Hyperglycemia Augments Macrophage Growth Responses to Colony-Stimulating Factor-1

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Pathologic changes produced (or potentiated) by the diabetic state include diabetic retinopathy, nephropathy, and atherosclerosis. There is evidence that the macrophage is implicated in the pathogenesis of these lesions. One of the growth factors known to exert a profound influence on macrophage physiology is colony-stimulating factor-1 (CSF-1). CSF-1 has previously been shown to be produced by endothelial cells, mesangial cells, and vascular smooth muscle cells, and it is reasonable to suggest that the interaction of this factor on infiltrating macrophages is an event that may have pivotal importance in the formation of diabetic lesions. We report on the modulating influence of hyperglycemia on the proliferative response of mouse splenic macrophages to CSF-1. Our studies showed that hyperglycemia enhanced the growth response of such macrophages to CSF-1. The mechanism underlying this enhanced response was examined, and it was demonstrated that hyperglycemia induced a threefold increase in CSF-1 receptor (CSF-1r) mRNA expression as visualized by Northern blot analysis. These investigations provide insight into one of the molecular mechanisms potentially relevant to the genesis of diabetic lesions in the host.

INVESTIGATIONS in several laboratories have demonstrated that the macrophage plays an important role in the genesis of many of the lesions found in the diabetic host. Specifically, the macrophage has been found to be involved in the genesis of the retinal lesion, the evolution of diabetic glomerulopathy, and the formation of the atherosclerotic lesion.

Elegant studies have been performed that have visualized macrophage sludging in the retinal capillaries of diabetic subjects at an early stage of the illness.[1] The interactions of advanced glycation end products (AGEs), which are increased in the diabetic state, with macrophages have been shown to result in the liberation of various cytokines such as tumor necrosis factor, interleukin-1, and platelet-derived growth factor, all of which have the capacity to modify the physiology of cells in the immediate environment.[2,3] This is especially important in the glomerulus, where there is a resident phagocytic macrophage-like cell[4] that can be activated by such substances in the diabetic milieu. The role of the macrophage in the establishment of the atherosclerotic lesion has been well demonstrated (reviewed in Ross[5]).

One of the most important substances known to affect macrophage physiology is colony-stimulating factor-1 (CSF-1). This cytokine has previously been shown to be elaborated by a variety of cell types, including endothelial,[6] mesangial,[7,8] and vascular smooth muscle[9,10] cells. CSF-1 has pleomorphic effects on macrophage function, activating them to produce an array of phlogistic substances, as well as inducing them to replicate. It is reasonable to state that the activity of such a substance on the behavior of the macrophage may play a critical role in the pathogenesis of the diabetic lesion.

There is scanty information on the physiology of the macrophage in the hyperglycemic environment. A comprehensive search of the extant literature has yielded two reports. One study reported that macrophages appeared to behave in an activated state as shown by their capacity to reduce Nitro Blue Tetrazolium.[1] The other study demonstrated that macrophages from the diabetic host had a decreased phagocytic capacity.[11]

The present study investigated the effect of increasing concentrations of glucose on the proliferative response of mouse splenic macrophages to CSF-1. Additionally, we studied the mechanism underlying the effect observed by evaluating expression of the macrophage receptor for CSF-1 (CSF-1r).

MATERIALS AND METHODS

Reagents

Recombinant human CSF-1 expressed in yeast with a specific activity of 2×10^5 U/mg (1 U is the amount of CSF-1 required to support half-maximal proliferation of nonadherent mouse bone marrow precursor cells, and is approximately equal to 5 to 10 colony-forming units) was obtained from Genzyme Laboratories (Cambridge, MA). For some experiments, CSF-1 isolated from mesangial cell supernatant, as described in detail previously,[8] and titrated to the recombinant material was also used.[8]

