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Differential effects of oncostatin M and leukaemia inhibitory factor expression in astrocytoma cells

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The effects of the production of two closely related cytokines, oncostatin M (OSM) and leukaemia inhibitory factor (LIF), by astrocytoma cells were investigated using the stable cell line human U373-MG, which expressed and secreted both biologically active polypeptides. The expression of LIF by these cells caused resistance to this cytokine due to loss of the LIF receptor (LIFR), from the cell surface, suggesting its retention. In contrast, cells expressing OSM were stimulated by this cytokine, utilizing an autocrine mechanism, and possessed receptors for OSM, but not LIF, on the cell surface. In these cells the continuous up-regulation of OSM-induced gene expression was found even though the Janus kinase–signal transducer and activator of transcription ('JAK/STAT') pathway was almost exhausted due to long-term autocrine stimulation of the cells by OSM. The amount of LIFR was down-regulated in both LIF- and OSM-producing cells and this effect was not found in wild-type U373-MG cells treated with externally added cytokines. To investigate the mechanism of autocrine stimulation by OSM we constructed a stable cell line expressing a form of OSM that is retained in the endoplasmic reticulum (ER). This biologically active cytokine was not secreted, but was localized in the ER. In addition, it did not stimulate the astrocytoma cells in an autocrine manner. We conclude that expression of LIF causes resistance of astrocytoma cells to this cytokine, whereas expression of OSM leads to autocrine stimulation.

Key words: autocrine stimulation, cytokine receptors, cytokines, signalling.

INTRODUCTION

Cytokines and growth factors are signalling molecules that are responsible for cell-to-cell communication. They regulate the expression of specific genes in target cells and, thus, lead either to cell proliferation or other specific responses that allow adaptation to changing environmental conditions. These secreted factors can stimulate a variety of cell types that possess the corresponding specific cytokine receptors. However, autocrine stimulation by cytokines and growth factors is also a common mechanism observed in many cell types, and the autocrine growth factor or cytokine loops ensure the continued growth of neoplastic cells [1]. Two major mechanisms of autocrine stimulation have been described [1]. In the 'public' mechanism growth factors or cytokines must be secreted which then bind to specific surface receptors not only on the cells of their origin but also on neighbouring cells. In the 'private' mechanism a signal can be transduced without either growth factor or cytokine secretion, indicating that these factors appear to interact with their receptors intracellulary. The known examples of 'public' and 'private' autocrine regulation are responses to erythropoietin (public) and interferon-γ, interleukin (IL)-3 and platelet-derived growth factor-related oncoprotein *sis* (private) [2–5]. Intracellular interaction of a secreted ligand and its receptor can, however, lead to retention of receptors without autocrine stimulation. In this case cells become unresponsive to an extracellular growth factor or cytokine due to the lack of functional receptors on the cell surface. This mechanism was demonstrated when IL-6 was overexpressed in hepatoma and melanoma cells normally responsive to this cytokine [6,7]. In both cell types resistance to IL-6 was observed due to the retention of the IL-6 receptor (IL-6R; also known as gp80).

The family of IL-6-type cytokines includes IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and novel neurotrophin-1 (NNT-1) [8]. These cytokines use the common receptor subunit gp130 for signal transduction and, therefore, elicit overlapping physiological activities. They either induce homodimerization of gp130 (IL-6 and IL-11), heterodimerization of gp130 with the LIF receptor (LIFR) (LIF, CT-1, CNTF, NNT-1 and OSM) or heterodimerization of gp130 with the OSM receptor (OSMR) (OSM). One of the multiple functions of these cytokines is induction of acute-phase protein (APP) synthesis in the liver, with IL-6 having a predominant role [9]. In contrast, extrahepatic production of several APPs found in the brain and lungs appears to be controlled by OSM, due to a limited amount of IL-6R present on lung epithelial cell and astrocyte surfaces [10,11]. Additionally, human astrocytes do not respond to LIF due to the lack of a functional LIFR system [11]. Since astrocytes are known to produce both IL-6 and LIF, but not OSM, their profile of responsiveness to the IL-6-type cytokines might result from the retention of IL-6R and LIFR. Furthermore, the

Abbreviations used: ACT, α_1-antichymotrypsin; APP, acute-phase protein; CNS, central nervous system; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; ER, endoplasmic reticulum; IL, interleukin; IL-6R, IL-6 receptor; JAK, Janus kinase; LIF, leukaemia inhibitory factor; LIFR, LIF receptor; LDLR, low-density lipoprotein receptor; NNT-1, novel neurotrophin-1; OSM, oncostatin M; OSMR, OSM receptor; PDI, protein disulphide-isomerase; RAP, receptor-associated protein; RT-PCR, reverse transcriptase-PCR; SIE, *sis*-inducible element of the c-*fos* gene; STAT, signal transducer and activator of transcription.

