Hepatocyte growth factor/scatter factor (HGF/SF) signals via the STAT3/APRF transcription factor in human hepatoma cells and hepatocytes

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Abstract Acute phase protein expression is regulated by a variety of cytokines such as IL-1, IL-6, IL-11, tumour necrosis factor α, interferon-γ, oncostatin-M, leukemia inhibitory factor, ciliary neurotrophic factor and cardiotrophin-1. Presently, IL-6 is regarded as the most potent mediator of acute phase protein (APP) synthesis. It was shown that IL-6 and IL-6-type cytokines activate the so-called JAK/STAT pathway and finally regulate APP expression in liver cells. Since HGF/SF is also capable of regulating APP expression, we asked whether it might also signal via the JAK/STAT pathway. Here we show that incubation of human hepatocytes as well as hepatoma cells (HepG2) with HGF/SF results in activation of the transcription factor STAT3. This STAT3 activation after HGF/SF did not occur before 5–7 h and was maintained up to 28 h. These observations are in contrast to the rapid and transient activation of STAT1 and STAT3 mediated by IL-6.

Key words: Hepatocyte growth factor/scatter factor; STAT3; α_1-Antichymotrypsin; Signal transduction; Hepatocyte; Acute phase protein

1. Introduction

Higher organisms respond in a complex reaction to a variety of injuries such as bacterial, viral or parasitic infections, mechanical or thermal trauma, ischaemic necrosis or neoplastic growth [1–3]. This so-called acute phase response consists of a local and a systemic reaction. The latter comprises endocrine and metabolic alterations, leucocytosis, increased hormone release, activation of blood clotting and the complement system as well as a marked change in a group of plasma proteins which have been designated as acute phase proteins (reviewed in [4,5]). Acute phase proteins have been shown to be synthesized mainly by hepatocytes and secreted into the circulation. It is a well-known observation that APPs are differently regulated in different species [5]. Cytokines such as IL-1, TNFα, IL-6 and the more recently described IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor and cardiotrophin-1 regulate the expression of APPs in liver cells [6]. Thus far, IL-6 seems to be the major mediator of APP expression in hepatocytes [7,8].

IL-6 and IL-6-type cytokines are known to exert their actions via surface receptors composed of specific low affinity receptor α-chains and the common signal transducer gp130 [9,10]. Previous results from our laboratory and others have shown that IL-6 mediated APP regulation occurs through the JAK/STAT signalling pathway [11,12]. Binding of IL-6 to its receptor induces dimerization of gp130 [13], activation of the gp130-associated protein tyrosine kinases JAK1, JAK2 and TYK2 [11,12] and phosphorylation of gp130 at tyrosine residues. Previously, we demonstrated that IL-6 triggers the rapid activation and tyrosine phosphorylation of a latent transcription factor, acute phase response factor (APRF), which plays an important role in the induction of multiple APPs [14]. APRF turned out to be a member of the STAT family, namely STAT3α [15–17].

Recent work has shown hepatocyte growth factor/scatter factor as being capable of regulating APP synthesis in human [18] and rat [19] primary hepatocyte cultures although – compared to IL-6 – the effects are weaker, and in some cases divergent.

In the light of these data, we addressed the question of whether the regulation of HGF/SF-mediated APP synthesis occurs via the same transcription factor activation as in the case of IL-6. Here we show that HGF/SF regulates the expression of the APP α_1-antichymotrypsin and activation of STAT3 in human hepatoma cells (HepG2) and in human hepatocytes in primary culture. The time course of STAT3 activation by HGF/SF, however, differs markedly from that observed for IL-6 and its related cytokines.

2. Materials and methods

2.1. Reagents

Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany), polyclonal antibodies to human ACT were from Dakopatts (Hamburg, Germany). Polyclonal antibodies raised against a C-terminal fragment of STAT 3α were kindly supplied by Dr. Werner Müller-Esterl (Mainz, Germany); DMEM/F12 mixture and MEM plus supplements leucine, arginine, glucose and inositol, Ham F-12 and Leibovitz L-15 culture media were from Gibco (Eggenstein, Germany), fetal calf serum from Seromed (Berlin, Germany). Recombinant human IL-6 was prepared as described in [20]. Its specific activity was 1×10^6 B-cell stimulatory factor-2 units/mg protein. Recombinant