Assay for Macrophage ^{3}H-Thymidine Uptake

Splenic macrophages were isolated from 4- to 8-week old C57BL/6J female mice, as reported in detail previously.[12] Macrophages were incubated with Dulbecco's modified Eagle's medium (DMEM) containing CSF-1 (150 U/mL), 10% fetal calf serum, and different concentrations of D-glucose for varying periods. Sixteen hours before the macrophages were harvested, they were pulsed with 1 μCi ^{3}H-thymidine (New England Nuclear, Boston, MA) and incubated further. At termination of the culture, the medium was aspirated and the cells were washed twice with Hanks balanced salt solution. An aliquot of 0.05% trypsin with 0.5 mmol/L EDTA (Gibco, Grand Island, NY) was added to each well; the cells were harvested onto glass-fiber filters and washed with distilled water using a semiautomatic cell harvester (Skatron, Sterling, VA). Filter

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discs were punched out and transferred to scintillation vials containing Aquasol-2 (New England Nuclear) and counted in a scintillation counter (LS-9800; Beckman Instruments, Fullerton, CA). For some experiments, L-glucose (which is not metabolized) was used instead of D-glucose to determine if the effect observed with the latter substance was mediated by changes in osmolality. The number of replicates and experiments is shown in each figure legend.

Cell Counts

To verify that the results obtained by ^{3}H-thymidine uptake were indicative of actual cell replication, limited experiments were performed in which aliquots of macrophages were incubated with CSF-1 (600 U/mL) and various concentrations of D-glucose for 144 hours, after which the cells were released and counted using a hemocytometer.

To determine if the results obtained were explicable on the basis of glucose consumption, glucose concentrations of culture media were measured by autoanalyzer at the beginning and end of the experiments. No significant differences were discerned (data not shown).

Northern Blot Analysis

The procedure for Northern blot analysis has been detailed previously.[13] In brief, RNA obtained from macrophages incubated for 144 hours in D-glucose 100 or 540 mg/mL was subjected to electrophoresis in 1% formaldehyde agarose gel and transferred to nylon membranes (Nytran; Schleicher and Schuell, Keene, NH) by standard methods. Specific transcripts were assessed by random priming using the following probes: a cDNA probe for CSF-1r[14] obtained from the American Type Culture Collection (Rockville, MD) and a 550-bp *Hin*dIII + *Xba*I–digested fragment from human glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[15] Hybridization was performed for 24 hours at 65°C in 7% sodium dodecyl sulfate, 1% bovine serum albumin, 1 mmol/L EDTA, 500 mmol/L PO_4 buffer, pH 7.2. Blots were successively washed three times in 0.1× SSC with 0.5% and sodium dodecyl sulfate at 65% before autoradiography. The relative amount of the transcript was assessed by densitometry scanning and normalized to the density of the GAPDH transcript.

Statistical Analysis

Statistical analysis was made by either ANOVA or by the unpaired *t* test.

RESULTS

The effect of different concentrations of D-glucose on CSF-1–stimulated macrophage proliferation as assessed by ^{3}H-thymidine uptake is shown Fig 1. Increasing amounts of glucose were shown to increase the proliferative response of macrophages to CSF-1. (Macrophages did not proliferate in the absence of CSF-1 at all concentrations of glucose.)

Figure 2 shows the temporal kinetics of the reaction. Macrophages cultured in medium containing D-glucose 540 mg/100 mL proliferated more readily (compared with macrophages cultured in medium containing D-glucose 100 mg/100 mL in response to CSF-1 by 96 hours, the effect persisting till 144 hours.

To control for osmolality, macrophage ^{3}H-thymidine uptake was assessed in experiments using a similar protocol but substituting L -glucose for D-glucose in the amounts

