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Oncostatin M up-regulates the ER chaperone Grp78/BiP in liver cells

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ABSTRACT

OSM, a cytokine of the IL-6-type cytokine family, regulates inflammatory processes (like the acute phase response), tissue remodeling, angiogenesis, cell differentiation and proliferation. Inflammation is discussed to favor carcinogenesis and the inflammatory cytokine OSM was lately described to up-regulate HIF-1α, whose up-regulation is also observed in many cancers. In this study we demonstrate that OSM, and to a lesser degree IL-6, induces the expression of Grp78/BiP, an ER chaperone associated with tumor development and poor prognosis in cancer. In contrast, IFN-γ or TNF-α had no effect on Grp78 expression. The up-regulation seems to be specific to liver cells, as it occurs in hepatocytes and hepatoma cells but not in prostate, melanoma, breast or kidney cells. OSM does not lead to up-regulation of Grp94, enhanced XBP-1 mRNA splicing or phosphorylation of eIF2α, indicating that it is not associated to a general ER stress response. Analysis of the underlying mechanism showed that Grp78 is up-regulated by transcriptional processes which are to the greater part, though not completely, dependent on MEK/Erk activation.

1. Introduction

The family of interleukin (IL)-6-type cytokines includes IL-6, IL-11, oncostatin M (OSM), leukemia inhibitory factor (LIF), cardiotrophin 1 and ciliary neurotrophic factor (CNTF). All of these cytokines bind to membrane receptor complexes containing the signal transducing receptor chain gp130 (glycoprotein 130). OSM signals either via an OSMR/gp130 or a LIFR/gp130 receptor complex [1]. Upon binding of OSM to its receptor complex, Janus kinases (Jaks) become activated. These Jaks (mainly Jak1) phosphorylate certain tyrosine residues within the receptor chains leading to the formation of docking sites for signaling molecules with matching SH2 domains, like signal transducers and activators of transcription (STAT) or adaptor proteins for the mitogen-activated protein kinase pathway (MAPK) [2,3]. STAT3 as well as the extracellular signal-regulated kinase (Erk) 1/2 and p38 are the major signaling cascades that are activated by OSM. OSM is a pleiotropic cytokine produced by various different cell types e.g. monocytes, macrophages and T cells. It is involved in cell differentiation, in proliferation, in inflammatory processes (e.g. acute phase response), in hematopoiesis and in angiogenesis [1,2,4]. In addition OSM functions in tissue remodeling and is an important factor for liver development and regeneration [5,6].

Grp78 (glucose-regulated protein 78) was discovered in 1977 [7]. Its alternative name BiP (immunoglobulin heavy chain binding protein) originates from the identification of Grp78 as cofactor of the immunoglobulin assembly [8]. It belongs to the heat shock protein 70 kDa (Hsp70) family and is its most abundant member in the endoplasmic reticulum (ER) [9]. Furthermore, a cytosolic form of Grp78 has been reported [10]. There are also studies describing extracellular and cell surface-bound BiP [11,12]. Like all Hsp70 family members, Grp78 binds and hydrolyzes ATP, which controls its chaperoning function. Hsp70 proteins are in an "open" configuration when ATP is bound. Via the substrate binding domain (SBD) of the chaperone, hydrophobic oligopeptides within more or less unfolded polypeptides are recruited to the folding complex. Binding of substrates to the SBD inhibits unproductive interactions of the polypeptide and favors productive folding and assembly that occurs concomitant with release from Hsp70 [13].

Besides its role in folding and assembly of newly synthesized proteins, Grp78 is a major regulator of ER homeostasis. Sensing critical amounts of unfolded proteins, Grp78 can trigger the unfolded protein response (UPR) via dissociation from its interaction partners PERK, ATF6 and IRE1α, which will subsequently lead to activation of ER stress responses involving an induced expression of ER chaperones to increase the folding capacity of the ER [14]. Known inducers of ER stress such as tunicamycin (an inhibitor of N-linked glycosylation) and thapsigargin (an inhibitor of the ER Ca^{2+}-ATPase)

Abbreviations: Erk, extracellular signal-regulated kinase; Grp, glucose-regulated protein; HIF, hypoxia-inducible factor; IFN, interferon; IL, interleukin; Jak, Janus kinase; MAPK, mitogen-activated kinase; OSM, oncostatin M; STAT, signal transducer and activator of transcription.

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result in a strong increase of Grp78 expression [15,16]. In tumor cells Grp78 expression is associated with proliferation, survival, and resistance against chemotherapeutics [17].

IL-6-type cytokine signaling via gp130 has been associated with the development of hepatocellular carcinoma [18,19]. Not much is known about possible interactions between IL-6-type cytokines and the ER chaperones Grp78 and Grp94. An increased expression of Grp78 or Grp94 induced by IL-6-type cytokines has not been shown previously. Chen et al. [20] could show an increase of Grp78 in burn-injured mice. They speculate that this effect could be mediated by IL-6 since this cytokine is also produced upon burn injury. OSM and IL-6 are known are both inducer of the acute phase response, a process where large amounts of proteins are produced and secreted by the liver [21,22]. Thus, an increase of ER chaperone levels would maybe be beneficial or even necessary for the acute phase response.

In this study we investigated whether inflammatory cytokines can induce the expression of Grp78 or Grp94. Our results show that the expression of Grp78 but not of Grp94 can be induced by OSM in hepatocytes/hepatoma cells and we analyze the underlying mechanism.

2. Materials and methods

2.1. Cell culture and reagents

HepG2 hepatoma cells were maintained in DMEM/NUT-MIX-F12 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (PAA, Dartmouth, MA, USA), 100 mg/L streptomycin, and 60 mg/L penicillin (Cytogen, Princeton, NJ, USA). The human hepatocyte cell line PH5CH8 [23] was kindly provided by Dr. Kato, Okayama. The human hepatoma cell line Huh-7 as well as HEK cells, the breast cancer cell line MCF-7 and DU-145 prostate cancer cells were cultured in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (PAA, Dartmouth, MA, USA), 100 mg/L streptomycin, 60 mg/L penicillin (Cytogen, Princeton, NJ, USA) and 25 mM Hepes (Lonza, Basel, Switzerland). The melanoma cell line FM55M1 and the human prostate cancer cell line PC-3 were cultured in RPMI medium (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (PAA, Dartmouth, MA, USA), 100 mg/L streptomycin, 60 mg/L penicillin (Cytogen, Princeton, NJ, USA) and 0.5 mM L-Glutamine (Lonza, Basel, Switzerland).

