EXPERIMENTAL STUDY

Suppressor of cytokine signaling gene expression in human pancreatic islets: modulation by cytokines

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Abstract

Objective: Suppressor of cytokine signaling (SOCS) proteins negatively regulate signal transduction of several cytokines. Since cytokines participate in the pancreatic islet damage in type 1 diabetes, the aim of our study was to investigate the expression of SOCS-1, -2 and -3 in isolated human islets, in basal conditions and after exposure, *in vitro*, to a combination of interferon (IFN)- γ , interleukin (IL)-1 β and tumor necrosis factor (TNF)- α cytokines and in control and in type 1 diabetic human pancreata, to establish (i) whether SOCS molecules are constitutively expressed in human pancreatic islets and (ii) whether their expression can be modulated *in vitro* by proinflammatory cytokines or *ex vivo* by an islet inflammatory process.

Methods: Gene expression of SOCS-1, -2 and -3 was evaluated by RT-PCR in untreated and cytokine-treated isolated human pancreatic islets and their protein expression by immunohistochemistry in control and in type 1 diabetic human pancreata paraffin-embedded sections.

Results: We found that SOCS-1, -2 and -3 mRNA is constitutively, although weakly, expressed in human pancreatic islets, similar to the expression observed in control pancreata by immunohistochemistry. SOCS-1, -2 and -3 mRNA expression was strongly increased in human islets after exposure, *in vitro*, to IFN- γ , IL-1 β and TNF- α . Accordingly, an intense and islet-specific immunohistochemical staining for all three SOCS was detected in pancreata from type 1 diabetic patients.

Conclusion: SOCS-1, -2 and -3 genes are constitutively expressed in human pancreatic islets; their expression increases after exposure to proinflammatory cytokines and during an autoimmune inflammatory process, raising the possibility that these molecules act as key regulators of cytokine signaling in pancreatic islets.

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Introduction

Human type 1 diabetes is caused by the autoimmune destruction of insulin-producing β -cells mediated by infiltrating T-lymphocytes, macrophages and their cytokine products (1). Increasing evidence indicates that proinflammatory and Th1 cytokines such as interferon (IFN)- γ , interleukin (IL)-1 β and tumor necrosis factor (TNF)- α , which are produced at the site of T-cell activation, play an important role in the development of the disease, both in rodents and in humans (2, 3). In particular, it has been shown that IFN- γ is an important mediator of β -cell death, both *in vitro* and *in vivo*, and is able to induce, in combination with IL-1 β , inducible nitric oxide (NO) synthase expression and β -cell apoptosis (4). Moreover, other reports showed deleterious effects of a combination of IL-1 β , IFN- γ and TNF- α on mouse NIT-1 β -cells and on intact mouse islets characterized by insulin release inhibition, Fas expression upregulation and NO production (5).

Cytokine-induced signal transduction pathways must be tightly regulated to avoid the harmful consequences of excessive stimulation. The suppressor of cytokine signaling (SOCS) family represents one of the inhibitors that contributes to the negative regulation of cytokine signaling. Data so far obtained indicate that SOCS proteins represent an important intracellular mechanism that defines the time and duration of the cellular response to cytokine stimulation, although cytokine-induced SOCS gene expression varies according to the cell type, to the experimental conditions and to the tissue being examined (6).

The SOCS family comprises at least eight members, SOCS-1 to SOCS-7 and cytokine-inducible SH2-containing protein, which share a central SH2 domain and a C-terminal SOCS box (6, 7). Cytokines activate several intracellular signaling pathways in order to exert their physiological effects. Receptors for several pro- and anti-inflammatory cytokines bind to members of the Janus kinase (JAK) family of tyrosine kinases and

activate downstream signaling molecules known as signal transducers and activators of transcriptions (STATs). Since SOCS genes are induced by cytokines and the corresponding SOCS proteins inhibit cytokine-induced signaling pathways, SOCS proteins are believed to form part of a classic negative feedback loop; therefore, cytokine activation of the JAK-STAT pathway leads to the induction of SOCS genes. SOCS proteins appear to inhibit signaling by different mechanisms: by association with JAKs and inhibition of their catalytic activity, by binding to activated cytokine receptor and by blocking the access of phosphorylated STAT to the cytokine receptor (8).

Although IFN- γ can induce the expression of SOCS-1, -2 and -3 mRNAs (7), SOCS-1 molecule is of particular importance in regulating the effects of IFN- γ (9), since its overexpression inhibits IFN- γ signaling in several cell lines (10), including NIT-1 β -cells and mouse islets (11).