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responsiveness of human astrocytes to OSM seems to be totally dependent on the gp130–OSMR complex [11]. To test the hypothesis that production of IL-6-type cytokines by cells can alter their ability to respond to these stimulatory agents we constructed stable cell lines of human astrocytoma cells U373-MG (normally responsive to LIF and OSM) overexpressing LIF and OSM. We found that expression of LIF abrogates signalling, whereas expression of OSM leads to strong autocrine stimulation. This autocrine stimulation was abolished when OSM was retained within the endoplasmic reticulum (ER) by the addition of the KDEL (single-letter amino acid code) signal from protein disulphide-isomerase (PDI).

MATERIALS AND METHODS

Cells and cell stimulation

Human astrocytoma U373-MG and human hepatoma HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % (v/v) fetal calf serum. Cells were stimulated with 25 ng/ml OSM, 25 ng/ml LIF or 5 ng/ml IL-1 (all obtained from R&D System Inc., Minneapolis, MN, U.S.A.) in the presence of 10 μM dexamethasone.

RNA preparation, Northern-blot analysis and reverse transcriptase-PCR (RT-PCR)

Total RNA was prepared using the phenol extraction method [12]. Samples of RNA (5 μg) were subjected to formaldehyde gel electrophoresis using standard procedures [13], and were transferred to Hybond-N membranes (Pharmacia Biotech, Little Chalfont, Bucks., U.K.) according to the manufacturer's instructions. The filters were prehybridized at 68 °C for 3 h in 10 % (w/v) dextran sulphate, 1 M NaCl and 1 % (w/v) SDS, and hybridized in the same solution with fragments of α_1-antichymotrypsin (ACT), OSM and LIF cDNAs labelled by random priming [14]. Following hybridization, non-specifically bound radioactivity was removed by washing in 2 × SSC (where 1 × SSC corresponds to 0.15 M NaCl/0.015 M sodium citrate) at room temperature, followed by two washes in 2 × SSC and 1 % (w/v) SDS at 68 °C for 20 min. RT-PCR was performed using the Ultra HF RT-PCR System (Stratagene, La Jolla, CA, U.S.A.). Specific intron-spanning primers for LIFR and β-actin, and the amplification conditions, were exactly as described previously [15]. The identity of the PCR products was confirmed by hybridization with specific cDNA probes. The RT-PCR experiments were performed four times using different amounts of total RNA (1–5 μg) and comparable results were obtained each time.

Plasmid construction

Plasmids pXMLIF/HILDA and pBluACT containing LIF and ACT cDNAs, respectively, were donated by Dr Stefan Rose-John (University of Mainz, Mainz, Germany) and Dr H. Rubin (University of Pennsylvania, Philadelphia, U.S.A.) respectively. Plasmid pBluOnc containing OSM cDNA was a gift from Immunex Corp. (Seattle, WA, U.S.A.). The expression plasmid pExLIF was generated by cloning the *Xho*I–*Xho*I fragment containing LIF cDNA into *Xho*I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA, U.S.A.). The expression plasmid pExOSM was constructed by cloning the *Eco*RI–*Eco*RI fragment containing OSM cDNA into *Eco*RI sites of pcDNA3.1. The pOSM–PDI plasmid coding for OSM fused with the 14-amino-acid residues from the C-terminus of PDI (containing the KEDL signal) was constructed as follows. Two primers 5′-GGGCGGGTCTTCA-GCAAGTGGGG-3′ and 5′-TGCTCTCGAGCTACAGTTCA-TCCTTCACGGCTTTCTGATCGTCGTCTTCTTCCATC-CGGGGCAGCTCTC-3′ were synthesized and used in the PCR with pExOSM as a template and *Pwo* polymerase (Roche, Indianapolis, IN, U.S.A.). The PCR product was digested with *Bsu*36I and *Xho*I and cloned into *Bsu*36I–*Xho*I-digested pExOSM. The obtained plasmid pstep1OSM was subsequently digested with *Bsu*36I and ligated with the *Bsu*36I–*Bsu*36I fragment of OSM cDNA to produce plasmid pstep2OSM. The plasmid pstep2OSM was then digested with *Xho*I and ligated with the *Xho*I–*Xho*I fragment of OSM cDNA, yielding the plasmid pOSM–PDI. The identity of the constructs was verified by sequencing.

Stable transfection

U373-MG cells were grown to 20 % confluence and transfected in DMEM supplemented with 10 % (v/v) fetal calf serum using calcium phosphate precipitates [16] with 5 μg of plasmid DNA/10 cm culture dish. Cells were incubated with precipitate for 6 h, washed twice and the medium was changed. Recipient cells were selected in the presence of 450 μg/ml G418 (also known as Geneticin). Several G418-resistant clones were isolated and probed for expression of LIF and OSM by Northern-blot analysis. Representative clones expressing either LIF or OSM were taken for further analysis.