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Abbreviations: ACT, α_1-antichymotrypsin; APP, acute phase protein; EMSA, electrophoretic mobility shift assay; HGF/SF, hepatocyte growth factor/scatter factor; IL, interleukin; JAK, janus kinase; STAT, signal transducer and activator of transcription

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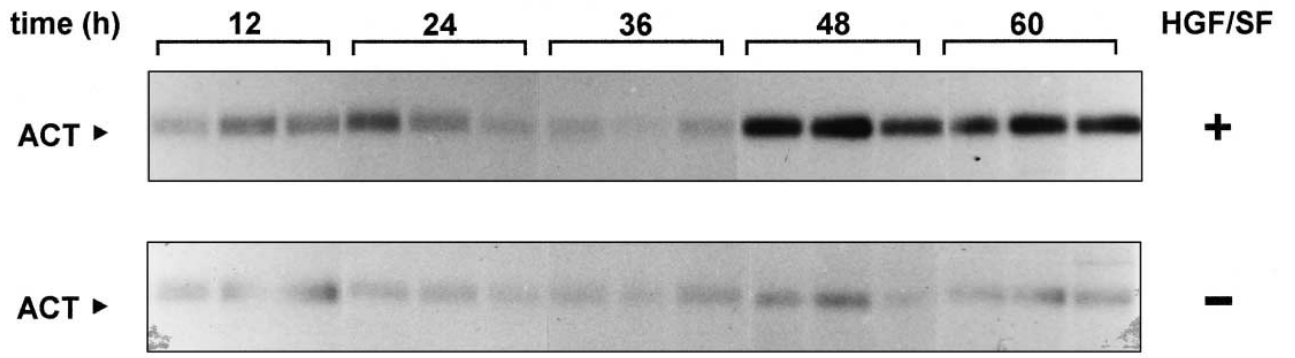

Fig. 1. Stimulation of ACT synthesis by HGF/SF in HepG2 cells. 1.2×10^5 HepG2 cells per cm^2 were incubated without (lower row) or with 10 ng/ml of HGF/SF (upper row) for the times indicated. 12 h before the end of the experiment, cells were labeled with 3.75 MBq/ml of TRAN^{35}S-LABEL in methionine/cysteine-free medium. ACT was immunoprecipitated from the conditioned medium with a specific antiserum and subjected to SDS-PAGE and autoradiography. Experiments were performed in triplicate.

human HGF/SF was prepared in the baculovirus expression system. Its specific activity was 3.3×10^6 units/mg of protein [21].

2.2. Cell cultures

HepG2-cells (ATCC HB 8065) were grown in DMEM/F12 medium at 5% CO_2 in a water-saturated atmosphere. Cell culture medium was supplemented with 10% fetal calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l).

Human hepatocytes were isolated from liver biopsies obtained in the course of a cholecystectomy after informed consent approved by the Ethics Committee of the hospital and cultured as described earlier [22].

2.3. Protein labelling and immunoprecipitation of ACT

After seeding into a 96-well plate, HepG2 cells were initially grown in DMEM/F12 medium supplemented with 10% fetal calf serum for 24 h, then cultured in serum-free medium and stimulated with 10 ng/ml of recombinant human HGF/SF for different times. For subsequent protein labelling, cells were shifted to MEM plus 0.75 MBq per well of TRAN^{35}S-LABEL (ICN) for 12 h. 180 μl of the ^{35}S-labelled cell culture supernatant were added to 96-well ELISA plates coated with 2.5 μg ACT antibody per well, followed by incubation at 37°C for 3 h. Plates were rinsed and bound proteins solubilized in SDS-PAGE loading buffer [23]. Proteins were separated by SDS-PAGE [23] on a 10% gel, the gels fixed in 40% methanol, 10% acetic acid and 50% water for 30 min, dried and autoradiographed. For quantification of radioactive protein, gels were analysed with the Image-Quant system (Molecular Dynamics).

2.4. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts of HepG2 cells and primary human hepatocytes were prepared as described [24]. Protein concentrations were measured by the Bio Rad protein assay. EMSAs were performed as described previously [25]. We used a double-stranded ^{32}P-labelled probe: a mutated SIE oligonucleotide of the *c-fos* promoter (m67 SIE: 5′-GAT CCG GGA GGG ATT TAC GGG GAA ATG CTG-3′) [26]. Protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25 TBE at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid and 80% water for 30 min, dried and autoradiographed. For supershift assays, nuclear extracts were pre-incubated with 2 μl of STAT3α antiserum for 15 min on ice and then used for EMSA.

