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Human monocytes and macrophages undergo M1-type inflammatory polarization in response to high levels of glucose

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ABSTRACT

Emerging data suggest that elevated glucose may promote inflammatory activation of monocytic lineage cells with the ability to injure vascular endothelial tissue of diabetic patients, however evidence in primary human monocytes and macrophages is still insufficient. We investigated the effect of high glucose concentration on the inflammatory capacity of human macrophages *in vitro* and examined whether similar responses were detectable in circulating monocytes from prediabetic patients. Primary monocytes were isolated from healthy blood donors and differentiated into macrophages. Differentiated macrophages were exposed to normal levels of glucose (NG), high glucose (HG) or high mannitol as osmotic pressure control (OP) for three days. Using PCR, ELISA and flow cytometry, we found that HG macrophages showed overexpression of CD11c and inducible nitric oxide synthase as well as down-regulation of arginase-1 and interleukin (IL)-10 with respect to NG and OP macrophages. Consistent with *in vitro* results, circulating monocytes from hyperglycemic patients exhibited higher levels of CD11c and lower expression of CD206 than monocytes from normoglycemic controls. In subjects with hyperglycemia, elevation in $CD11c^+$ monocytes was associated with increased obesity, insulin resistance, and triglyceridemia as well as low serum IL-10. Our data suggest that human monocytes and macrophages undergo M1-like inflammatory polarization when exposed to high levels of glucose on *in vitro* culture conditions and in patients with hyperglycemia. These results demonstrate that excess glucose has direct effects on macrophage activation though the molecular mechanisms mediating such a response remain to be elucidated.

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1. Introduction

Hyperglycemia is a metabolic alteration characterized by abnormally high levels of blood glucose (blood glucose ≥ 5.5 mM or 100 mg/dl) [1]. Hyperglycemia is considered a key component of the metabolic syndrome and hallmark of *Diabetes Mellitus*, a chronic disease with high prevalence and mortality rates worldwide [2–4]. In diabetic patients, hyperglycemia has been shown to adversely affect both large and small blood vessels, leading to macro- and microvascular complications that include coronary artery disease as well as diabetic nephropathy, neuropathy, and retinopathy [5–7].

Abbreviations: AGEs, advanced glycation end products; PKC, protein kinase C; ROS, reactive oxygen species; CD, cluster of differentiation; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor alpha; IL, interleukin; Arg-1, arginase 1; LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; ELISA, enzyme-linked ImmunoSorbent Assay; WBCs, white blood cells; BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin resistance; M, male; F, female; NG, normal glucose levels; HG, high glucose levels; OP, osmotic pressure control; MDM, monocyte-derived macrophages; M-CSF, macrophage-colony stimulating factor; M1, classically activated macrophage; M2, alternatively activated macrophage.

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Numerous studies have proposed three main mechanisms through which high levels of glucose may damage vascular endothelial tissue: intracellular glucose toxicity, production of advanced glycation end products (AGEs), and protein kinase C (PKC) activation [8–10]. Intracellular glucose toxicity (also referred to as the polyol pathway) is able to elicit endothelial release of reactive oxygen species (ROS), while AGEs and PKC activation are involved in promoting leukocyte adhesion to vascular endothelial tissue and subsequent blood vessel injury [10–12]. Interestingly, emerging data have now pointed to macrophage inflammatory activation as a possible additional mechanism through which high levels of glucose may also damage vascular endothelium.

Macrophages are white blood cells that originate from monocytes [13]. In the bone marrow, hematopoietic stem cells are capable of differentiating into monocytes. Monocytes are believed to persist in blood circulation for 4–7 days and afterward migrate into tissues where they will differentiate into tissue resident macrophages [13,14]. Depending on the extracellular milieu, macrophages are capable of carrying out inflammatory or anti-inflammatory actions [15–17]. Macrophages displaying a pro-inflammatory phenotype of activation (also referred to as M1, or classically activated) are characterized by expression of specific cell surface markers such as the cluster of differentiation (CD) 11c [18,19]. Functionally, M1 macrophages are characterized by production of inflammatory mediators including inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), and interleukin (IL)-1β [20]. In contrast, macrophages exhibiting anti-inflammatory activation (also referred to as M2, or alternatively activated) typically show expression of CD206 and arginase 1 (Arg-1) as well as increased production of IL-10, a cytokine with potent immunoregulatory actions [20–22]. A growing body of experimental evidence has recently suggested that excessive amounts of glucose may be capable of acting directly on monocyte-macrophage cell lines by shifting the polarization of these immune cells toward a pro-inflammatory state that resembles an M1 phenotype [17,23–25]. Indeed, RAW264.7 cells (a monocyte-macrophage cell line derived from murine leukemia) significantly increase TNF-α production after being exposed to high levels of glucose [23]. Similarly, exposure to high glucose levels results in stimulation of IL-6 and IL-8 secretion in lipopolysaccharide (LPS)-stimulated U937 monocytes [17]. Furthermore, murine peritoneal macrophages show increased mRNA levels of TNF-α, IL-1β, IL-6, and IL-12 in response to elevated concentrations of glucose [24]. Human peripheral blood mononuclear cells (PBMCs) have been demonstrated to exhibit decreased production of IL-10 when exposed to high glucose conditions [25]. These findings suggest that excess glucose may be associated with an M1/M2 imbalance; however, such a phenomenon has not been explored in primary human monocytes and macrophages to date. To this end, we chose to investigate the effect of high levels of glucose on the inflammatory and anti-inflammatory capacity of human macrophages *in vitro*, while also examining whether a similar response could be seen in circulating monocytes from patients with hyperglycemia.