Cells were grown at 37 °C in a water-saturated atmosphere at 5% CO_2. For glucose starvation the cells were cultured in glucose-free DMEM medium. HepG2 cells were transiently transfected using the TransIT LT1 reagent (Mirus, Madison, WI, USA) according to the manufacturer's recommendations. Stable knockdowns of STAT3 or HIF-1α in HepG2 cells were generated using pGENEClip vectors encoding shRNA against STAT3 or HIF-1α (SAB Biosciences, Frederick, MA, USA).

Human recombinant OSM, IL-6, IFN-γ and TNF-α were from Peprotech (Rocky Hill, NJ, USA). Actinomycin D, cycloheximide, tunicamycin, thapsigargin, Jak inhibitor I and U0126 were from Calbiochem (San Diego, CA, USA).

2.2. Western blot analysis and antibodies

Cells were lysed on the dish with lysis buffer containing 30 mM Tris/HCl pH6.7, 5% glycerol, 2.5% mercaptoethanol, 1% SDS. The lysates were further analyzed by SDS-PAGE and Western blotting. All experiments were performed at least three times. A representative of these independent biological experiments is shown. Quantification of Western blots was performed using ImageJ software. Antibodies against HIF-1α, phospho-STAT1, phospho-STAT5, STAT1, STAT3 and FIN-13 were from BD Transduction Laboratories (San Diego, CA, USA). Antibodies against Grp78, Grp94, phospho-STAT3, phospho-Erk1/2, phospho-p38, p38, phospho-eIF2α and eIF2α were from Cell Signaling (Danvers, MA, USA). The antibodies against Erk1/2 and STAT5 were purchased from Santa Cruz (Santa Cruz, CA, USA). ECL signals were detected as described [24]. Before re-probing, blots were stripped as described before [3].

2.3. Reporter gene assays

HepG2 cells were transfected with 1 μg of the β-galactosidase control plasmid (pCH110, GE Healthcare, Piscataway, NJ, USA) and 1.5 μg of the Grp78 promoter reporter gene construct comprising 374 bp of the human Grp78 promoter [25] kindly provided by Dr. Kyoungsook Park. 24 h after transfection, the cells were treated with the different stimuli as described in the figure legends. Cell lysis and luciferase assays were performed using the Promega luciferase assay system (Promega, Madison, WI, USA). All experiments were performed at least in triplicate and technical triplicates were also performed within one experiment. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector. For all experiments the luciferase activity values after OSM stimulation were additionally normalized to values from control unstimulated cells.

2.4. Quantitative real-time PCR

Total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction with additional on-column DNase I digestion. Quantity and purity of RNA samples was assessed using a NanoDrop ND2000 spectrophotometer. 500 ng of total RNA was reverse transcribed in a 10 μL reaction volume containing 50 μM oligo$(dT)_{20}$ (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (NEB, Ipswich, MA, USA), 0.1 M DTT (GE Healthcare, Piscataway, NJ, USA), 5× RT buffer (250 mM Tris–HCl pH = 8.3, 375 mM KCl, 15 mM $MgCl_2$), 7.5 units MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA) and 3 units RNase inhibitor (Applied Biosystems, Carlsbad, CA, USA). Thermal cycling conditions included an initial denaturation step of the RNA template and oligo$(dT)_{20}$ primer at 65 °C for 5 min followed by the addition of RT buffer, DTT and enzymes and incubation at 42 °C for 1 h. MultiScribe was heat-inactivated at 85 °C for 5 min. The cDNA was additionally treated with 2 units of RNase H (NEB, Ipswich, MA, USA) at 37 °C for 20 min. Quantitative real-time PCR (qPCR) was carried out on a CFX96 Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Standard curves using four 10-fold dilutions (1×, 0.1×, 0.01×, 0.001×) were produced to ensure that the amplification efficiencies were similar and in the range of 95–105%. The mRNA level of each target gene was normalized to the relative amount of the housekeeping gene TBP. The comparative threshold cycles (C_T) method, $2^{-\Delta C_T}$, was used to calculate the changes in gene expression for each target gene.

2.5. XBP-1 mRNA splicing

Total RNA from HepG2 cells was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The concentration of isolated RNA was measured using a NanoDrop ND2000 spectrophotometer. Constant amounts of 500 ng of total RNA were reversely transcribed as described for qPCR. The PCR was performed using Platinum Taq polymerase under the following conditions: 95 °C for 5 min, followed by 35 cycles of 50 s 95 °C, 50 s 50 °C and 30 s at 72 °C. The last step was incubation at 72 °C for 10 min. The XBP-1 primers (forward: CCTTGTAGTTGAGAACCAGG; reverse: GGGGCTTGGTATATATGTGG) amplify the unspliced XBP1 mRNA resulting in a 442 bp band and the spliced form with a band at 416 bp on an agarose gel [26].