Rocket immunoelectrophoresis

ACT secreted into the media was measured using rocket immunoelectrophoresis as previously described [17], using anti-ACT antibodies (Dako, Carpinteria, CA, U.S.A.).

Whole-cell extract preparation and electrophoretic mobility-shift assay (EMSA)

Cell extracts were prepared exactly as described previously [18]. The SIEm67 (where SIE corresponds to the *sis*-inducible element of the c-*fos* gene) oligonucleotide [18] was labelled by filling in 5′-protruding ends with Klenow enzyme and [α-^{32}P]dCTP using standard procedures [13]. Gel-retardation assays were carried out according to published procedures [19,20]. Nuclear extracts (5 μg) and approx. 10 fmol (10000 c.p.m.) of probe were used.

Immunostaining

Approximately 0.25×10^6 U373-MG cells were grown on cover glasses for 24 h. Cells were fixed with 3.5 % (v/v) formaldehyde and either permeabilized with 0.1 % Triton X-100 (detection of OSM–PDI) or used directly for immunostaining (detection of cytokines bound to surface receptors). Cells fixed on cover glasses were treated with either the rabbit polyclonal anti-OSM antibody or the goat polyclonal anti-LIF antibody (both at 1:150 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) for 20 min. Immune complexes were detected with fluorescein-conjugated anti-rabbit or anti-goat antibodies (1:75 dilution) and analysed using fluorescence confocal microscopy. The Bio-Rad MRC 1024 laser scanning confocal system was used. Fluorescein was detected using 488 nm excitation, a 527DRLP beam splitter and a 540 ± 30 nm emission filter. Simultaneous fluorescence (512 × 512 pixels) and transmitted light images were collected using Kallman averaging. Image processing was performed using LaserSharp version 3.2 (Bio-Rad) and Scion (NIH Image for PC; Scion Corporation, Frederick, MD, U.S.A.) software. In order to measure binding

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of cytokines, cells were incubated on ice with 25 ng/ml OSM or 25 ng/ml LIF for 2 h.

Cell lysis and Western-blot analysis

Cells growing in 6 cm dishes were lysed in 400 μl of boiling 10 mM Tris/HCl (pH 7.5), 1 % (w/v) SDS and 1 mM sodium orthovanadate. Samples (30 μl) were subjected to SDS/PAGE and blotted on to nitrocellulose. LIFR and gp130 were detected using polyclonal anti-LIFR (C-19) and anti-gp130 (C-20) antibodies (Santa Cruz Biotechnology, Inc.).

RESULTS

Construction of U373 cells expressing biologically active LIF and OSM

The astrocytoma U373-MG cells express gp130, LIFR and OSMR [21], thus allowing LIF and OSM to activate signal transducer and activator of transcription (STAT)1 and STAT3, and up-regulate expression of the ACT gene in these cells [11]. The current model of signalling by these cytokines reveals that LIF signals via the receptor containing gp130 and LIFR, whereas OSM signals via heterodimers of gp130 with either LIFR or OSMR. Upon cytokine stimulation both receptor systems can activate the expression of the ACT gene [11, 22]. In order to investigate the effect of *de novo* production of LIF and OSM on the responsiveness to these cytokines, by cells normally capable of stimulation by these cytokines, we expressed LIF and OSM in U373-MG cells. LIF and OSM cDNAs were cloned into the expression vector pcDNA3.1, and the constructs were transfected into U373-MG cells. Stable transfectants were selected in medium containing G418, and were assayed for the expression of LIF and OSM mRNA (results not shown). Representative colonies of U373-LIF and U373-OSM cells expressing LIF and OSM, respectively, were further analysed for the biological activity of each secreted cytokine. Media from U373-LIF and U373-OSM cells were collected and used to stimulate human hepatoma HepG2 cells, which respond to either OSM or LIF by enhanced expression of positive APPs, including ACT [9]. The expression of both ACT mRNA (results not shown) and protein (Figure 1) was measured in HepG2 cells in response to media from control and transfected U373-MG cells. Medium from wild-type U373-MG cells had no effect on ACT expression in HepG2 cells. However, the effect of LIF and OSM present in the media from U373-LIF and U373-OSM cells, respectively, was clearly demonstrated. We assume that the secreted OSM and LIF are biologically active and are produced at approximately 25 ng of OSM/10^6 cells per 24 h and 60 ng of LIF/10^6 cells per 24 h, as judged by stimulation of ACT synthesis.

