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Short-term sustained hyperglycaemia fosters an archetypal senescence-associated secretory phenotype in endothelial cells and macrophages

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ABSTRACT

Diabetic status is characterized by chronic low-grade inflammation and an increased burden of senescent cells. Recently, the senescence-associated secretory phenotype (SASP) has been suggested as a possible source of inflammatory factors in obesity-induced type 2 diabetes. However, while senescence is a known consequence of hyperglycaemia, evidences of SASP as a result of the glycaemic insult are missing. In addition, few data are available regarding which cell types are the main SASP-spreading cells *in vivo*.

Adopting a four-pronged approach we demonstrated that: i) an archetypal SASP response that was at least partly attributable to endothelial cells and macrophages is induced in mouse kidney after *in vivo* exposure to sustained hyperglycaemia; ii) reproducing a similar condition *in vitro* in endothelial cells and macrophages, hyperglycaemic stimulus largely phenocopies the SASP acquired during replicative senescence; iii) in endothelial cells, hyperglycaemia-induced senescence and SASP could be prevented by SOD-1 overexpression; and iiii) *ex vivo* circulating angiogenic cells derived from peripheral blood mononuclear cells from diabetic patients displayed features consistent with the SASP.

Overall, the present findings document a direct link between hyperglycaemia and the SASP in endothelial cells and macrophages, making the SASP a highly likely contributor to the fuelling of low-grade inflammation in diabetes.

1. Introduction

Type 2 diabetes, obesity, and metabolic syndrome are associated with an increased inflammatory score [1,2]. The term 'metaflammation' was coined some years ago to indicate metabolically triggered inflammation [2]. The condition, which is mainly fostered by nutrient and metabolic surplus, is characterized by a set of molecules and signalling pathways similar to those involved in classic inflammation [2]. Recently, the senescence-associated secretory phenotype (SASP) has been suggested to be a major contributor to metaflammation in obesity as well as in type 2 diabetes [3–8]. The SASP consists of a transcriptional pro-inflammatory program that is activated following senescence growth arrest and involves the secretion of a variety of molecules including cytokines, chemokines, and growth factors [9]. In mouse models, removal of senescent cells (SCs) induced an increase in lifespan and healthspan and reduced inflammation in various organs, including kidney [10]. These findings suggest that the SASP plays a major role in inflammaging, the chronic, low-grade inflammatory state that accompanies aging [10,11]. There is substantial evidence that the SASP is an early event preceding insulin sensitivity loss in adipose tissue of obese

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mice and humans [6,12,13]. Moreover, in aged mice SASP inhibition improved glycaemic parameters by increasing insulin sensitivity [14]. Hyperglycaemia (HG) is known to induce senescence *in vitro* [15], but *in vivo* evidence of accelerated senescence in diabetic mice and humans has also been provided [15–17]. These data suggested the existence of a feedback loop between type 2 diabetes and the SASP [3,4]. However, there is no evidence that HG is a direct cause of SASP acquisition *in vivo*. It is also unclear whether it can trigger a secretory profile comparable to the one induced by classic pro-senescence stimuli, *e.g.* replicative exhaustion [4]. Moreover, little is known regarding which cell types are the main SASP-spreading cells *in vivo* [18,19]. In this study, a mouse model of short-term sustained HG was used to investigate senescence and inflammation in kidney, a prototypical organ damaged by diabetes. Moreover, since *in vivo* data suggest that endothelial cells (ECs) and macrophages are key SASP-carrying cells, human cell lines were used to dissect the secretome/phenotype induced by HG in these cell types *in vitro*. Finally, circulating angiogenic cells (CACs) isolated from healthy subjects and age-matched diabetic patients were studied *ex vivo* to gain preliminary insights into the significance of these phenomena in humans.

2. Materials and methods

2.1. STZ treatment and tissue sampling

Male C57BL/6 mice kept in a standard light/dark cycle (12 :12 h) with free access to standard chow and water were studied at the age of 25 weeks. Diabetes was induced by a single intraperitoneal injection (150 mg kg − 1) of STZ in 0.05 M citrate buffer (pH 4.5) vehicle. Animals were fasted for 4 h before and 30 min after the injection. Control mice received the vehicle alone. No acute tubular cytotoxicity was detected [20] (Fig. 1B). All STZ-treated mice developed sustained HG but experienced no episode of severe hyperglycaemia (blood glucose > 600 mg/dl) (Supplementary Table 1). Animals were not randomized to the experiments. Twelve mice were used for each experimental condition. All animals were included in data analysis. Sample size was selected on the basis of previous publications [7,12,14] and it was not calculated by statistics. Mice were sacrificed 7 days after STZ injection. Kidneys were immediately extracted and snap-frozen for RNA and protein analysis, fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) for immunohistochemistry, or stained directly for SA β-gal. *In vivo* studies were performed with approval of the University of Barcelona Ethics Committee, complying with current Spanish and European legislation.