3. Results

Previous studies have shown that IL-6 is a potent inducer of the acute phase protein α_1-antichymotrypsin in hepatocytes and hepatoma cells [18,27]. Interestingly, HGF/SF has also been described as being a regulator of ACT expression in liver

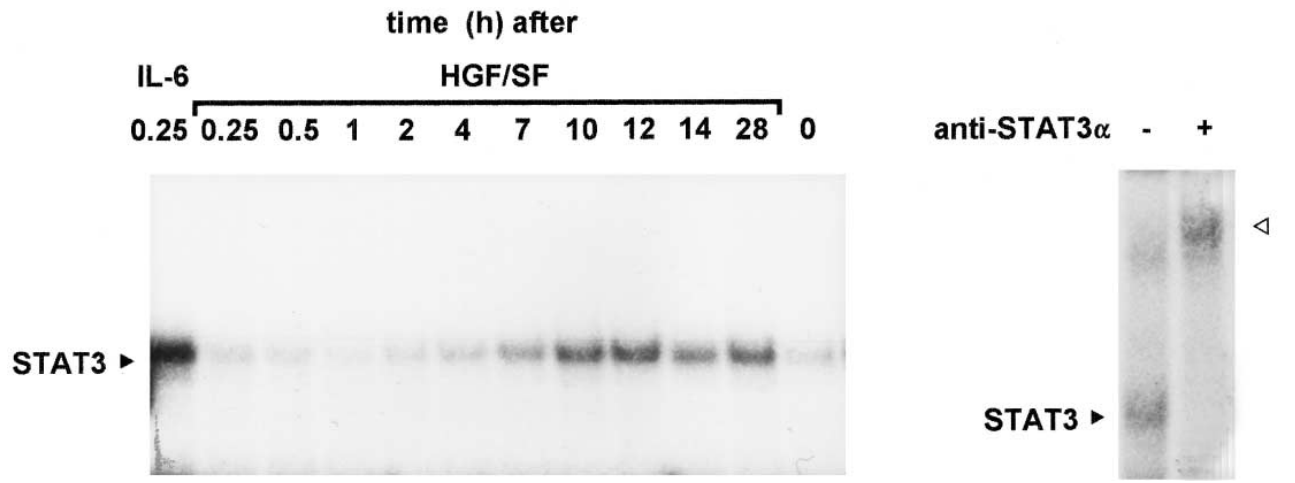

Fig. 2. Time dependence of STAT activation after incubation of HepG2 cells with HGF/SF. All HepG2 cells were cultivated for a period of 28 h. Before the end of the experiment, cells were stimulated without or with either 10 ng/ml of HGF/SF or 100 pg/ml of IL-6 for the times indicated. Nuclear extracts were prepared and analysed by EMSAs using an m67SIE probe specific for STAT1/STAT3 as previously described [25]. The panel on the right shows a supershift experiment with an antiserum specific for STAT3α (Müller-Esterl, Schaper, Küster, Heinrich, unpublished work). Nuclear extracts were prepared from HepG2 cells stimulated with 10 ng/ml of HGF/SF for 15 h.

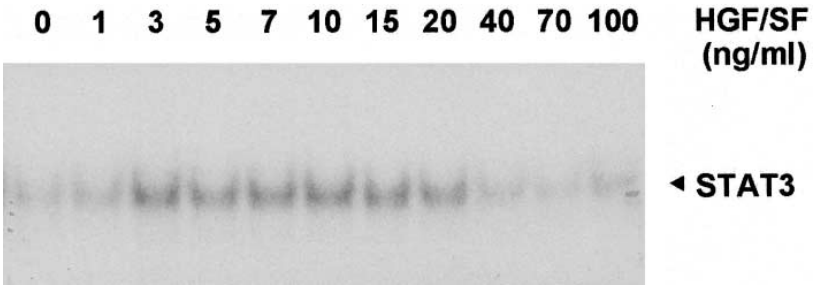

Fig. 3. Dose dependence of STAT3 activation by HGF/SF in HepG2 cells. HepG2 cells were incubated with increasing amounts of HGF/SF for 15 h. As described in the legend to Fig. 2, nuclear extracts were prepared and EMSAs performed.

cells [18]. Fig. 1 shows the effect of HGF/SF on the synthesis of ACT in the human hepatoma cells HepG2. A marked increase (3–5-fold) between 36 and 48 h after addition of HGF/SF was observed. The high rate of ACT synthesis remained unchanged up to 60 h (Fig. 1) and even up to 72 h (not shown) after HGF/SF administration.