SOCS-2 appears to play an important role in regulating both growth hormone (GH) and insulin-like growth factor (IGF)-I signaling (6); however, its overexpression can be induced also by proinflammatory cytokines such as IL-1 β in human tonsillar cells and IFN- γ in human bone marrow cells and granulocytes (12). SOCS-2 gene is expressed in non-obese diabetic severe combined immunodeficient (NOD-SCID) pancreatic islets and in NIT-1 insulinoma cells and up-regulated by IFN- γ , IL-1 β and TNF- α (10).

Studies in mouse bone marrow cells showed that SOCS-3 was up-regulated by IL-1 β and TNF- α (7). Moreover, overexpression of SOCS-3 has a protective effect in the rat β -cell line INS-1 exposed to IL-1 β and/or IFN- γ (13).

To date a few reports have been published (10, 11, 13) on SOCS genes expression in rodent pancreatic islets, showing that SOCS overexpression could be a useful strategy to protect β -cell from the deleterious effect of proinflammatory cytokines. At the present time, no data are available about SOCS expression in human pancreatic islets.

In the present study we investigated, at the mRNA level the expression of SOCS-1, -2 and -3 in isolated human islets, in basal conditions and after exposure, *in vitro*, to a combination of IFN- γ , IL-1 β and TNF- α cytokines, and at the protein level, by immunohistochemistry, in control and type 1 diabetic human pancreata, in order to establish (i) whether SOCS molecules are constitutively expressed in human pancreatic islets and (ii) whether their expression can be modulated *in vitro* by proinflammatory cytokine or *ex vivo* by an islet autoimmune inflammatory process.

Materials and methods

Isolation and culture of human islets

In the present study we used vital purified human pancreatic islets obtained from three cadaveric multiorgan donors and prepared as previously described (14).

Within 7–10 days from isolation, aliquots of approximately 300 hand-picked islets were cultured free-floating for 10 h in Petri dishes containing 6 ml supplemented Medium 199 (M199) culture medium under the following condition: (i) control M199; (ii) M199 containing 50 U/ml IL-1 β + 1000 U/ml TNF- α + 1000 U/ml IFN- γ . All cytokines were of human recombinant origin (Roche Diagnostics).

RT-PCR analysis of SOCS mRNAs

The expression of SOCS-1, -2 and -3 genes in human pancreatic islets, in all experimental conditions, was evaluated by RT-PCR. Total RNA was extracted from each sample with Trizol (Invitrogen) according to the manufacturer's instructions. Residual DNA was removed by treatment with DNase-I (Invitrogen) for 15 min at room temperature. DNase-I was inactivated by adding 25 mmol/l EDTA and heating for 10 min at 65 °C. First-strand cDNA synthesis was performed in a total volume of 20 μ l, using 2 μ g of each RNA sample primed with random hexamers with 200 U Superscript II (Invitrogen); cDNA aliquots corresponding to 200 ng RNA were subsequently amplified in a 100 μ l reaction volume containing 20 pmol of sense and antisense specific primers and 2.5 U Taq DNA polymerase (Invitrogen). The following primers were used: SOCS-1, sense 5'-CAC GCA CTT CCG CAC ATT C-3' and antisense 5'-AGC AGC TCG AGG AGG CAG TC-3' (297 bp product) (15); SOCS-2, sense 5'-AGA CAG GAT GGT ACT GGG GAA G-3' and antisense 5'-CTT GTT GGT AAA GGC AGT CCC-3' (446 bp product); SOCS-3, sense 5'-ACC TTC AGC TCC AAG AGC GAG TA-3' and antisense 5'-CGG AGT AGA TGT AAT ATG GCT CT-3' (439 bp product). The primers used for SOCS-2 and -3 amplification were constructed from the human cDNA sequences (SOCS-2 GeneBank: AF037989; SOCS-3 GeneBank: AF159854). Expression of β -actin as RNA control was analyzed employing the following primers, sense 5'-ACC AAC TGG GAG GAG ATG GAG-3' and antisense 5'-CGT GAG GAT CTT CAT GAG GTA AGT C-3' (354 bp product) (16).

cDNA was amplified in serial cycles ranging from 20 to 40 cycles in order to observe a linear amplification of PCR products. According to the results, after an initial denaturation step (2 min at 95 °C), β -actin was amplified by 25 cycles (30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C) with 1.5 mmol/l MgCl₂ and SOCS-1, -2 and -3 by 35 cycles (30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C) with 1.5, 2.5 and 1.5 mmol/l MgCl₂ respectively. The final extension period was 3.5 min at 72 °C. Reaction conditions were standardized in order to observe a linear amplification of PCR products. All PCR products were electrophoresed on 1.2% ultrapure agarose 'Separide' (Invitrogen) and the bands visualized by ethidium bromide staining.