2. Materials and methods

2.1. Subjects

For *in vitro* studies, ten healthy male volunteers with no metabolic disease were included. For *in vivo* studies, 101 women and men attending the Blood Bank or the Internal Medicine Department of the General Hospital of Mexico were included and divided into normoglycemic or hyperglycemic groups. All of the participants provided written informed consent, previously approved by the institutional ethical committee of the General Hospital of Mexico, which guaranteed that the study was conducted in accordance with the principles described at the Helsinki Declaration. For both *in vitro* and *in vivo* studies, subjects were excluded if they had previous diagnosis of non-communicable or infectious diseases. We also excluded pregnant or lactating women as well as subjects taking any kind of anti-inflammatory, anti-aggregant, or anti-hypertensive medication.

2.2. Monocyte isolation and cell culture

For *in vitro* studies, buffy coat samples were collected from each donor (n = 10) and separately diluted 1:2 with phosphate saline buffer 1X (PBS 1X, Sigma-Aldrich, Mexico) for posterior isolation of PBMC by density gradient centrifugation using histopaque-1077 (Sigma-Aldrich, Mexico). Monocyte cells were then isolated from PBMC by CD14-negative selection using magnetic columns (Miltenyi Biotec, Germany). Purified monocytes were placed in glucose-free RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml gentamicin, 5.5 mM D-glucose, and 10 μg/ml macrophage-colony stimulating factor (M-CSF) in 6-well cell-culture plates (Costar, USA), at a density of 3×10^6 monocytes per well. Culture media and M-CSF were replaced every other day for six days.

2.3. High glucose stimulation

After six days of *in vitro* culture differentiation, monocyte-derived macrophages (MDM) were exposed to a high glucose environment. Control MDM (NG) were incubated in glucose-free RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5.5 mM D-glucose for three days designed to resemble normal glucose levels observed in healthy subjects. High glucose MDM (HG) were incubated in glucose-free RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, and 15 mM D-glucose for three days designed to resemble sugar levels seen in diabetic patients with uncontrolled hyperglycemia. Control for osmotic pressure (OP) was achieved by incubating MDM in glucose-free RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, 5.5 mM D-glucose, and 9.5 mM D-manitol for three days. After three days in culture, MDM were harvested using sterile cell scrapers (Corning, USA) and supernatant was collected from each culture well. Collected MDM were equally divided into 1 ml of PBS 1X (Sigma-Aldrich, Mexico) or 500 μl of TRIzol Reagent (Life Technologies, USA) for being used in flow cytometry and RT-PCR assays, respectively.

2.4. Characterization of cell surface markers by flow cytometry

After collecting MDM, 1×10^6 cells were resuspended in 50 μl of sterile PBS 1X (Sigma-Aldrich, Mexico). Immediately after, 3 μl of Human TruStrain (BioLegend, Inc., USA) was added and incubated for 5 min on ice. Then, MDM were simultaneously incubated with anti-CD45 FITC, anti-CD14 PE/Cy7, anti-CD11c PE/Cy5, and anti-CD206/Cy7 APC (BioLegend, Inc., USA) for 20 min for posterior analysis on a FACSCanto II flow cytometer (BD Biosciences, Mexico) by means of BD FACSDiva™ software 6.0, acquiring 50,000 events per test in triplicate. FITC mouse IgG1, PE/Cy7 mouse IgG2, APC/Cy7 mouse IgG1, and PE/Cy5 mouse IgG1 (BioLegend, Inc., USA) were used as isotype control antibodies for cell surface staining of CD45, CD14, CD206, and CD11c, respectively.

2.5. Cytokine production by ELISA

In vitro production of TNF-α and IL-10 were measured in MDM culture supernatants by the Enzyme-Linked ImmunoSorbent Assay

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