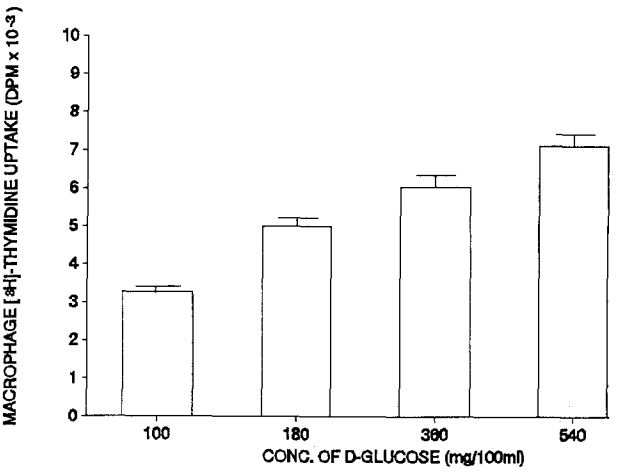

Fig 1. Effect of D-glucose concentration on macrophage proliferation. Cells were incubated in DMEM containing CSF-1 (150 U/mL) and D-glucose at varying concentrations for 144 hours. Values are the mean ± SE of 4 determinations and are representative of 4 separate experiments. ANOVA showed that the mean values for ^{3}H-thymidine uptake by macrophages at different D-glucose concentrations were significantly different ($P < .0008$).

shown in Fig 3. Results of the assays show that higher concentrations of L-glucose had no demonstrable effect on macrophage growth response to CSF-1.

Table 1 shows actual cell counts for macrophages cultured in different concentrations of D-glucose, verifying that cell numbers were increased under those conditions.

Figure 4 shows the Northern blot analysis for expression of CSF-1 mRNA and GAPDH mRNA by macrophages cultured in medium containing D-glucose 100 and 540 mg/100 mL. There was an increase in the expression of

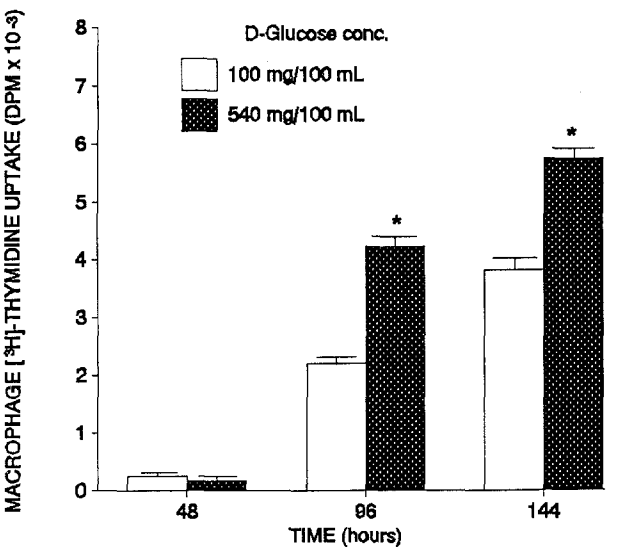

Fig 2. Temporal kinetics of stimulation of macrophage proliferation by a high D-glucose concentration. Cells were incubated in media containing CSF-1 (150 U/mL) and D-glucose at concentrations shown for the designated period. Values are the mean ± SE of 4 determinations and are reflective of 3 separate experiments. Statistical comparisons were made by the unpaired *t* test. *$P < .005$.

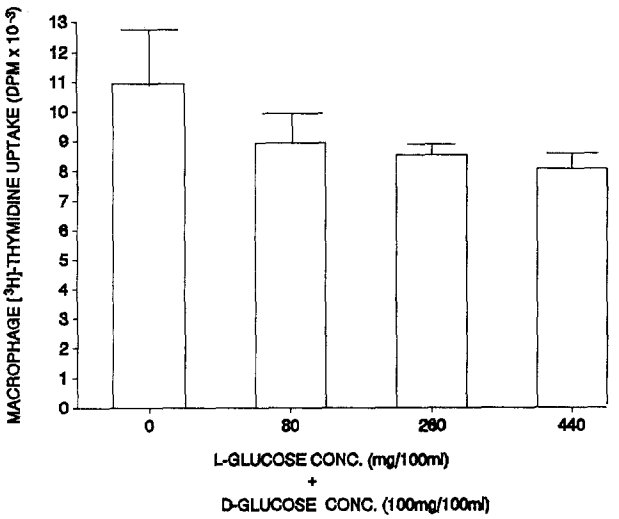

Fig 3. Effect of L-glucose on macrophage proliferation. Cells were incubated in DMEM containing CSF-1 (150 U/mL) and D-glucose 100 mg/100 mL plus L-glucose in the amounts shown for 144 hours. Values are the mean ± SE of 3 determinations and are reflective of 3 separate experiments. ANOVA showed that the mean values for ^{3}H-thymidine uptake by macrophages at different L-glucose concentrations were not significantly different ($P < .4$).