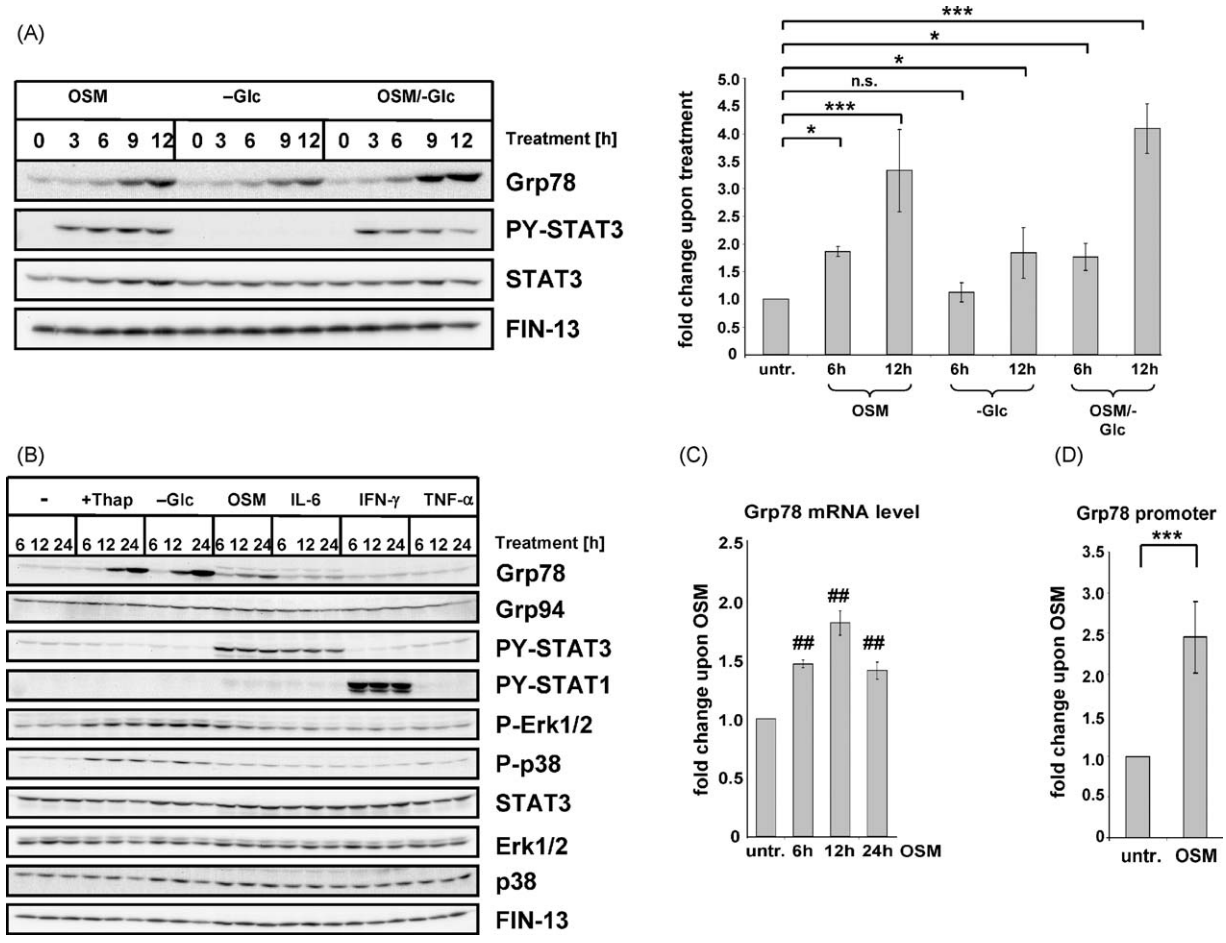

Fig. 1. OSM up-regulates Grp78 at the transcriptional and protein level. (A) HepG2 cells were treated for the indicated periods of time with OSM (10 ng/mL) or cultured in glucose-free medium (-Glc). Lysates of the cells were separated by SDS-PAGE, and Western blots of the membranes were detected with Grp78, phospho-STAT3, STAT3 and FIN-13 antibodies. Protein levels were quantified using ImageJ software, Grp78 levels were normalized to FIN-13. $^{*}P < 0.05$, $^{***}P < 0.001$. (B) HepG2 cells were treated for the indicated periods of time with OSM, IL-6, IFN-γ or TNF-α (10 ng/mL each), with thapsigargin (Thap) (1 μM) or cultured in glucose-free medium (-Glc). Lysates of the cells were separated by SDS-PAGE, and Western blots of the membranes were detected with antibodies directed against Grp78, Grp94, phospho-STAT3, STAT3, phospho-STAT1, P-Erk1/2, Erk1/2, phospho-p38, p38, and FIN-13. (C) HepG2 cells were stimulated for the indicated periods of time with OSM (10 ng/mL). RNA was prepared and Grp78 mRNA levels were analyzed by quantitative PCR. $^{\#\#}P < 0.001$, statistically significant difference with respect to untreated control cells. (D) HepG2 cells were transfected with the luciferase reporter gene plasmid pGL3-Grp78-Luc and the β-galactosidase expression vector pCH110. 24 h post-transfection the medium was exchanged and the cells were treated for additional 16 h with OSM (10 ng/mL) before lysates were prepared and the reporter gene activity was measured. $^{***}P < 0.001$.

2.6. Statistical analysis

Each experiment was performed at least three times. Representative data are shown. Statistical analysis (one-way ANOVA) with Tukey–Kramer multiple comparison test or paired *t* test (promoter study in Fig. 1D) were performed with GraphPad InStat version 3.00 software. Error probabilities <0.05 were considered to be significant.

3. Results

3.1. OSM induces the expression of Grp78 in HepG2 cells

Withdrawal of glucose or stimulation of HepG2 hepatoma cells with OSM leads to an increase in Grp78 protein levels. For both treatments the effect can be seen after 6 h and Grp78 protein levels continuously increase until 12 and 24 h (Fig. 1A and B). As a control for the activity of OSM the phosphorylation of STAT3 was monitored. To investigate whether other cytokines are also able to induce the expression of Grp78, HepG2 cells were treated with OSM, IL-6, IFN-γ and TNF-α. Thapsigargin and the withdrawal of glucose were used as positive controls for the induction of Grp78 (Fig. 1B). OSM and to a lesser extent also IL-6 are the only cytokines tested that lead to an increase in Grp78 protein levels. Interestingly, the levels of Grp94 were unaffected by IFN-γ treatment in HepG2 cells although it was previously reported to up-regulate Grp94 [27].

OSM treatment also affected Grp78 mRNA levels in HepG2 cells (Fig. 1C): a slight (approx. 1.8-fold), but reproducible increase in Grp78 mRNA can be observed which peaks at 12 h treatment. In contrast, OSM did not change mRNA levels of cytosolic Hsp70 (data not shown). To investigate whether OSM affects the activity of the Grp78 promoter we transfected HepG2 cells with a human Grp78 reporter gene construct (Fig. 1D). OSM treatment increased the luciferase activity about 2.5-fold. Thus, in HepG2 hepatoma cells OSM leads to an up-regulation of Grp78 protein, mRNA and to an increased activity of a reporter gene under the control of the human Grp78 promoter.