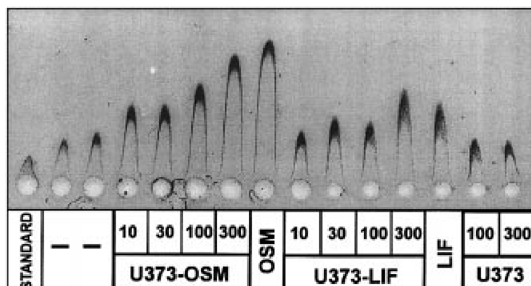

Figure 1 OSM and LIF expressed in U373-MG cells are biologically active and stimulate ACT production

HepG2 cells were stimulated with media from U373-MG, U373-LIF or U373-OSM cells, or with recombinant OSM and LIF (25 ng/ml). After 24 h media were collected and the amount of secreted ACT was measured by rocket immunoelectrophoresis. Purified ACT (150 ng) was used as a standard. The minus symbols indicate control cells that were untreated. A representative example of three experiments is shown.

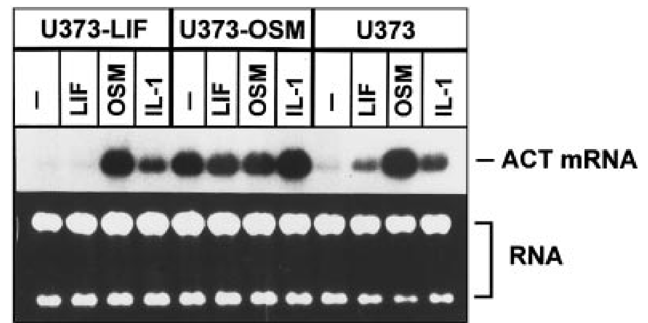

Figure 2 Regulation of ACT expression in wild-type and cytokine-expressing cells

U373-MG, U373-LIF and U373-OSM cells were stimulated with 25 ng/ml OSM, 25 ng/ml LIF or 5 ng/ml IL-1. RNA was isolated after 18 h and subjected to Northern-blot analysis using ACT cDNA as a probe. The bottom panel shows a ribosomal RNA stained with ethidium bromide on the membrane. A representative example of two experiments is shown.

Effect of LIF and OSM on endogenous gene expression

OSM and LIF, when expressed, could, theoretically, either stimulate the cells by an autocrine mechanism or cause their resistance. In order to identify which of the mechanisms applied to this model we measured activation of the ACT gene using Northern-blot analysis. The results are presented in Figure 2. As expected, the expression of ACT mRNA in control U373-MG cells was up-regulated by both OSM and LIF. The magnitude of stimulation by LIF, however, was much lower in comparison with that observed after OSM stimulation, but this result has already been reported for ACT and other APP genes [23]. In contrast, cells expressing LIF could only respond to OSM, but not to LIF, and the magnitude of the response to OSM was only slightly diminished in comparison with that found in wild-type U373-MG cells. Because IL-1 is known to activate ACT gene expression in astrocytes and astrocytoma cells we used this cytokine in our studies as an additional control. The response in both wild-type and U373-LIF cells was identical.

In contrast with both of the cell types described above, the U373-OSM cells had a very high basal level of ACT mRNA without any stimulation. Both OSM and LIF could not further stimulate expression of the ACT gene, whereas IL-1, known to activate the ACT gene by a distinct mechanism from that used by OSM and LIF, further up-regulated ACT expression. We conclude that expression of OSM results in autocrine stimulation, whereas expression of LIF causes resistance to this cytokine.

Activation of the JAK/STAT pathway in wild-type and transfected U373-MG cells

Binding of cytokines of the IL-6 family, including LIF and OSM, to their receptors activates the JAK/STAT pathway, with STAT3 being the major transcription factor involved in the activation of APP genes by these cytokines. In addition, while STAT5 is known to be activated in response to OSM and LIF [24,25], ACT is strictly STAT3 responsive and is not regulated by STAT5 [11]. For this reason we compared activation of STAT3 (and STAT1)

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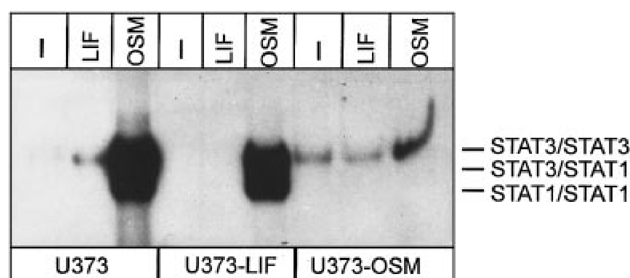

Figure 3 Activation of STAT3 and STAT1 in wild-type and cytokine-expressing cells

U373-MG, U373-LIF and U373-OSM cells were stimulated with 25 ng/ml OSM or 25 ng/ml LIF. Whole-cell extracts were prepared after 15 min and were analysed by EMSA using an SIE probe. Positions of STAT3 and STAT1 complexes are indicated.