Semiquantitative analysis was performed by densitometric gel scanning utilizing a Bio-Rad 'Gel Doc 2000' video image system and results have been expressed as SOCS-1, -2 or -3/ β -actin density % ratio in each sample analyzed.

Immunohistochemistry

Human pancreatic paraffin sections were obtained from ten healthy (four females and six males, aged 14–45 years with no family history of type 1 or type 2 diabetes) and three type 1 diabetic organ donors (a 19-year-old male and a 14-year-old female who accidentally died 9 and 8 months after diabetes onset respectively, and a new-onset 5-year-old boy who died due to severe brain edema developing after diabetic ketoacidosis). Sections were dewaxed, treated with 0.3% hydrogen peroxidase in methanol for 30 min to quench endogenous peroxidase activity then processed in a microwave oven in 10 mmol/l citrate buffer pH 6 followed by cooling at room temperature and finally incubated for 1 h at room temperature with purified goat polyclonal antibody to SOCS-1 (2 μ g/ml; C-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal antibody to SOCS-2 (2 μ g/ml; H-74; Santa Cruz Biotechnology) and rabbit polyclonal antibody to SOCS-3 (2 μ g/ml; H-103; Santa Cruz Biotechnology). Immunoreactivity was revealed using the streptavidin-biotin-peroxidase system and diaminobenzidine chromogen (LSAB + Kit, HRP; DAKO Corporation). Sections were counterstained with Mayer's hematoxylin. In order to assess specificity of the immunohistochemical staining, experiments in the absence of primary antibodies were performed as well.

Results

Expression of SOCS mRNA in cultured human islets before and after cytokine treatment

The presence of SOCS mRNA was analyzed by RT-PCR in healthy isolated human pancreatic islets in the resting condition and after 10 h *in vitro* treatment with a combination of IFN- γ + IL-1 β + TNF- α . Low levels of SOCS-1, -2 and -3 mRNA were found to be constitutively expressed in untreated isolated human islets (Fig. 1A, lane A). We then investigated in human islets SOCS gene expression after 10 h incubation *in vitro*, with a combination of IFN- γ + IL-1 β + TNF- α . The expression of SOCS-1, -2 and -3 genes was markedly increased (Fig. 1A, lane B) by such a treatment. Semiquantitative analysis performed by densitometric gel scanning showed (Fig. 1B) a significant upregulation ($P < 0.05$ for SOCS-1 and -3 and $P < 0.01$ for SOCS-2 by Mann–Whitney test) of SOCS expression in cytokine-treated islets.

Figure 1 Expression of SOCS mRNA in cultured human islets before and after cytokine treatment. (A) RT-PCR for SOCS-1, -2 and -3 genes in human control islets (lane A) and human islets (lane B) exposed, *in vitro*, for 10 h to a combination of IFN- γ , IL-1 β and TNF- α cytokines. (B) Data illustrated on the graph bar represent the means $\pm$ s.d. of three different experiments. * $P < 0.05$, ** $P < 0.01$ vs control.

Expression of SOCS in human normal and type 1 diabetic pancreas:

Immunohistochemical analysis of pancreatic sections from six control multiorgan donors, revealed that SOCS-1 was virtually undetectable and SOCS-3 were weakly expressed in pancreatic islet cells (Fig. 2A and E). SOCS-2 showed immunopositivity with only few cells with a cytoplasmic staining (Fig. 2 C). No specific staining for any of the SOCS molecules analyzed could be detected in exocrine pancreas.