CSF-1r mRNA (5.2-kb transcript) by cells incubated in the higher concentration of D-glucose versus the lower concentration. When normalized to GAPDH, the relative expression of CSF-1 mRNA was elevated approximately threefold in cells grown in the higher concentration of glucose compared with those grown in D-glucose 100 mg/100 mL.

DISCUSSION

Studies in several laboratories have demonstrated a pivotal role for the macrophage in the pathophysiology of diabetes mellitus. Specifically, the macrophage has been implicated in several areas of pathology in this disease-state: (1) the destruction of islet cells of the pancreas, (2) the genesis of microvascular lesions of the retina, (3) the evolution of diabetic nephropathy, and (4) the pathogenesis of an important complication of diabetes mellitus, atherosclerosis.

Evidence has been obtained both from experimental models and from studies of patients to show that the macrophage plays an important role in the destruction of islet cells of the pancreas in type I diabetes mellitus (reviewed in Kolb-Bachofen and Kolb[16]). The lines of evidence may be summarized as follows: (1) identification of macrophages in the pancreas in the prediabetic stage of the disease[17-20] and (2) abrogation of development of the disease if the animals (which are predisposed to develop diabetes) are first pretreated to deplete them of macrophages.[21-24] The results of the current study on the altered physiology of the macrophage in hyperglycemia are not relevant to the pertubations resulting in the onset of diabetes, but they are certainly germane to the ongoing process of destruction of islet cells of the pancreas when diabetes and hyperglycemia have become established.

Table 1. Effect of D-Glucose Concentration on Macrophage Cell Number

D-Glucose Concentration (mg/100 mL)	Cell Count ($\times 10^{-4}$)
100	2.4 ± 0.3
180	4.5 ± 0.5
360	9.3 ± 0.3
540	12.2 ± 0.7

NOTE. Cells were incubated with DMEM containing CSF-1 (600 U/mL) and D-glucose at the concentrations shown for 144 hours. Values represent the mean ± SE of triplicate cultures and are representative of 2 separate experiments. ANOVA showed that differences between mean cell counts at various D-glucose concentrations were highly significant ($P < .0001$).

With respect to the role of the macrophage in the pathogenesis of the lesions found in diabetes mellitus, namely diabetic retinopathy, diabetic nephropathy, and atherosclerosis, studies have shown an accumulation of macrophages in the kidney at an early stage of the disease[25] and sludging of such cells in diabetic retinal vessels.[1] In vitro studies have also shown alterations in the physiology of fibroblasts, endothelial cells, renal tubular cells, and mesangial cells when these cells were incubated in a hyperglycemic environment (reviewed in Kreisberg[26]). Thus, all four cell types could be shown to elaborate increased amounts of fibronectin and other matrix components when they were exposed to high glucose concentrations[27-35]; activation of autocrine transforming growth factor-β has been shown to be the responsible mechanism in some cases.[36,37] However, the growth response of such cells to a hyperglycemic stimulus was found to vary depending on the type of cell. For example, endothelial cells underwent decreased cell replication and eventual cell death when exposed to glucose at a concentration of 30 mmol/L.[38,39] However, mesangial cells behaved less predictably—one study showed no effect of hyperglycemia on the proliferation of such cells, but another demonstrated a biphasic reaction, with cells exhibiting increased cell growth in early cultures but being suppressed in later cultures, the latter effect mediated by transforming growth factor-β.[26,40]