in wild-type and cytokine-expressing cells using EMSA (Figure 3). Massive activation of STAT3 (and STAT1) was observed in wild-type cells after stimulation with OSM, whereas LIF could only weakly activate STAT3. These observations confirm previous findings from studies on other cell types, including human hepatoma HepG2 and human astrocytoma CCF-STGG1 cells [11,23]. In CCF-STGG1 cells, which are very similar to U373-MG cells in terms of both ACT induction and STAT activation, LIF very weakly activates STAT3, regardless of the fact that LIFR mRNA is more abundant then OSMR or gp130 mRNA [21]. STAT3 (and STAT1) were also strongly activated by OSM in U373-LIF cells but, in contrast with wild-type cells, LIF was completely ineffective in these cells. A unique pattern of STAT activation was found in U373-OSM cells, with the JAK/STAT pathway being almost exhausted due to long-term over-stimulation. We observed continuous low-level activation of STAT3 in untreated U373-OSM cells, whereas treatment with exogenous OSM had only a minor effect. These results provide further evidence supporting an autocrine mechanism of stimulation by OSM, but resistance to LIF.

Detection of LIF and OSM surface receptors on wild-type and cytokine-expressing cells

The lack of LIF responsiveness in U373-LIF cells not only suggested that this cytokine could interact with LIFR at some stage of the protein secretory pathway but also indicated that the complexes of LIF–LIFR might be removed and degraded before reaching the cell surface. As a result these cells should not possess any functional LIFRs on their surfaces. In contrast, autocrine stimulation by OSM could be achieved either by intracellular or extracellular signalling by OSM ('public' or 'private' mechanism respectively). In both of these models detection of functional receptors on the cell surface was a prerequisite for further analysis. We utilized antibodies directed against LIF and OSM, together with a fluorescein-conjugated secondary antibody, to detect ligand binding on the cell surface using confocal microscopy. Wild-type, LIF- and OSM-expressing cells were incubated with OSM or LIF, and cytokines bound to cell surfaces were visualized by confocal microscopy. As shown in Figure 4, wild-type cells bound both cytokines; however, U373-LIF cells could only bind OSM, whereas U373-OSM cells bound OSM and a marginal amount of LIF. These results support the hypothesis that a lack of functional LIFRs on the cell surface of U373-LIF cells is responsible for their resistance.

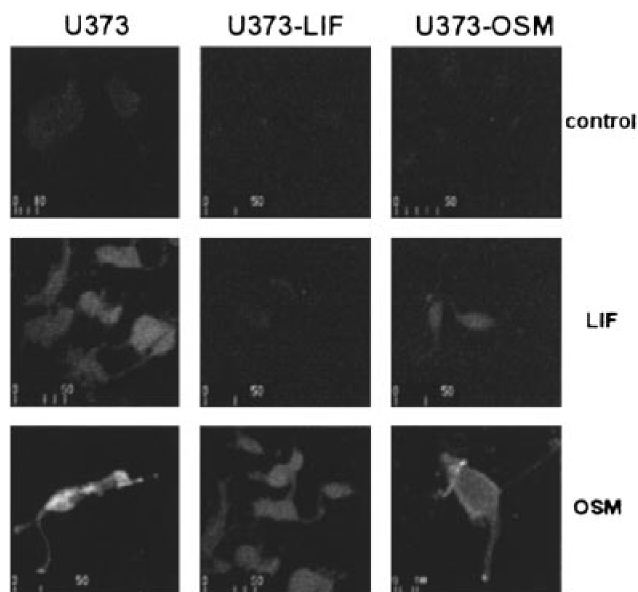

Figure 4 Binding of LIF and OSM to U373-MG, U373-LIF and U373-OSM cells

Cells were grown on cover glasses for 24 h, 25 ng/ml OSM and 25 ng/ml LIF were added, and incubated on ice for 2 h. They were then extensively washed with PBS, fixed and cytokines bound to the cells were detected using anti-OSM, anti-LIF and fluorescein-conjugated antibodies. Immune complexes were visualized using fluorescence confocal microscopy.

Analysis of LIFR and gp130 expression in wild-type and cytokine-producing cells

The lack of response to LIF of cytokine-producing cells could theoretically result from the down-regulation of LIFR mRNA expression. To test this possibility we analysed LIFR mRNA expression by Northern-blot analysis; however, the accumulation of LIFR mRNA was hardly detectable (results not shown). Therefore we analysed the expression of LIFR by RT-PCR using previously described primers and conditions [15]. Figure 5 shows that LIFR was detected in wild-type and cytokine-producing

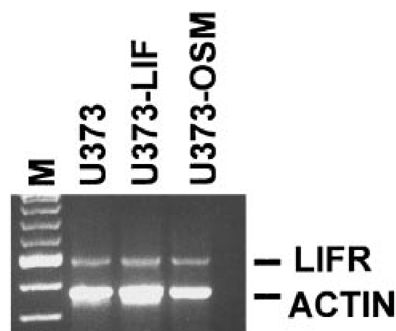

Figure 5 Detection of LIFR mRNA in U373-MG, U373-LIF and U373-OSM cells

Total RNA was isolated from U373-MG, U373-LIF and U373-OSM cells. RT-PCR was performed using 1 μg of total RNA and intron-spanning primers for LIFR and β-actin as described in the Materials and methods section. PCR products were separated in 1.5% (w/v) agarose gels containing ethidium bromide. The positions of PCR products specific for LIFR and β-actin are marked. M indicates a 1 kb ladder of DNA markers (New England Biolabs, Beverly, MA, U.S.A.).