2.2. SA β-gal analysis

Freshly isolated kidneys were cut into 4 portions to maximize penetration. After 5 min fixation and overnight staining with Cellular Senescence Assay Kit solutions (CBA 230, Cell Biolabs), 2 portions were included in paraffin and sections 5 µm in thickness were cut, stained with Nuclear Fast Red (Sigma-Aldrich), and subjected to histological examination. The remaining 2 portions of each kidney were weighed and homogenized in an equal volume of PBS and the solutions were read with a spectrophotometer at OD 595. A fluorescence-based protocol [19] was used to assess SA β-gal activity in protein lysates from kidney and cell-derived samples.

2.3. Immunohistochemistry

Nine non-consecutive sections (5 µm thick) of each kidney portion were mounted on microscope slides. Prior to immunohistochemical staining, sections were deparaffinized, rehydrated, permeabilized, processed for antigen retrieval, and blocked in 3% BSA. Primary antibody used p16 (C-7: sc-377412; Santa Cruz) was dissolved in PBS 1X/0.1 BSA buffer and stined overnight at 4oC). Alexa Fluor 555–donkey anti-mouse IgG (A-31570) was used as secondary antibodies and negative control. Slides were mounted with Dako fluorescent mounting medium. Images were acquired with a Leica TCS SPE confocal microscope.

2.4. Macrophages and endothelial cells isolation from kidneys

Mice kidneys were digested with Multi Tissue Dissociation Kit 2 (Miltenyi Biotec) to form a cell suspension. Then, F4/80 positive macrophages were sorted with Anti-F4/80 MicroBeads UltraPure (Miltenyi Biotec) according to manufacturer instructions for manual separation. Negatively selected cells were further sorted to collect endothelial cells with CD31 MicroBeads (Miltenyi Biotec). Negatively selected cells were collected as well and the three cell populations were directly subjected to mRNA extraction, cDNA synthesis and RT–PCR analysis.

2.5. Cell cultures

HUVECs from three batches (1 from single donor and 2 from pooled donors) were cultured in EGM-2 endothelial growth medium (Lonza). Replicative senescence was studied by culturing cells up to the 15/16th or the 20th passage, depending on the batch. CPD was calculated as the sum of all population doubling (PD) changes. Cells were divided into young (SA β-gal < 10%) and senescent (SA β-gal > 50%).

Human THP-1 and U937 cells were purchased from ATCC and maintained in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine (Euroclone). A published protocol [34] was used for macrophages derived from THP-1 and U937 cells. The proportion of SA β-gal+ cells was assessed as described previously [21,50].

2.6. Total RNA extraction

Total RNA was purified using an RNA purification kit (Norgen Biotek) according to the manufacturer's instructions.

2.7. RT qPCR for mRNA expression

Total RNA (1–2 µg) was reverse-transcribed with Superscript III RT kit according to the manufacturer's instructions. Real-time PCR (RT-PCR) was performed in an ABI Prism 7900 sequence detection system using SybrGreen reagents (Takara Bio Company) and TaqMan® Gene Expression Master Mix (Life Technologies). Values were normalized to GAPDH RNA levels for human cells and mouse kidney. Bcl-2, p21 and p53 were analysed with the TaqMan Gene Expression assay (Applied Biosystems). Thermal profiles used were previously published [51,52]. The primers for all the other mRNAs are listed in Table 4 of supplemental material.

2.8. MiRNA quantification by RT qPCR

MiRNA expression was quantified using a modified real-time approach using TaqMan MicroRNA RT kit and a miRNA assay (both from Applied Biosystems). Procedures and thermal profiles were previously published [50]. MiRs expression in HUVECs was evaluated using RNU44 as the reference; for miRs expression in kidneys RNU 6b was used as the reference.

2.9. Senescence mRNA profiling

Three different HUVEC batches were exposed to hyperglycaemic medium for a week, subcultured until the onset of replicative senescence (> 50 PD and SA β-gal positivity > 50%), or left untreated (control). Total RNA from treated cells was isolated using Master Script RT-PCR System (5 PRIME). cDNA synthesis was performed using SA