3.2. OSM-mediated up-regulation of Grp78 protein levels is due to de novo transcription but not due to regulation of protein stability

To further investigate whether *de novo* transcription of the Grp78 gene is involved in the induction of Grp78 by OSM we treated HepG2 cells with the transcriptional inhibitor actinomycin D (Fig. 2A and Supplementary Fig. 1). As previously observed, the treatment with OSM or tunicamycin or glucose starvation induces the expression of Grp78 protein. However, this up-regulation was

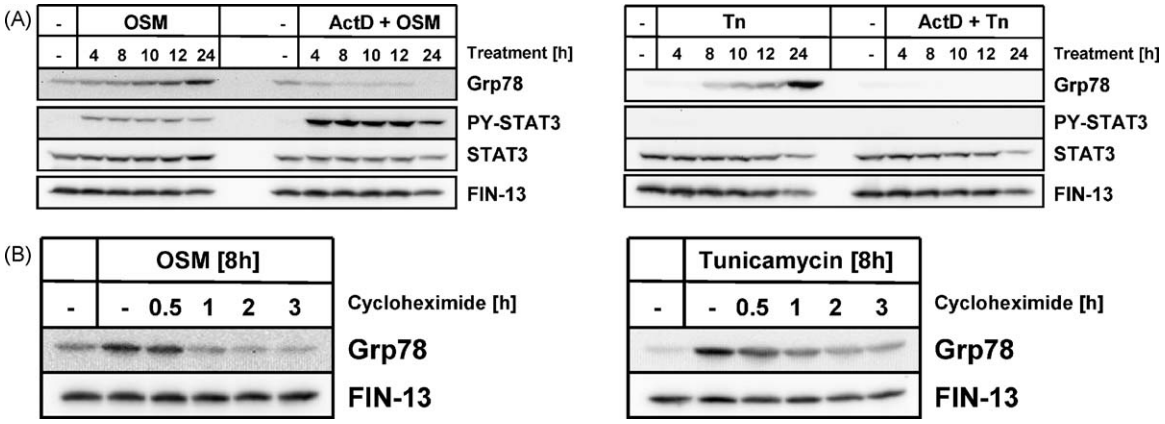

Fig. 2. OSM up-regulates Grp78 by transcriptional processes and does not induce a stabilization of Grp78 protein. (A) HepG2 cells were treated for the indicated periods of time with OSM (10 ng/mL) or tunicamycin (Tn) (1 μM) in the presence of DMSO alone or actinomycin D (ActD) (5 μg/mL). Western blots of lysates separated by SDS-PAGE were detected with Grp78, phospho-STAT3, STAT3 and FIN-13 antibodies. (B) HepG2 cells were stimulated for 8 h with tunicamycin (1 μM) or OSM (10 ng/mL). Then, cycloheximide (10 μg/mL) was added for the indicated periods of time before lysates were prepared. Western blots were detected with Grp78 and FIN-13 antibodies. For the Grp78 detection the Western blots were exposed so that the band intensity for both treatments was comparable.

abolished if the cells were treated with actinomycin D. Grp78 protein levels slowly declined over time with kinetics similar to the one for Grp78 mRNA which was reported to be between 4 and 5.5 h [28,29]. This indicates that the induction of Grp78 by OSM as well as by glucose starvation and by tunicamycin (Fig. 2A and Supplementary Fig. 1) involves gene transcription, thereby corroborating the data of the promoter assay (Fig. 1D).

A change in protein stability is another possible mechanism leading to an increase of protein levels. Therefore we used cycloheximide, an inhibitor of protein translation, to roughly evaluate the half-life of Grp78 (Fig. 2B). We used OSM and tunicamycin to induce the expression of Grp78 and followed the decrease of Grp78 protein levels over time after treatment with cycloheximide. No significant difference was observed between the protein stabilities of Grp78 induced by OSM or by tunicamycin (the protein half-life was estimated to be between 30 and 60 min), indicating that OSM does not affect the protein half-life of Grp78.

3.3. OSM-mediated induction of Grp78 is restricted to hepatocytes/hepatoma cells

We then performed OSM stimulations of 8 different cell lines to investigate if OSM induces Grp78 also in other cells. Therefore we treated HepG2, Huh-7 (both hepatoma cell lines), PH5CH8 (primary hepatocyte cells), PC-3, DU-145 (both prostate cancer cell lines), FM55M1 (melanoma cell line), MCF-7 (breast cancer cell line), and human embryonic kidney cells (HEK) for 6, 12 or 24 h with OSM (Fig. 3A). The activity of OSM was determined by monitoring STAT phosphorylation (Supplementary Fig. 2). Interestingly, OSM leads to an increase in Grp78 protein levels only in the hepatoma or hepatocyte cell lines HepG2, Huh-7 and PH5CH8 cells. In the other cell lines the amount of Grp78 is only increased by the treatment with tunicamycin (data not shown) but not upon OSM stimulation. Similar to HepG2 cells (Fig. 1C), the Grp78 mRNA level is also elevated by OSM in Huh-7 and PH5CH8 cells (around 2-fold induction) (Fig. 3B). Taken together, the OSM-mediated up-regulation of Grp78 is not a general effect but seems to be specific to hepatocytes and hepatoma cells.

3.4. OSM does not induce a classical ER stress response that could be responsible for the increase of Grp78 protein levels

The up-regulation of Grp78 expression under stress conditions is known to be mediated by pathways of the ER stress response. We tested if OSM can induce such signaling cascades. Therefore we analyzed the status of XBP-1 mRNA (Fig. 4A). As shown previously [26], the ER stress inducer tunicamycin is able to induce the splicing of XBP-1 mRNA which results in a PCR product of 416 bp. For OSM no splicing of XBP-1 mRNA was observed, indicating that OSM does not activate the ER stress signaling cascade via IRE1α/XBP-1.