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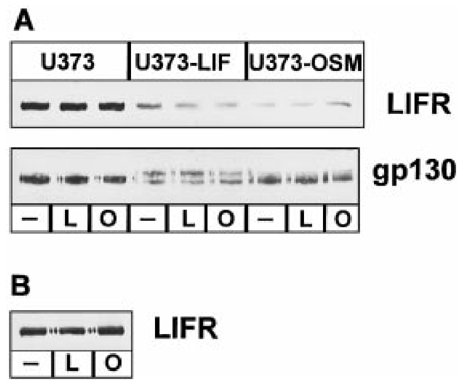

Figure 6 Western-blot analysis of LIFR and gp130

U373-MG, U373-LIF and U373-OSM cells were stimulated with 25 ng/ml OSM (O) or 25 ng/ml LIF (L) for 3 h (**A**), or U373-MG cells were stimulated with 25 ng/ml OSM or 25 ng/ml LIF for 24 h (**B**). Cells were subsequently lysed under denaturing conditions, and cell lysates were resolved by SDS/PAGE [8% (w/v) polyacrylamide] and electroblotted on to nitrocellulose. LIFR and gp130 were detected using anti-LIFR and anti-gp130 antibodies, respectively, and visualized by ECL® (Amersham).

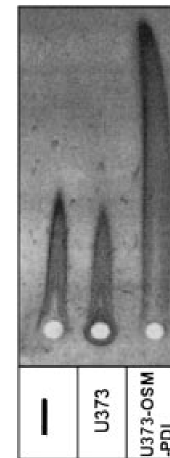

Figure 7 OSM–PDI is biologically active and stimulated ACT production

U373-MG or U373-OSM-PDI cells were collected in PBS, frozen twice and sonicated. Insoluble material was removed by centrifugation. Cell extracts were then used to stimulate HepG2 cells. Media were collected after 24 h and secreted ACT was measured using rocket immunoelectrophoresis.

cells, and that the amounts of PCR product were comparable between all three cell types analysed. This semi-quantitative analysis led us to the conclusion that LIFR mRNA is present not only in wild-type cells but also in cytokine-producing cells. The amounts of LIFR mRNA found in cytokine-producing cells were not greatly diminished in comparison with wild-type cells.

Western-blot analysis was used to study the amounts of LIFR and gp130 in wild-type and cytokine-producing U373-MG cells. Both receptor subunits were detected in all cell types tested. As shown in Figure 6(A), the amount of LIFR was strongly diminished in LIF- and OSM-producing cells in comparison with wild-type U373-MG cells. This down-regulation was not observed after prolonged treatment of wild-type cells with exogenously added cytokines (Figure 6B), suggesting that simultaneous secretion of both cytokines together with LIFR is necessary for the observed effect. Furthermore, in cytokine-producing cells a new protein band with slower mobility was detected with anti-gp130 antibodies. The identity of this band is unclear; however, it may represent a phosphorylated gp130 molecule. Since LIFR was not detected at cell surfaces of LIF-producing cells we conclude that the LIF–LIFR–gp130 complex was formed while these proteins passed through various compartments of the secretory pathway. Formation of these complexes results in phosphorylation of gp130; however, signalling does not occur. In OSM-producing cells LIFR is also down-regulated, most probably by a similar mechanism that involves formation of OSM–gp130–LIFR complexes.

Construction of a cell line producing biologically active OSM retained in the ER

Since autocrine stimulation could be the result of either the intracellular or extracellular interaction of OSM with its receptors we engineered this cytokine so that it contained an additional 14-amino-acid residues at its C-terminus, which was derived from PDI. This C-terminal extension included a KDEL sequence responsible for the retention of proteins in the ER, which has previously been shown to be more effective than the KDEL motif by itself [6]. An expression plasmid encoding OSM–PDI was transfected into U373-MG cells and stable transfectants were selected as described above. Expression of OSM–PDI mRNA was confirmed by Northern-blot analysis (results not shown). The biological activity of OSM–PDI was tested using HepG2 cells. Whereas the medium from U373-OSM–PDI cells did not increase ACT expression in HepG2 cells (results not shown), extracts prepared from these cells did stimulate secretion of this APP (Figure 7), indicating that the biologically active cytokine is retained within cells. To identify the localization of OSM–PDI, cells were permeabilized and the cytokine was visualized using an anti-OSM antibody and fluorescein-conjugated secondary-antibody binding. Wild-type and U373-OSM cells were also included in these studies for comparison. The results, shown in Figure 8, indicate that wild-type cells do not express OSM; however, this cytokine was found throughout the entire cell body of U373-OSM cells, indicating its efficient production and secretion. In contrast OSM–PDI synthesized by U373-OSM–PDI cells was retained within the ER and was localized around the nucleus.

