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SHORT COMMUNICATION

Cytokine-induced osteoprotegerin expression protects pancreatic beta cells through p38 mitogen-activated protein kinase signalling against cell death

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Abstract
Aims/hypothesis Pro-inflammatory cytokines play a crucial role in immune-mediated beta cell destruction, an essential mechanism in the pathogenesis of type 1 diabetes mellitus. Microarray analysis recently identified osteoprotegerin (*OPG*; now known as tumour necrosis factor receptor superfamily, member 11b [TNFRSF11B]) as a cytokine-induced gene in beta cells. The aim of the present study was to characterise the functional role and signalling pathways of OPG that are involved in cytokine-induced beta cell death.
Materials and methods As cellular models, the rat beta cell line INS-1E and human primary pancreatic islets were employed. The effects of IL-1β and TNF-α on *OPG* expression were characterised by northern blot and immunoassay. The effect of OPG on beta cell survival was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Signalling pathways were evaluated by western blot analysis using antibodies against p38 mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase and extracellular signal-regulated kinase 1/2.
Results The INS-1E cell line and primary pancreatic islets expressed *OPG* mRNA and secreted OPG protein, both of which were enhanced by IL-1β and TNF-α. Exposure to IL-1β resulted in sustained phosphorylation of p38 MAPK in INS-1E cells and subsequent cell death. Administration of exogenous OPG prevented both IL-1β-induced beta cell death and sustained p38 MAPK phosphorylation.
Conclusions/interpretation Our data indicate that cytokine-induced production of OPG may protect beta cells from further damage. This protective effect is, at least in part, mediated through inhibition of p38 MAPK phosphorylation. Thus OPG is an autocrine or paracrine survival factor for beta cells.

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Abbreviations

ECL	electrochemoluminiscence
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OPG	osteoprotegerin
RANK	receptor activator of nuclear factor-κB
RANKL	receptor activator of nuclear factor-κB ligand
TRAIL	TNF-related apoptosis-inducing ligand

Introduction

T cells and pro-inflammatory cytokines play a crucial role in the pathogenesis of type 1 diabetes mellitus [1]. While islet cell transplantation has emerged as a therapy for selected patients [2], cellular stress provoked by cytokines may limit the outcome of this procedure [3]. Several studies indicate that cytokines act through phosphorylation of stress kinases, including c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) [1, 4]. Osteoprotegerin (OPG; now known as tumour necrosis factor receptor superfamily, member 11b [TNFRSF11]) is a glycoprotein that neutralises receptor activator of nuclear factor κB ligand (RANKL; now known as tumour necrosis factor [ligand] superfamily, member 11 [TNFSF11]) [5]. RANKL promotes the differentiation and activation of osteoclasts and dendritic cells, and is essential for bone biology and immune functions [5].

We have shown that *OPG* is expressed in the pancreas [6]. In addition, a microarray identified *OPG* as a cytokine-induced gene in beta cells [7]. Here, we tested the hypothesis that cytokines regulate *OPG* expression in beta cells and evaluated the signalling pathways of OPG in cytokine-induced beta cell death. We found that cytokines enhance OPG production and that exposure to IL-1β induced beta cell death and prolonged p38 MAPK activation, which was prevented by OPG.

Materials and methods

Material Antibodies were from Cell Signaling (Frankfurt, Germany) or from Santa Cruz (Heidelberg, Germany), electrochemoluminescence (ECL) detection reagents were from Amersham Bioscience (Little Chalfont, UK).

Cell culture INS-1E cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, ciprofloxacin (20 μg/ml), sodium pyruvate (1 mmol/l) and HEPES (10 mmol/l). Primary human islets were from the islet cell transplantation programme at the University of Giessen [2]; they were used after Institutional Review Board approval and informed consent had been obtained and were cultured in tissue culture medium-199 and identical supplements.

Northern blot analysis RNA was isolated with a kit (RNeasy; Qiagen, Hilden, Germany), analysed on a denaturing agarose gel and transferred to nylon membranes. The cDNAs were radiolabelled using random primer DNA labelling [6].

RT-PCR analysis RANKL and receptor activator of nuclear factor-κB (*RANK*; now known as tumour necrosis factor receptor superfamily, member 11a [TNFRSF11A]) gene expression by human islets was analysed by RT-PCR using 30 and 35 cycles, respectively, [8]. As positive controls, RNA from SaOS-2 osteosarcoma cells, buffy coat cells and dendritic cells was used.