Since the expression of Grp78 can be induced via the ER stress pathway PERK/eIF2α/ATF4, we investigated whether OSM activates this pathway. In HepG2 cells we could not observe an increase in eIF2α phosphorylation by OSM (Fig. 4B) compared to a significant increase upon tunicamycin treatment (data not shown). Also in Huh-7 and PH5CH8 cells no induction of eIF2α phosphorylation was observed (data not shown). These data were corroborated by studying the status of down-stream molecules of eIF2α signaling activation: The mRNA level of the transcription factor ATF4 and its target gene CHOP remained unaffected by OSM treatment whereas tunicamycin induces a significant increase (Fig. 4C).

Taken together, the absence of eIF2α phosphorylation, XBP-1 mRNA splicing and Grp94 up-regulation (Fig. 1B) indicates that the up-regulation of Grp78 by OSM is a rather selective process and does not seem to be associated to a general ER stress response.

3.5. Investigation of the involvement of Jaks, Erk1/2, HIF-1α and STAT3 in the OSM-mediated up-regulation of Grp78

OSM is known to induce the Jak1/STAT3- and the MAP kinase pathways (Erk1/2 and p38) and is an inducer of HIF-1α [30]. We have recently reported that OSM can induce the expression of transcriptionally active HIF-1α and thus we wondered whether HIF-1α might contribute to OSM-mediated up-regulation of Grp78, as hypoxia is known to lead to ER stress. Therefore, we generated HepG2 cells stably expressing shRNA directed against HIF-1α. Compared to "scrambled" control transfectants, OSM only marginally induces HIF-1α in these cells (Fig. 5A, left panel). However, OSM-induced Grp78 levels are not affected by HIF-1α suppression, indicating that HIF-1α is not involved in the up-regulation of Grp78.

To study the implication of STAT3 in the induction of Grp78 by OSM, stable HepG2 cells were generated in which STAT3 levels were suppressed by a shRNA plasmid targeting STAT3. OSM-induced expression of γ-fibrinogen, a known STAT3-dependent acute phase protein, was totally abrogated in these cells (Fig. 5A, right panel). In contrast, the STAT3 knockdown cells express similar levels of Grp78 as the control cells. It should be noted,

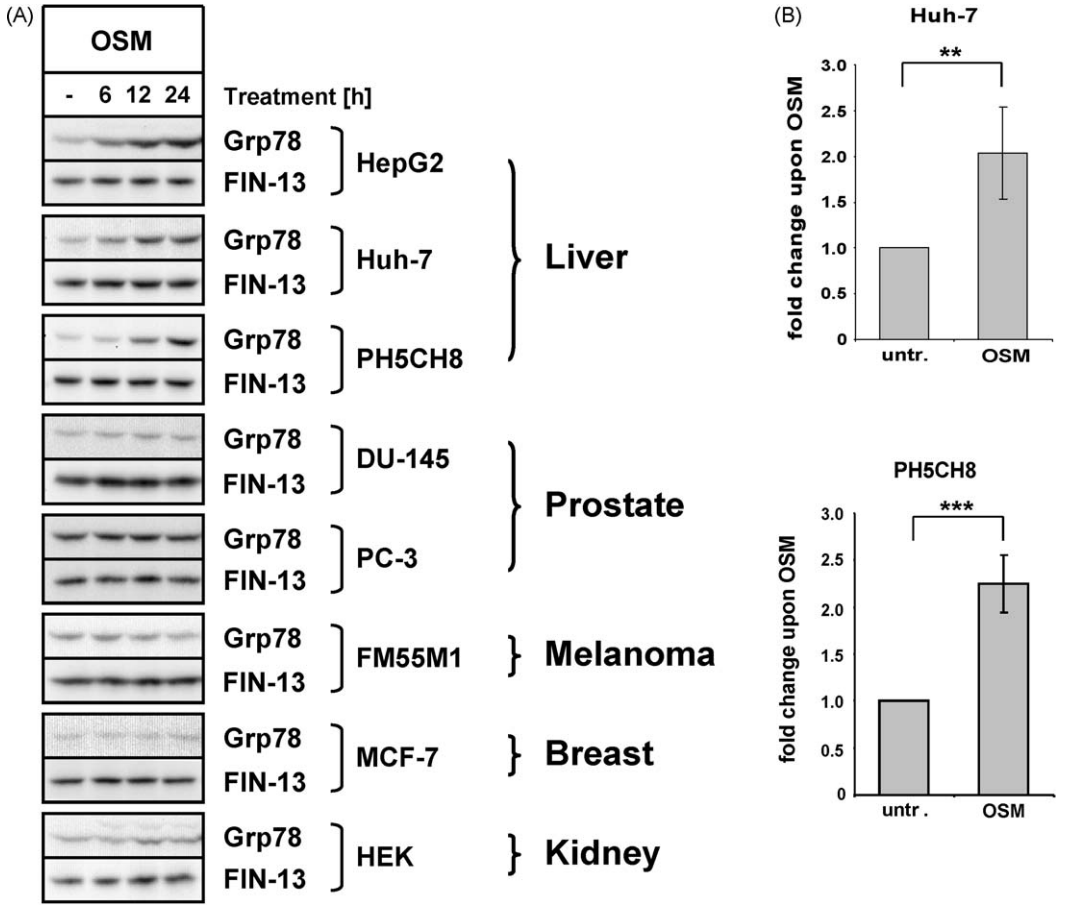

Fig. 3. OSM up-regulates Grp78 in liver cells. (A) The various cells were treated with OSM (10 ng/mL) for the indicated periods of time or were left untreated. Lysates of the cells were separated by SDS-PAGE, and Western blots were detected with Grp78 and FIN-13 antibodies. (B) Huh-7 and PH5CH8 cells were stimulated for 12 h with OSM (10 ng/mL). RNA was prepared and Grp78 mRNA levels were analyzed by quantitative PCR. $^{**}P < 0.01$, $^{***}P < 0.001$.