Results of the current study provide information on one aspect of the behavior of macrophages exposed to a hyperglycemic milieu. We examined the responses of macrophages to CSF-1, since it is likely that these cells receive pulses of this cytokine when they are in the proximate vicinity of endothelial, mesangial, mesangial, and vascular smooth muscle cells, since this substance is produced by all three cell types. We were able to show that increasing the amount of glucose in the culture media increased the proliferative response of these cells and also increased the expression of their CSF-1r mRNA. These data are consonant with previous investigations that have demonstrated alterations in the physiology of macrophages incubated in hyperglycemic media.[1,11] The finding that hyperglycemia may induce enhanced expression of a cell receptor also has precedence in a recent study that showed increased expression of mRNA for integrins, which are the receptors for various matrix proteins.[41]

The potential role of the macrophage in the establishment of pathological changes in many target organs has

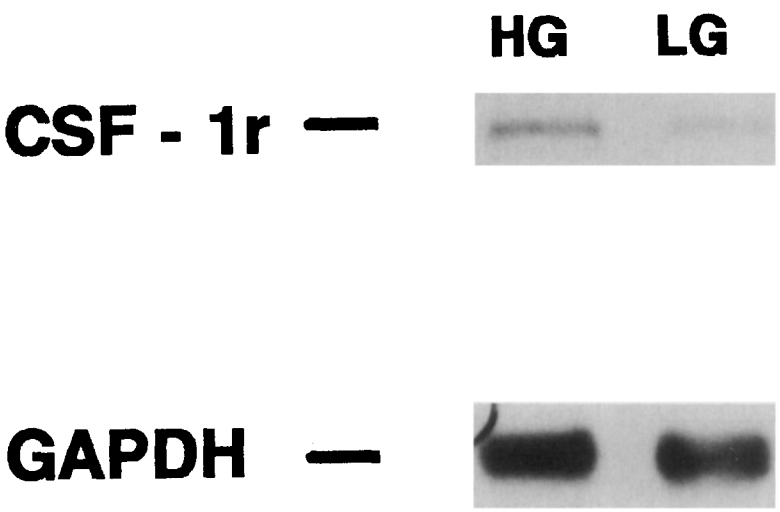

Fig 4. Northern blot analysis of CSF-1r from macrophages cultured with DMEM containing D-glucose 540 and 100 mg/100 mL. Equivalent loading was verified by rehybridization with a probe for the constitutive transcript GAPDH. Blots are representative of 4 individual experiments. By densitometric scanning, the transcript for CSF-1r (5.2 kb) was increased threefold in macrophages cultured at the higher glucose (HG) concentration versus lower glucose (LG). The results are reflective of 3 separate experiments.

been ascribed to interactions between this cell type and AGEs formed as a result of the extended interaction between proteins and aldose sugars such as glucose (reviewed in Makita et al[42]). Thus, specific receptors for AGEs have been identified in monocytes/macrophages[43-45]; additionally, such modified proteins have also been shown to exert a chemoattractant effect on macrophages,[46] and to induce production of various cytokines such as platelet-derived growth factor, tumor necrosis factor, and interleukin-1,[2,3] all of which have the capacity to modulate many aspects of cell function. In the kidney, the role of macrophages may be even more significant, since there are populations of resident glomerular macrophages, as well as interstitial macrophages.[27,47] Previous investigations have shown the modulation of renal function by these populations of phagocytes, which secrete vasoactive substances such as thromboxane that are known to regulate renal blood flow.[47]

The macrophage has also been implicated in the pathogenesis of one of the most important complications of diabetes mellitus, namely the atherosclerotic plaque. Numerous studies have defined the involvement of this cell type in the evolution of this lesion, from the formation of the early fatty streak to its interaction with oxidized lipoproteins (reviewed in Ross[5]).

The results of the present study contribute to our understanding of the role of the macrophage in the pathogenesis of complications resulting from the hyperglycemia of diabetes mellitus. It may reasonably be speculated that our findings suggest that hyperglycemia will tend to lead to an increase in the accumulation of macrophages at tissue sites that have cells producing CSF-1, and that this will lead to an amplification of the events resulting from the disordered macrophage physiology.

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