ELISA measurement of secreted OPG OPG protein from rat INS-1E cells was measured using a murine OPG antibody pair (R & D Systems, Minneapolis, MN, USA) and a streptavidin-horseradish peroxidase construct (CV: 5.1%, lower limit of detection: 62 pg/ml). Human OPG protein was determined as described [6]. Protein values were normalised for total protein content.

Assessment of cell survival INS-1E cells were cultured for 24 h in the presence of IL1β with or without recombinant mouse OPG (R & D Systems). Cell survival was measured by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h. The supernatant fraction was removed, the tetrazolium dissolved in DMSO and absorbance was measured at 570 nm.

Western blot analysis Cells were lysed and 20 μg of protein were separated by SDS-PAGE and transferred on to nitrocellulose. Membranes were incubated at 4°C with antibodies to p38 MAPK, JNK, extracellular signal-regulated kinase (ERK)-1/2 and β-actin. Signals were detected using horseradish peroxidase-conjugated anti-rabbit IgG antibody and ECL detection reagent.

Statistical analysis Values are expressed as the mean ± SD of triplicate (OPG secretion) or duplicate (MTT assay) measurements. Student's paired *t* test and multiple measurement ANOVA corrected by Student–Newman–Keul's test were employed. A p value <0.05 was considered statistically significant.

Results

Our finding of pancreatic *OPG* expression did not distinguish between endocrine or exocrine expression [6]. Since a recent microarray suggested that OPG is induced by cytokines in beta cells [7], we focussed on the endocrine pancreas using INS-1E cells. Exposure to the pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ enhanced *OPG* mRNA levels (Fig. 1a). The induction of *OPG* mRNA by cytokines was paralleled by an increase of OPG protein from 0.96±0.03 to 1.61±0.07 ng/ml after 6 h ($p<0.00001$). We also employed human pancreatic islets and found a similar induction of *OPG* mRNA levels (Fig. 1a), while OPG protein levels increased from 20.2±0.36 to 154±4.57 pmol/l ($p<0.00001$).

Next we characterised IL-1β, TNF-α and IFN-γ separately in dose–response and time-kinetic studies using INS-1E cells. IL-1β caused a dose- and time-dependent increase of *Opg* mRNA levels with a maximum effect at 1 ng/ml and after 24 h of exposure (Fig. 1b). At a dose of 10 ng/ml, IL-1β increased OPG secretion in INS-1E cells from 1.1±0.09 ng/ml at baseline to 5.7±0.39 ng/ml after 48 h ($p<0.00001$) (Fig. 1c). TNF-α enhanced *Opg* mRNA levels with a maximum effect at 10 ng/ml and after 6 h (Fig. 1b). Moreover, TNF-α transiently induced an increase of OPG secretion from 0.73±0.02 ng/ml at baseline to 2.72±0.11 ng/ml after 6 h ($p<0.00001$) (Fig. 1d). IFN-γ slightly enhanced *Opg* mRNA levels after 48 h (Fig. 1b), whereas IL6 had no