Since IL-6-induced expression of ACT, a typical class II APP, involves the activation of the transcription factors STAT1 and STAT3 [11,25,28], we investigated whether HGF/SF-mediated ACT expression also correlates with the activation of STAT1 and/or STAT3. When a STAT1/3-specific DNA probe was used to test nuclear extracts of HGF/SF-treated HepG2 cells in an EMSA, we found activation of STAT3 beginning at 7 h after addition of the growth factor (Fig. 2). As shown by the supershift with a STAT3α-specific antiserum (right panel of Fig. 2) the gel shift band seen after HGF/SF treatment is completely shifted to lower mobility proving that it indeed represents STAT3α.

When HepG2 cells were stimulated with HGF/SF at increasing concentrations for 15 h, a STAT3 activation was detected between 3 and 20 ng/ml HGF/SF, concentrations >20 ng/ml resulted in a drastic reduction of signal intensity reaching control levels at a dose of 100 ng/ml (Fig. 3).

It was of great interest to determine whether STAT3 activation is only a phenomenon occurring in hepatoma cells or whether it is also relevant in human hepatocytes. Fig. 4 clearly demonstrates the activation of STAT3 in human hepatocytes in primary culture upon stimulation with HGF/SF. The delayed STAT3 activation is similar to that observed in HepG2 cells (Fig. 2).

4. Discussion

Hepatocyte growth factor and scatter factor are identical αβ-heterodimeric glycoproteins mainly produced by mesenchymal cells that act predominantly on cells of epithelial origin which express the HGF/SF receptor. HGF/SF mediates all biological effects via its high-affinity cell-surface receptor c-Met [29,30]. c-Met (190 kDa) is encoded by the *c-met* proto-oncogene and composed of an extracellular α-chain (50 kDa) and a membrane-spanning β-chain (145 kDa). The intracellular part of the β-subunit contains a tyrosine kinase domain and several phosphorylation sites [29]. The Ras [31,32], phosphatidylinositol-3-kinase [33,34] and phospholipase Cγ [35] pathways are known to be activated after ligand-induced receptor phosphorylation. Up to now, there has been no evidence for a link between HGF/SF signalling and the JAK-STAT pathway.

The diversity of HGF/SF-used signalling pathways might explain the multitude of its biological effects: HGF/SF stim-

