solution hybridization (66). For
the purpose of cellular attribution, immunohistochemical
detection of protein products (even when reasonably appli-
cable) may need to yield to detection of mRNAs by molecular
hybridization (in situ hybridization) with its greater sensitivity
(detection limit <5 mRNA copies/cell) (98). Accuracy of cel-
lar attribution may be optimized by combining in situ hy-
bridization with immunocytochemical identification of the
specific cell type through the use of known markers (e.g.,
von Willebrand factor for endothelial cells) (99).

The expectations generated by new thoughts and investi-
gative tools are readily tempered at the workbench, and
issues of availability of informative specimens, signal-noise
ratio in the study of rare transcripts, and individual variability
in gene expression are certain to make the studies discussed
labor intensive. However, as much as the investigation of
cellular events cannot substitute for other lines of study on
the vascular complications of diabetes, it appears to rep-
resent a necessary tool for their accurate interpretation.

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