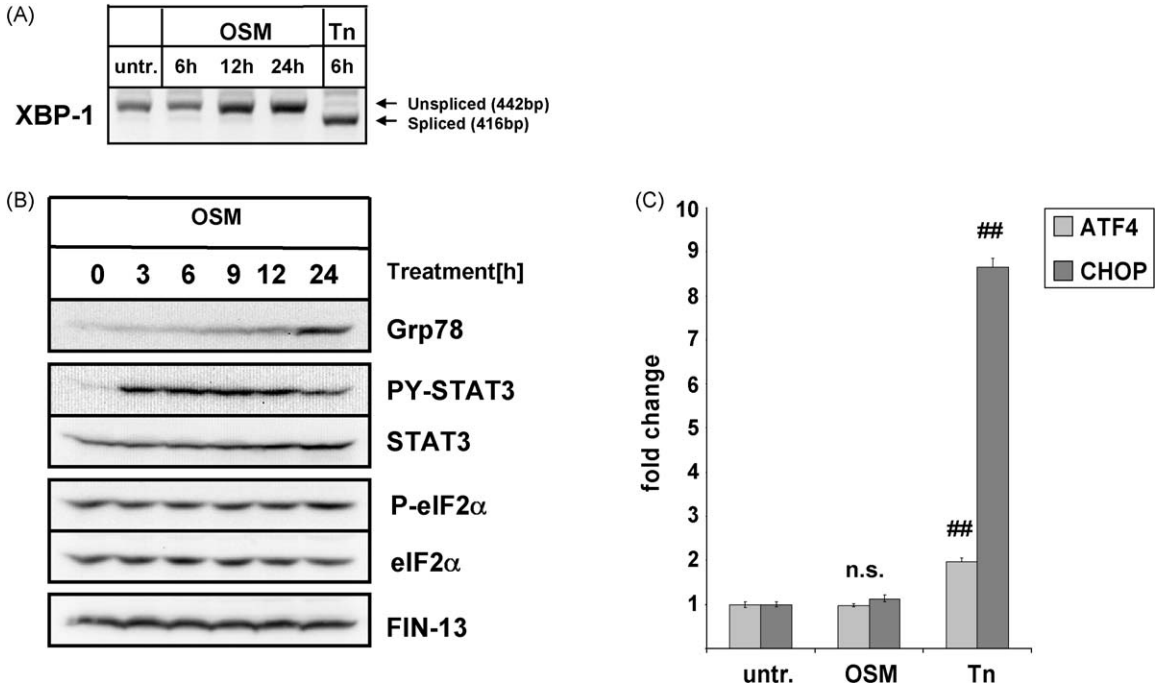

Fig. 4. OSM does not induce XBP1 mRNA splicing and eIF2α phosphorylation. (A) HepG2 cells were treated with OSM (10 ng/mL) or tunicamycin (Tn) (1 μM) for the indicated periods of time. RNA was prepared and the spliced and unspliced levels of XBP-1 were analyzed by semi-quantitative PCR. (B) HepG2 cells were treated with Oncostatin M (10 ng/mL) for the indicated periods of time. Lysates of the cells were separated by SDS-PAGE, and Western blots were detected with Grp78, phospho-STAT3, STAT3, phospho-eIF2α, eIF2α and FIN-13 antibodies. (C) HepG2 cells were treated with OSM (10 ng/mL) or tunicamycin (Tn) (1 μM) for 12 h. RNA was prepared and ATF6 and CHOP mRNA levels were determined by quantitative PCR. $^{\#\#}P < 0.001$, statistically significant difference with respect to untreated control cells.