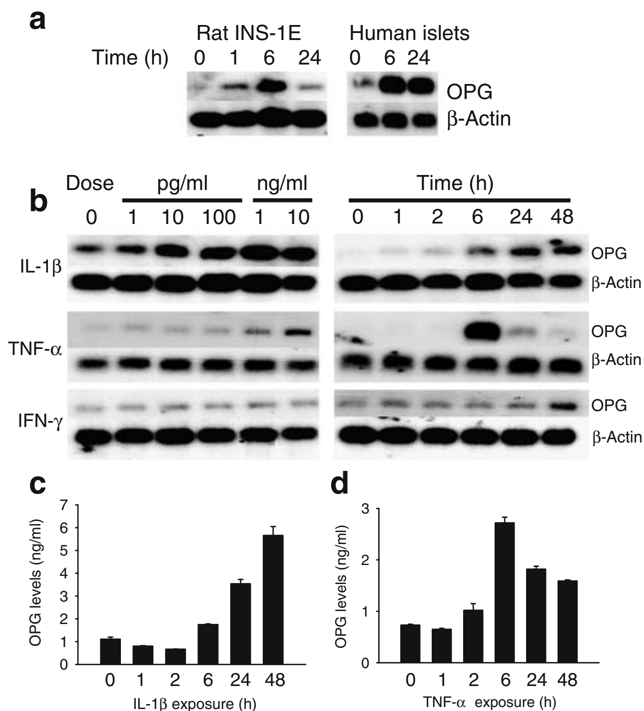

Fig. 1 OPG expression by pancreatic beta cells is upregulated by pro-inflammatory cytokines. **a** Rat INS-1E cells and primary human islets were stimulated with a cytokine combination consisting of IL-1β (10 ng/ml), TNF-α (10 ng/ml) and IFN-γ (50 ng/ml) for up to 24 h. *OPG* mRNA levels were assessed by northern blot analysis along with the housekeeping gene β-actin. **b** For dose responses, rat INS-1E cells were stimulated with IL-1β, TNF-α and INF-γ at varying doses ranging from 1 pg/ml to 10 ng/ml for 24 h. For time kinetics, rat INS-1E cells were stimulated with IL-1β, TNF-α and INF-γ for different time intervals (up to 48 h) at a constant concentration of 10 ng/ml. *Opg* and β-actin mRNA levels were assessed by northern blot analysis. **c**, **d** OPG secretion into the beta cell supernatant fraction was measured from the time kinetics experiment **b** by an ELISA. OPG protein values were normalised for total protein content and are expressed as the mean±SD of triplicate measurements, $p<0.00001$ by ANOVA

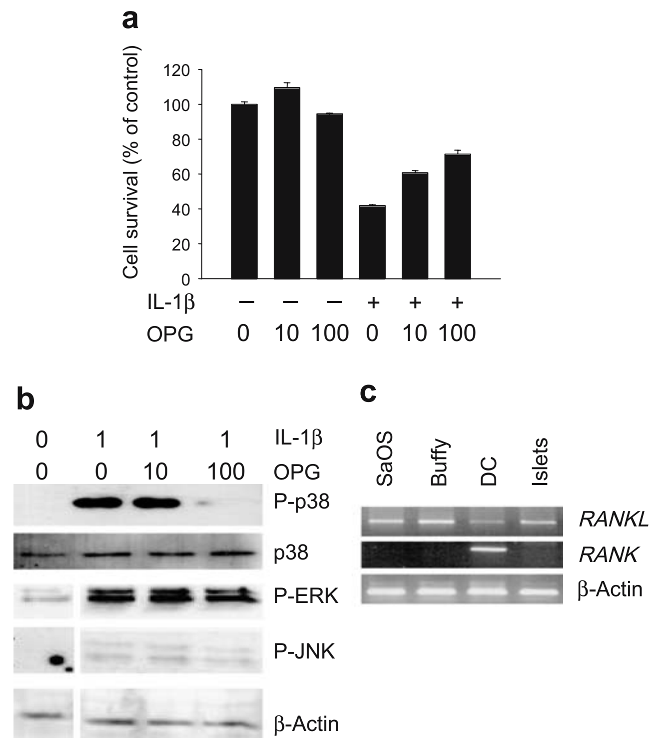

Fig. 2 OPG protects pancreatic beta cells from IL-1β-induced cell death and abrogates IL-1β-induced p38 MAPK activation. **a** Rat INS-1E cells were incubated for 24 h with IL-1β (0.1 ng/ml) and with or without OPG at 10 or 100 ng/ml; cell survival was measured by an MTT assay. Values are expressed as the mean±SD of triplicate measurements ($p<0.05$ by t test for control vs IL-1β treatment; $p<0.05$ by ANOVA for various OPG concentrations in IL-1β-treated cells). **b** Western blot analysis demonstrating p38 MAPK in its phosphorylated (P-) and non-phosphorylated form, phosphorylated JNK and ERK-1/2 MAPK, and β-actin. INS-1E cells were exposed to IL-1β (1 ng/ml) with or without OPG at 10 or 100 ng/ml for 30 min. Note that IL-1β phosphorylated all three MAPK stress kinases, but only p38 MAPK activation was specifically blocked by OPG. **c** RT-PCR analysis of *RANKL* and *RANK* gene expression in human islets. RNA was isolated from unstimulated primary human islets (Islets) and 2 µg of total RNA were subjected to RT-PCR. As positive controls, RNA was analysed from SaOS-2 osteosarcoma cells (*SaOS*), human buffy coat cells (*BC*) or dendritic cells (*DC*). Expression of *RANKL* (486 bp), *RANK* (497 bp) and the housekeeping gene β-actin (117 bp) is shown on an agarose gel stained with ethidium bromide

effect. These data suggest that IL-1β and TNF-α were the main inducers of OPG in beta cells.