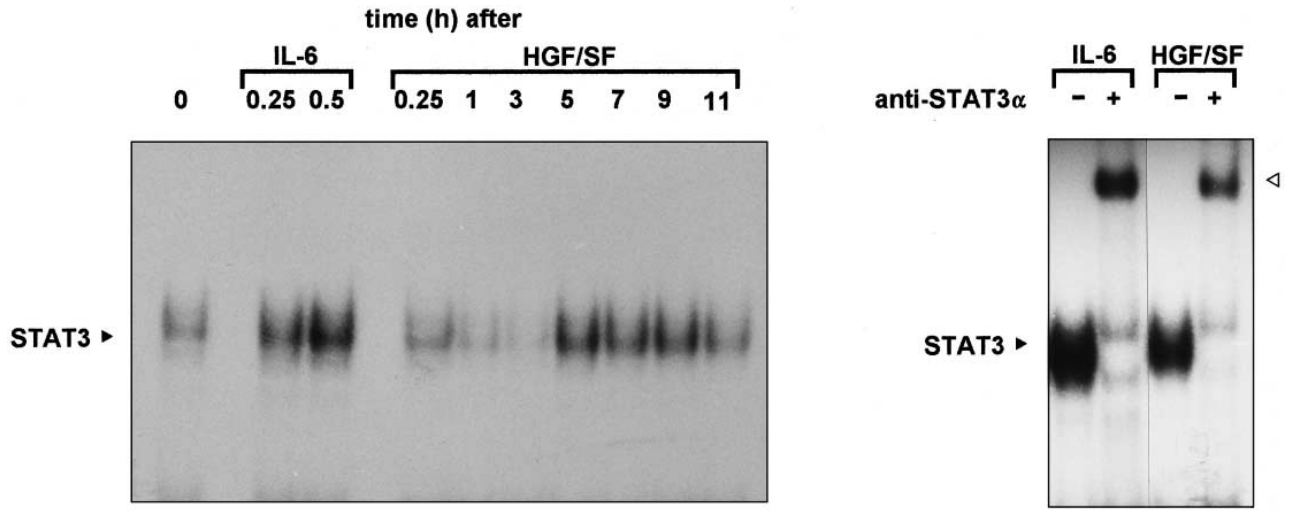

Fig. 4. STAT3 activation after stimulation of human hepatocytes in primary culture with HGF/SF. Human hepatocytes in primary culture were incubated without or with either 100 pg/ml of IL-6 or 10 ng/ml of HGF/SF for the times indicated. STAT3 activation is demonstrated by EMSAs (see legend to Fig. 2). Supershift experiments using the STAT3α-specific antiserum are shown in the panel on the right: human hepatocytes were stimulated with 100 pg/ml of IL-6 for 30 min (lanes 1,2) or 10 ng/ml of HGF/SF for 7 h (lanes 3,4). Exposure times of the two autoradiographs differ.

ulates growth of various cell types, e.g. hepatocytes, kidney tubular epithelium, keratinocytes, endothelial cells and melanocytes (reviewed in [29,30,36]). For hepatocytes, HGF/SF is the most potent mitogen, and it is believed to play an important role in liver regeneration in vivo [36–38]. Moreover, HGF seems essential during embryonic development [39,40], particularly of the liver [41]. Angiogenesis and wound healing are also influenced by the action of HGF/SF [42,43]. Overexpression of HGF/SF and/or c-Met occurs in a wide variety of human tumors. The generation of an autocrine stimulatory loop is assumed to be crucial in tumor formation as well as in invasiveness and in vivo metastatic potential of tumor cells (reviewed in [40]). Other well-described features of the HGF/SF are the induction of 'scattering' and increased motility as well as branching and other types of morphogenesis [44–49].

Although IL-6 is regarded as the major regulator of APP synthesis in liver [7,8], HGF/SF has recently also been found to be a regulator of APP expression [18,19].

Since IL-6 signalling involves the JAK/STAT pathway [11,12], we examined whether HGF/SF also uses components of this pathway. This could indeed be shown in the present work where we demonstrate HGF/SF-mediated STAT3 activation. In contrast to the rapid IL-6 signalling events [11,14], we observed a surprisingly delayed STAT3 activation after HGF/SF: In IL-6-stimulated HepG2 cells (Fig. 2) and hepatocytes (Fig. 4), the tyrosine phosphorylation of STAT transcription factors and their subsequent translocation to the nucleus – detectable by binding to an IL-6-responsive element – takes place between 5 and 30 min, leading to the transcriptional activation of APP genes. In the case of HGF/SF, we did not observe STAT3 activation before 5–7 h after stimulation with the growth factor. Further experiments should clarify this phenomenon. The retarded STAT3 activation could be due to de novo synthesis of signalling molecules acting upstream of STAT3.

A similar delay in STAT activation has been reported for the G-protein-coupled angiotensin II receptor system [50].

A further difference between the STAT3 activation after IL-6 and HGF/SF was the long-lasting activation state of the transcription factor in the case of HGF/SF compared to the transiently activated transcription factor after stimulation with IL-6. Since STAT3 activation after IL-6 is due to tyrosine phosphorylation [11,12], it is possible that (a) tyrosine phosphatase(s) – acting as negative regulators of STAT activity – is (are) differently regulated by the two cytokines.

Obviously there is need to identify the mechanisms responsible for these kinetic features and the kinase(s) responsible for the HGF/SF-mediated tyrosine phosphorylation of STAT3. Recently, it has been demonstrated that the intrinsic tyrosine kinase of the epidermal growth factor receptor directly activates STAT3 [51]. Since c-Met is also a receptor tyrosine kinase, further work is required to show the possible role of this receptor kinase in STAT3 activation.

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