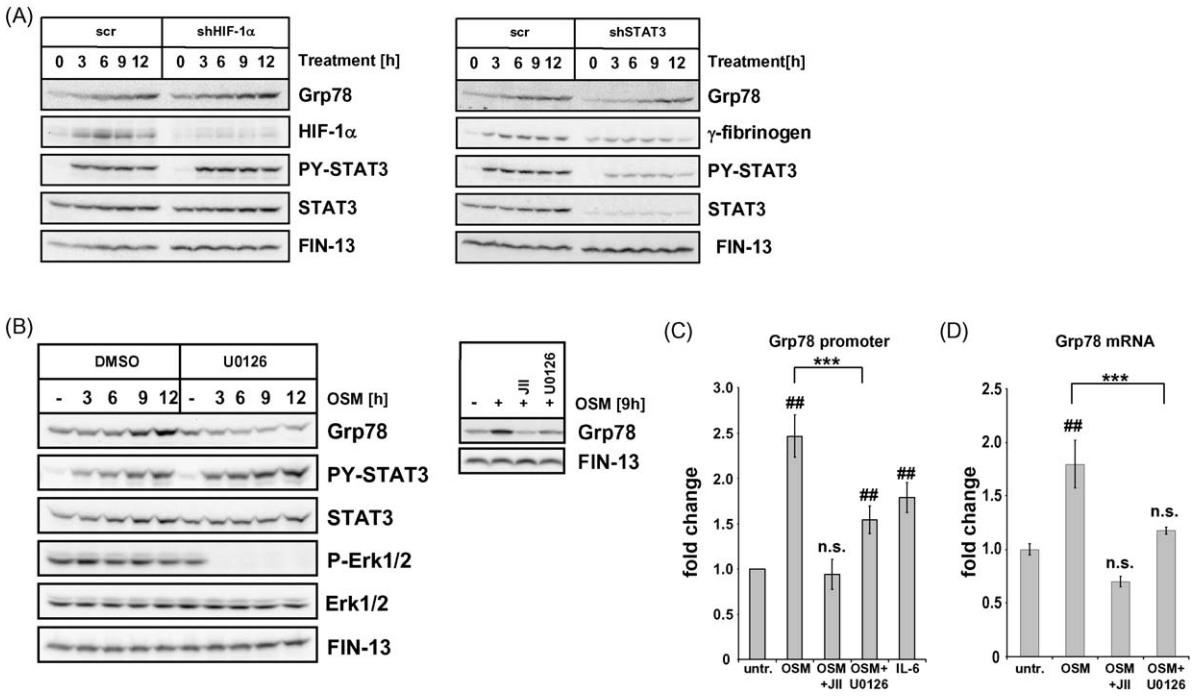

Fig. 5. Jak and Erk1/2 activation plays a role in OSM-induced Grp78 expression in liver cells. (A) HepG2 cells stably expressing either a scrambled RNA control plasmid, a shRNA plasmid targeting HIF-1α or a shRNA plasmid targeting STAT3 were stimulated with OSM (10 ng/mL) for the indicated periods of time. Lysates of the cells were separated by SDS-PAGE, and Western blots of the membranes were detected with HIF-1α, Grp78, phospho-STAT3, STAT3, γ-fibrinogen and FIN-13 antibodies as indicated. (B) HepG2 cells were cultured in FCS-free medium overnight. Right panel: cells were pretreated for 30 min with DMSO alone, U0126 (2.5 μM) or Jak inhibitor I (1 μM). Cells were then stimulated for 9 h with OSM (10 ng/mL). Left panel: after a 30 min pretreatment with U0126 (5 μM) or DMSO alone, cells were treated with OSM for 3, 6, 9 or 12 h. The lysates were used to prepare Western blots which were detected with antibodies directed against Grp78, phospho-STAT3, STAT3, phospho-Erk1/2, Erk1/2, and FIN-13. (C) HepG2 cells were transfected with the luciferase reporter gene plasmid pGL3-Grp78-Luc and the β-galactosidase expression vector pCH110. 24 h post-transfection the medium was exchanged and the cells were pretreated for 30 min with DMSO alone, Jak inhibitor I (2 μM) or U0126 (2.5 μM). Afterwards the cells were additionally treated for 16 h with OSM or IL-6 (10 ng/mL each) before lysates were prepared and the reporter gene activity was measured as described in experimental procedures. $^{\#\#}P < 0.001$, statistically significant difference with respect to untreated control cells. $^{***}P < 0.001$. (D) HepG2 cells were cultured overnight in FCS-free medium. Afterwards the cells were pretreated with either Jak inhibitor I (1 μM) or U0126 (5 μM) for 30 min prior to an OSM stimulation (10 ng/mL) for 12 h. Grp78 mRNA levels were quantified by quantitative PCR. $^{\#\#}P < 0.001$, statistically significant difference with respect to untreated control cells. $^{***}P < 0.001$.

however, that these stable cells responded to OSM treatment with stronger signals for P-STAT1, P-STAT5, P-Erk1/2 and P-p38 (Supplementary Fig. 3), so the data have to be viewed with caution.

This is why we also took advantage of known pharmacological inhibitors targeting Janus kinases and MEK1/2 (the upstream kinases of Erk1/2) to investigate Grp78 induction by OSM. The MEK1 inhibitor U0126 has been shown to specifically inhibit the Erk1/2 activation upon OSM (without affecting STAT3 activation) while Jak inhibitor I suppresses all signaling pathways activated by OSM [30]. Interestingly, the treatment of HepG2 cells with U0126 decreases the induction of Grp78 (Fig. 5B). This finding could be confirmed in Huh-7 and PH5CH8 cells (data not shown). The treatment with Jak inhibitor I showed that OSM-induced Grp78 expression crucially depends on Jak activity. The inhibition of OSM-induced Grp78 up-regulation with Jak inhibitor I was only slightly more efficient compared to the one with the MEK inhibitor U0126, which is an indication that Erk1/2 activation is the most important factor and that STAT3 is involved to a lesser degree.

To corroborate the involvement of Erk1/2 we also performed reporter gene experiments using the Grp78 promoter construct (Fig. 5C). The luciferase activity of cells pretreated with U0126 prior OSM stimulation is reduced almost totally compared to the cells only treated with OSM. The residual activity is again indicative of a slight involvement of STAT3, an incomplete inhibition of the MEKs, and/or the involvement of additional signaling pathways. Moreover, the induction of the Grp78 promoter activity is weaker for IL-6 than for OSM, which is in line with our observation in Fig. 1B. We could confirm these results on the mRNA level (Fig. 5D). The OSM-induced increase of Grp78 mRNA is diminished in the presence of Jak inhibitor 1 as well as U0126.

Taken together, our data point at a major involvement of the Jak-activated MEK/Erk pathway in OSM-mediated Grp78 up-regulation, with a minor involvement of STAT3 while OSM-induced HIF-1α is dispensable. We cannot, however, exclude the possibility that other signaling pathways are also involved in this regulation.

4. Discussion

The key findings of the present manuscript are: (1) OSM leads to the selective up-regulation of the ER chaperone Grp78/BiP, which was not mediated by IFN-γ or TNF-α. (2) This up-regulation seems to be specific to liver cells, and does not occur in prostate, melanoma, breast or kidney cells. (3) Grp78 is up-regulated by transcriptional processes which are (4) mostly dependent on MEK/Erk activity, although other pathways may also be involved.

We could show Grp78 up-regulation by OSM to be stronger than by IL-6 and the up-regulation was corroborated in a number of hepatic cell lines. None of the other tested cytokines (IFN-γ or TNF-α) up-regulated Grp78. OSM is known to activate many signaling pathways. The cytokine receptor-bound Janus kinases (Jaks) are the first intracellular relay station of a cytokine signal [31]. Upon cytokine receptor binding the Jaks get activated, transphosphorylate and subsequently phosphorylate tyrosine residues in the cytokine receptor intracellular part, to which SH2 domain-containing signaling proteins can now bind and mediate further signal transduction. Not surprisingly, our studies with the pan-Jak inhibitor (Fig. 5B–D) clearly show that Jak activity is crucial for the OSM-mediated effect on Grp78 expression.

While our data obtained with the STAT3-shRNA cells pointed at an only minor role for STAT3 we had to be cautious since in these cells STAT3 suppression was correlated with a stronger activation of other signaling pathways (e.g. STAT1, STAT5, MAPKs), possibly due to the lack of STAT3-induced SOCS3 feedback regulation, as observed before for STAT3-deficient MEFs [32]. However, as STAT1 activation by IFN-γ was not associated with Grp78 up-regulation (see Fig. 1), it is unlikely that the increased Grp78 expression in these cells is mediated by the compensatory stronger STAT1 response. Experiments with pharmacological inhibitors against Janus kinases and MEK-1/2 (the kinases upstream of Erk1/2) showed that Erk1/2 are of crucial importance for Grp78 up-regulation upon OSM and that if STAT3 is involved it only plays a minor role. MEK inhibitors abrogated most of the Grp78 mRNA and protein up-regulation seen after OSM treatment (Fig. 5B and D) and had deleterious effect in promoter studies (Fig. 5C). Preliminary evidence derived from experiments with siRNA directed against Erk1 and/or Erk2 implies involvement particularly of Erk1 (data not shown).