Retention of OSM in the ER prevents autocrine stimulation

U373-OSM cells were continuously activated by constitutively produced OSM. Hence, we analysed the expression of the ACT gene and activation of STATs in U373-OSM–PDI cells in order to determine if retention of OSM in the ER would result in autocrine stimulation. Both the regulation of ACT gene expression and the activation of STATs were similar to those found in wild-type cells (Figure 9). We observed neither continuous up-regulation of ACT gene expression in non-treated cells nor diminished STAT activation in response to exogenous OSM as observed in U373-OSM cells. The responses to exogenously added LIF and OSM were comparable with those found in wild-type cells. Thus we conclude that retention of OSM within the ER abolishes autocrine stimulation by this cytokine. Whereas the mechanism of autocrine stimulation is not clear, it seems that OSM either needs to be secreted and bind to cell surface receptors (a 'public' mechanism) or to interact with receptor subunits in post-ER compartments of the secretory pathway.

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Figure 8 Localization of OSM and OSM–PDI

U373-MG, U373-OSM and U373-OSM-PDI cells were grown on cover glasses for 24 h. Cells were fixed and permeabilized as described in the Materials and methods section. OSM and OSM–PDI were detected using anti-OSM and fluorescein-conjugated antibodies. Immune complexes were visualized by fluorescence confocal microscopy. The images shown are approx. 80 μm across.

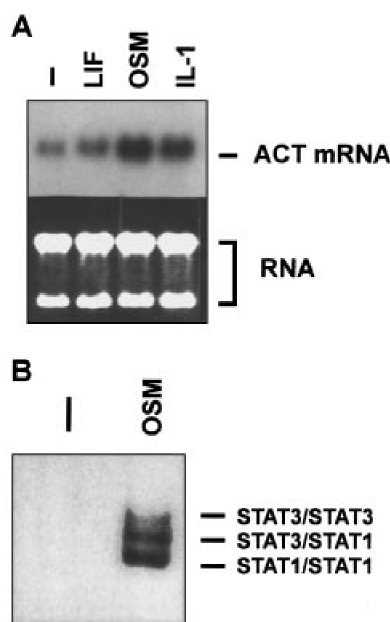

Figure 9 Responsiveness of U373-OSM–PDI cells to cytokines

U373-OSM–PDI cells were stimulated with 25 ng/ml OSM, 25 ng/ml LIF or 5 ng/ml IL-1. (**A**) RNA was isolated after 18 h and analysed by Northern blotting using ACT cDNA as a probe. The bottom panel shows ribosomal RNA stained with ethidium bromide on the membrane. (**B**) Whole-cell extracts were prepared after 15 min and analysed by EMSA using an SIE probe. Positions of STAT3 and STAT1 complexes are indicated.

DISCUSSION

Cytokines of the IL-6 family have been shown to control a variety of physiological processes, from cell proliferation to regulation of specific sets of genes in terminally differentiated cells [8]. In particular the unique role of these cytokines within the central nervous system (CNS) has been well documented [26]. While LIF and CNTF have attracted most of the attention, due to their effects on different types of neurons [27], IL-6 and OSM have also been proposed as mediators of the 'cerebral' acute phase response, which is similar to the 'hepatic' acute phase response that occurs within the liver [28]. Expression of these cytokines has been reported within the CNS, both in astrocytes, which express IL-6 and LIF, and in microglia, which, in addition, produce OSM [11,26]. Thus the responsiveness of cells from the CNS to the IL-6-type cytokines has become an important issue. Astrocytes appear to be of particular interest since they (1) represent a major cell type within the CNS, (2) are capable of producing IL-6 and LIF, and (3) can be efficiently activated by OSM.

The autocrine mechanism of astrocyte activation by LIF and IL-6 produced constitutively could hypothetically exist. This is supported by findings that murine astrocytes produce nerve growth factor in response to IL-6 [29], and astrocyte progenitor cells differentiate in response to LIF [30]. However, astrocytes have recently been shown to express limited amounts of IL-6R, so that their response to IL-6 can be enhanced by soluble IL-6R [31]. In addition, we have previously reported that human astrocytes lack a functional receptor system for LIF [11]. In the present study we clearly show that expression of LIF by astrocytoma U373-MG cells, normally responsive to LIF, but not expressing this cytokine (results not shown), results in the resistance of these cells to this cytokine. This might be of particular importance, since continuous low-level expression of LIF by differentiated astrocytes has been reported [32]. Recently, stimulation of LIFR degradation by extracellular signal-regulated kinase has been reported in hepatoma, NIH3T3 and HeLa cells in response to OSM and LIF [33]. However, we did not observe similar down-regulation of LIFR by exogenously added LIF or OSM in U373-MG cells treated with these cytokines for 24 h (see Figure 6B). Furthermore, LIF is known to be an autocrine growth factor for several cell types, as discussed below, and this suggests that the observed degradation may not be found in many cell types. In the present study we propose that the observed resistance of astrocytoma cells to LIF is the result of its expression, followed by interaction with LIFR within compartments of the secretory pathway and retention of the receptor itself.