All three type 1 diabetic pancreata analyzed showed an intense SOCS-1, -2 and -3 staining that could be

Figure 2 Expression of SOCS-1, -2 and -3 in human normal and type 1 diabetic pancreata. Immunohistochemistry for SOCS-1 (A, B), SOCS-2 (C, D) and SOCS-3 (E, F) was performed on pancreatic paraffin sections from healthy (A, C, E) and type 1 diabetic organ donors (B, D, F). Magnification $\times 400$ (A, C–F) or $\times 250$ (B). A similar expression pattern was observed in all subjects analyzed.

detected in most pancreatic islets. As shown in Fig. 2, SOCS-1 (Fig. 2B), SOCS-2 (Fig. 2D) and SOCS-3 (Fig. 2F) were markedly expressed in the cytoplasm of a portion of islet cells. Single scattered acinar cells expressing SOCS-1 (Fig. 2B) or SOCS-3 (Fig. 2F) were also detected. The staining intensity and the number of islet cells expressing SOCS proteins did not appear to correlate with the intensity of the islet infiltration process. Control experiments in the absence of primary antibodies produced no staining (data not shown).

Discussion

In this study we show, for the first time, that SOCS-1, -2 and -3 genes are constitutively expressed in human pancreatic islets, and that their expression increases following exposure, *in vitro*, to a combination of proinflammatory cytokines such as IFN- γ , IL-1 β and TNF- α . Moreover, we have demonstrated that type 1 diabetic pancreata showed a strong islet-specific immunostaining for all three SOCS proteins tested, in contrast to control pancreatic islets, showing only a weak positivity. The differences between normal and type 1 diabetic pancreas are likely due to the mononuclear cell infiltrate as a possible source of proinflammatory cytokines.

SOCS-1 upregulation observed in human islets treated, *in vitro*, with IFN- γ + IL-1 β + TNF- α and in

type 1 diabetic pancreata is in line with a number of reports showing that SOCS-1 is a key regulator and critical inhibitor of IFN- γ signaling and is also able to suppress TNF- α -induced cell death (17).

Taking into account that overexpression studies have pointed out that only the constitutive and continuous expression of SOCS-1 was able to inhibit cellular damage resulting from the combination of IFN- γ and IL-1 β (4, 10), our data strengthen the regulatory role of SOCS-1, and that its overexpression could be an attempt to protect human β -cells from cytotoxic cytokines known to be important mediators in the progression of autoimmune diabetes (11).

The increase of SOCS-2 mRNA expression in cytokine-treated human islets associated with a strong immunopositivity of SOCS-2 protein in diabetic human islets provides evidence for an involvement of this molecule in the modulation of cytokine signaling during the insulinitic process.

Although SOCS-2 overexpression is induced by proinflammatory cytokines in different human tissues (12), its regulatory role is less defined and perhaps more complex: SOCS-2 appears to play a primary role in regulating both GH and IGF-I signaling in humans and rodents (6) and in addition to SOCS-1 and -3 it is differentially regulated at the onset of labor thought to be initiated by inflammatory cytokines (18).

Moreover, results obtained from cotransfection studies showed that increasing concentrations of SOCS-2 inhibited, in a dose-dependent manner, the actions of SOCS-1 on cytokine signaling, suggesting that SOCS-2 at high concentrations might act to inhibit the function of other SOCS family members (19).

Our finding that exposure of human islets to inflammatory cytokines was associated with upregulation of SOCS-3, both *in vitro* and *in vivo*, is in line with studies indicating a role of this molecule in the modulation of IL-1 β and TNF- α cytokine signaling (7). Furthermore SOCS-3 overexpression has a protective effect in the rat β -cell line INS-1 exposed to IL-1 β and/or IFN- γ (11) and it is able to drastically reduce the severity of arthritis in autoimmune rheumatoid arthritis (20).

Our data point to the existence of a complex cross-talk occurring among SOCS proteins and probably between SOCS and other proteins to regulate the action of different cytokines in diabetic human pancreatic islets.

The fact that SOCS-1, -2 and -3 expression is up-regulated, in human islets, in response to cytokines both *in vitro* and more importantly during islet inflammation *in vivo*, strongly suggests a possible involvement of all these three SOCS in the modulation of cytokine action(s) in human pancreatic islets.

In conclusion, we have shown that human islet cells express a class of cytokine modulators such as SOCS proteins and that the expression of such molecules is increased by proinflammatory cytokines *in vitro* and during islet inflammation in type 1 diabetes. These

findings suggest that modulation of SOCS expression, as suggested in animal models (10, 11, 13), may represent a useful strategy to counteract the deleterious effects of proinflammatory cytokines in order to protect islet β -cells from immune-mediated destruction which takes place during type 1 diabetes development as well as during allograft rejection or recurrence of islet autoimmunity in type 1 diabetic patients transplanted with a whole pancreas or with purified islets.

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