Hypoxia has been known to affect the ER stress response, including Grp78 expression [25,33]. In addition, we have recently shown that OSM leads to up-regulation of HIF-1α in hepatoma cells and hepatocytes even under normoxic conditions [30]. Therefore we tested whether OSM-mediated Grp78 up-regulation could be mediated by HIF-1α. However, and similar to Song et al. [25], we did not find evidence for an involvement of HIF-1α in the regulation of Grp78/BiP expression: Grp78 was readily induced in HepG2 transfectants in which expression of HIF-1α was stably suppressed by shRNA (Fig. 5A).

The fact that we find an involvement of the MEK/Erk pathway in OSM-mediated Grp78 induction fits well to data obtained in very different systems. Grp78 up-regulation in gastric cancer cells upon thapsigargin- and tunicamycin treatment [34] or upon chronic hypoxia treatment also depended on MEK/Erk activity [25]. OSM is a stronger inducer of Grp78 compared to IL-6, although both types of cytokines belong to the family of IL-6-type cytokines and both are known to induce the expression of acute phase genes. OSM however has been described to lead to a stronger activation of MAP kinases (e.g. Erk1/2 and p38) than IL-6. This has been explained by the fact that the OSM receptor can induce the MAP kinase cascade via the SHP2 and Shc adaptor proteins, while IL-6 uses only SHP2 [35]. The fact that Erk1/2 activation is involved in Grp78 activation may also explain that IL-6 induces less Grp78 although it induces a STAT3 response of comparable strength as OSM (see Fig. 1B). However, further studies are needed to address the potential contribution of other signaling pathways.

We only observed an OSM-dependent up-regulation of Grp78 in liver-derived cells: in hepatoma cells (HepG2, Huh-7) and in non-transformed hepatocytes (PH5CH8), but not in any other cell type investigated so far (Fig. 3). The reason for this cell type-specific up-regulation is currently unknown. For cytokine-driven expression of hepatic acute phase response genes complex formation of c-Fos, STAT3 and the liver-enriched transcription factor HNF-1 have been reported to be essential [36–38]. Thus, it is well conceivable that liver-specific transcription factors also collaborate with OSM-induced transcription factors to regulate Grp78 gene expression. Moreover, OSM might modulate the DNA binding activity or transactivation potential of liver-specific transcription factors, as described for HNF-4 upon combined treatment with IL-6, IL-1, and TNF [39].

Increased Grp78 expression helps cells to better cope with ER stress. Thus, OSM as an inflammatory cytokine likely acts on hepatocytes in a way that helps them to be protected under inflammatory conditions characterized by microenvironmental stress such as hypoxia. OSM is a known inducer of the acute phase response, characterized by the massive production of proteins in the ER of hepatocytes to be secreted into the serum such as γ-fibrinogen, α1-antichymotrypsin, and haptoglobin [22]. We discarded the possibility that Grp78 expression is simply a "byproduct" of an ongoing acute phase response (and an associated ER stress response) for the following reasons: (1) Grp78 up-regulation upon OSM treatment seems to be selective, as other characteristics associated to an ER stress response were not observed (XBP-1 mRNA splicing, increased levels of eIF2α phosphorylation, increased Grp94 expression, induction of ATF4 and CHOP mRNA (Fig. 4), increased ATF6 cleavage (data not shown)). Of note, in preliminary studies an up-regulation of (unspliced) XBP-1 mRNA could be observed upon OSM stimulation (see also Fig. 4A). (2) Grp78 was also up-regulated in HepG2 hepatoma cells stably expressing a shRNA targeting STAT3 thereby precluding the acute phase response as exemplified by the lack of up-regulation of γ-fibrinogen (Fig. 5A).

Due to poor vascularization, the tumor microenvironment also promotes the development of ER stress in response to nutrient (e.g. glucose) starvation and hypoxia. One characteristic of the ER stress response is the induced expression of ER chaperones to increase the folding capacity of the ER. Hypoxia and ER stress in human cancer have been associated with the up-regulation of chaperones, including Grp78 [33,40]. It is known that Grp78 is widely expressed in cancer cells and seems to favor tumor development, proliferation and survival of cancer cells and tumor angiogenesis [17,41,42]. Moreover, Grp78 also protects tumor cells against therapy as it promotes their resistance to various chemotherapeutic agents [17].

Also in the liver, Grp78 and the ER stress response seem to be pro-oncogenic: The ER stress response has been implicated in hepatocarcinogenesis [43] and in resistance of hepatocellular carcinoma cells against drugs such as camptothecin, etoposide and doxorubicin [44,45]. Since a few years, Sorafenib has been successfully applied in the treatment of patients with advanced HCC [46,47]. Sorafenib is a multi-kinase inhibitor but is also known to induce apoptosis with the involvement of an ER stress response [48]. Interestingly, Grp78 expression negatively correlated in two hepatoma cell lines with responsiveness to Sorafinib treatment: those cells expressing higher levels of Grp78 were more resistant to Sorafenib-induced apoptosis [49].

Inflammation is discussed to favor carcinogenesis and the inflammatory cytokine OSM not only leads to an up-regulation of HIF-1α [30] but also of Grp78 as shown in the present study. Both proteins protect cells under conditions of microenvironmental stress, as present under conditions of inflammation and within a tumor. It will be interesting to find out whether a selective up-regulation of Grp78 by the inflammatory cytokine OSM could favor tumor development and resistance against chemotherapeutic drugs. It is possible that the OSM signaling pathway may represent an additional target in a co-treatment strategy that could increase the efficacy of Sorafenib treatment in HCC patients. Since Grp78 promotes resistance of tumor cells to various chemotherapeutic agents, gaining knowledge about the factors and mechanisms leading to the enhancement of Grp78 expression is of importance in order to improve current cancer treatments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.015.

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