We did not address the issue of responsiveness of astrocytes to constitutively produced IL-6 in the present study; however, it has been previously shown in other models (melanoma and hepatoma cells) that the simultaneous production of IL-6 and IL-6R causes resistance to this cytokine [6,7]. Thus we suspect that similar mechanisms exist in astrocytes and astrocytoma cells.

In contrast with IL-6 and LIF, OSM has never been shown to be synthesized by astrocytes or astrocytoma cells. We cannot exclude that these cells express this cytokine under conditions yet to be defined. However, if this was the case astrocytes would be expected to be stimulated by an autocrine mechanism, and one would also note the up-regulation of expression of genes, including those coding for ACT and IL-6. The autocrine mechanism of stimulation by OSM has already been reported for spindle cells from AIDS-associated Kaposi's sarcoma [34]. These cells produce OSM, and the gp130–OSMR complex mediates their activation by this cytokine [35]. This observation correlates with findings presented in the current study. In addition, we clearly demonstrate that retention of OSM in the ER abolishes autocrine stimulation.

OSM-induced signal transduction from the surface receptors of OSM-expressing cells indicates that both components of the type II receptor (gp130 and OSMR) are present on the cell surface. In contrast, LIF-expressing cells do not show any LIF binding or response to this cytokine, but are still responsive to OSM, suggesting that only the LIFR is missing from the cell surface. Since LIFR, OSMR and gp130 are highly homologous molecules, these results, at first, might be somewhat unexpected. However, there is an important difference in the binding properties of these receptor subunits. LIFR can bind LIF by itself, but OSMR cannot bind OSM without gp130 [21]. OSM is first bound to gp130, followed by association with either OSMR or

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LIFR. We speculate that in LIF-expressing cells the intracellular interaction of LIF with LIFR occurs, a complex of LIF–LIFR–gp130 is then formed, and this results in retention of LIFR. Because down-regulation of LIFR protein was also observed in OSM-producing cells we suggest that a complex of OSM–gp130–LIFR is also formed in these cells and LIFR is retained. Since the retention of OSM–PDI within the ER results in a normal phenotype we speculate that secretion is necessary for the autocrine action of OSM; however, formation of the OSM–gp130–OSMR complex in post-ER compartments of the secretory pathway cannot be excluded. The last question then remains 'Why is OSMR not retained in cells producing OSM?' We suggest a model in which OSM–gp130 cannot bind to OSMR intracellulary. Lack of this binding might be either a result of different conditions (for example differences in pH, incomplete folding or glycosylation of OSMR) or a highly specific mechanism involving, as yet unrecognized, additional protein(s), as described for members of the low-density lipoprotein receptor (LDLR) family [36]. The receptor-associated protein (RAP), a chaperone responsible for proper folding of LDLR within the ER, binds to LDLR and blocks binding of its ligands. This association is transient, since RAP dissociates from LDLR in downstream compartments of the secretory pathway and is retained in the ER, because of an HNEL sequence in its structure [37]. A similar mechanism could apply to the OSM–gp130 complex and OSMR; however, this is only speculation and requires further investigation.

The autocrine stimulation of astrocytoma cells by OSM correlates with reports involving other cell systems. In contrast with the resistance to stimulation by LIF described in the present study, this cytokine has been reported as an autocrine growth factor for kidney (A-498), metastatic kidney (ACHN) and prostate (DU145) cell lines [38]. These cells produce LIF, and anti-LIF antibodies suppressed their proliferation. LIF has also been reported as an autocrine factor for sympathetic neurons and Schwann cells [39,40]. Autocrine stimulation of the neurons and Schwann cells suggests that LIF expression does not result in the retention of LIFR. We suggest that a mechanism preventing binding of LIF to LIFR or LIF–LIFR to gp130 may exist in these cells, similar to that which we propose for OSM–gp130 and OSMR, but is not present in astrocytes and astrocytoma cells. Recently [41], the formation of autocrine loops by OSM, LIF and IL-6 has been analysed in human cerebral meningioma cells producing these cytokines and all receptor components. Resistance to IL-6 and LIF was found, whereas OSM caused growth inhibition [41]. Such data support our findings with astrocytoma cells in which expression of LIF causes resistance, whereas expression of OSM leads to autocrine stimulation.

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