To define the biological role of OPG in beta cells, INS-1E cells were pre-incubated with recombinant mouse OPG (which is biologically active in rat cells) for 1 h prior to 24 h of stimulation with IL-1β, which reduced cell survival by 58% ($p<0.05$) (Fig. 2a). OPG protected beta cells from IL-1β-induced cell death in a dose-dependent fashion (increase by 70%, $p<0.05$) (Fig. 2a). To elucidate how OPG protects beta cell survival, we studied the signal transduction involved in cytokine-induced beta cell death and its modulation by exogenous OPG. IL-1β phosphorylated the stress kinase p38 MAPK, as well as other MAPKs including JNK and ERK-1/2 after 30 min (Fig. 2b). While OPG alone had no effect on the phosphorylation of p38 MAPK, JNK and ERK-1/2 MAPK, it prevented prolonged p38 MAPK activation by IL-1β in a dose-dependent fashion without affecting the activation of JNK and ERK-1/2 MAPK (Fig. 2b). To characterise gene expression of other components of the RANKL-RANK-OPG system, we performed RT-PCR and found that *RANKL*, but not *RANK* was expressed by human islets (Fig. 2c).

Discussion

Our data verify the results of a microarray study [7] and demonstrate that *OPG* expression in beta cells is stimulated by IL-1β and TNF-α. Upregulation of *OPG* expression was detected at the mRNA and protein level, occurred in a dose- and time-dependent fashion and was substantial in magnitude (eightfold). Similar results were obtained in the rat cell line INS-1E and human islets, demonstrating that this regulation is independent of species and disease model. These findings indicate that stimulation of OPG by pro-inflammatory cytokines is a physiologically relevant response. IL-1β was most effective in inducing OPG, while TNF-α only transiently upregulated OPG. Therefore, further cytokine experiments were conducted with IL-1β.

In search of the function of beta-cell-derived OPG, we found that OPG abrogated IL-1β-induced p38 MAPK activation in INS-1E cells and prolonged cell survival. These data are consistent with earlier findings that p38 MAPK activation is required for IL-1β-induced beta cell death [4]. In contrast to RT-PCR findings from the type 1 diabetes base (http://t1dbase.org/), we detected RANKL, but not RANK in beta cells.

RANKL inhibition by OPG is the major mechanism in the maintenance of bone metabolism [5]. However, alternative pathways independent of RANKL binding have been described for OPG. First, OPG may also bind and neutralise TNF-related apoptosis-inducing ligand (TRAIL), thus preventing TRAIL-induced apoptosis in susceptible cells [9]. This mechanism has been demonstrated in tumour cells and beta cells [10]. However, we did not observe TRAIL-induced apoptosis in INS-1E cells (data not shown). Second, OPG binds to cells that express syndecan-1, probably via its heparin-binding domain [11]. Syndecan-1 expression by myeloma cells has been identified as a mechanism to sequester and degrade OPG [11]. Since we did not characterise expression of syndecans in pancreatic beta cells, we cannot exclude this possibility. Third, as yet unidentified receptors for OPG may exist on the cell surface, which confer direct OPG effects. Here, we identified inhibition of IL-1β signalling via p38 MAPK as a novel mechanism of OPG in pancreatic beta cells. IL-1β-induced beta cell death required sustained p38 MAPK activation [6], which in our study was abrogated by OPG, indicating that OPG is a crucial modulator of beta cell integrity. Our data do not exclude the possibility that other MAPKs contribute to beta cell death.

A potential limitation of this study was that we were unable to perform extensive signalling studies in human islets due to limited cell supply. In addition, the effect of RANKL on MAPK and survival of beta cells was not evaluated, because pancreatic islets did not express *RANK* mRNA. Because of its autocrine or paracrine mode of action, OPG released upon cytokine-induced stress may act both as a local and systemic safeguard within the pancreas to limit beta cell damage. This potential protective effect should be evaluated to optimise cell survival in the clinical setting of islet cell transplantation.

In conclusion, IL-1β and TNF-α induced OPG production by beta cells, which may limit cell damage, indicating that OPG is an autocrine or paracrine survival factor for beta cells. This protective effect is mediated through inhibition of the p38 MAPK pathway.

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Duality of interest The authors are not aware of any conflict of interest.

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