Non-autocrine, constitutive activation of Met in human anaplastic thyroid carcinoma cells in culture

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Summary Activation of Met by its ligand HGF has been shown to elicit both mitogenic and motogenic responses in thyrocytes in vitro. In the present study we have investigated the expression of Met in human anaplastic thyroid carcinoma cells in culture. There was a variation in expression level and size of Met in the different cell lines; high Met expression was found in four cell lines, compared to non-neoplastic human thyrocytes. Treatment with glucoproteinase F showed that the size differences observed were due to variances in the degree of glycosylation. Interestingly, in cell lines with high expression of Met, the receptor proteins were found to be constitutively tyrosine phosphorylated. None of these cell lines expressed HGF mRNA, and addition of suramin did not affect the level of tyrosine phosphorylation of Met in unstimulated cells, suggesting the absence of autocrine stimulatory pathways. Furthermore, we did not observe MET gene amplification, activating mutations or phosphatase defects. The tyrosine phosphorylated receptors appeared functionally active since the receptors associated with the adaptor molecule Shc. In summary, we have found ligand-independent constitutively activated Met in four out of six anaplastic thyroid carcinoma cell lines.

Keywords: Met; thyroid; anaplastic carcinoma; receptor activation

The receptor tyrosine kinase Met consists of two disulphide-linked polypeptide chains, α and β, with molecular masses of 50 and 145 kDa respectively (Giordano et al, 1989). Hepatocyte growth factor (HGF), also known as scatter factor (SF), has been demonstrated to be the ligand for Met (Bottaro et al, 1991; Naldini et al, 1991). Met is expressed in a variety of cell types, mainly of epithelial origin (Zarnegar and Michalopoulos, 1995). Activation results in a number of cellular responses, such as growth, motility and morphogenesis, depending on cell type (Stoker et al, 1987; Higashio et al, 1990; Rubin et al, 1991; Weidner et al, 1991). In the cytoplasmic domain of the receptor protein there are two tyrosine phosphorylation sites (Y1234 and Y1235) needed for activation of the kinase domain (Longati et al, 1994; Zhen et al, 1994), and two others for association with different SH2-domain containing signalling proteins (Y1349 and Y1356) (Ponzetto et al, 1994). Met has been shown to interact with Grb2, phospholipase C-γ (PLC-γ), phosphoinositol (PI) 3-kinase, Shc and Src (Bardelli et al, 1992, 1994), as well as Gab 1 (Weidner et al, 1996) and a tyrosine phosphatase (SHP2/Syp 2) (Villa-Moruzzi et al, 1993; Fixman et al, 1996). Recently, it was demonstrated that a member of the ets family of transcription factors, ets 1, positively regulates the expression of Met (Gambaratto et al, 1996).

Overexpression of Met has been reported in several different types of carcinomas, including ovarian (Di Renzo et al, 1994), pancreatic (Di Renzo et al, 1995α), colorectal (Di Renzo et al, 1991; Prat et al, 1991) and thyroid carcinomas (Di Renzo et al, 1991, 1992, 1995β). High expression of Met protein was found in approximately 70% of papillary thyroid carcinomas. The overexpression was not related to gene amplification, but the histotype of the tumours correlated to bad prognosis for the patients (Di Renzo et al, 1992). In normal thyroid epithelium in vivo Met is only expressed at a very low level (Di Renzo et al, 1991, 1992). However, HGF has been shown to be a very potent mitogen for both human and dog thyroid follicle cells (Dremier et al, 1994; Eccles et al, 1996). Besides the growth stimulatory effect, HGF also induced de-differentiation of the thyrocytes, as have been observed after stimulation with other thyroid mitogens (Westermark et al, 1983). Furthermore, expression of HGF has also been found within the human thyroid (Zarnegar et al, 1990), making a paracrine stimulation possible in the regulation of thyroid follicle cell growth and function.

In the present study we have investigated the expression and activity of Met in human anaplastic thyroid carcinoma cell lines, as well as in non-neoplastic human thyrocytes in primary culture. We found an overexpression of Met in a majority of the carcinoma cell lines, viz. in HTh 83, C 643, SW 1736 and KAT-4, compared to the level found in normal thyrocytes. In these thyroid carcinomas, Met appeared to be constitutively activated in a ligand-independent fashion.

MATERIALS AND METHODS

Cell lines

The human anaplastic thyroid carcinoma cell lines HTh 7 (Carlsson et al, 1983), HTh 74 (Heldin et al, 1991), HTh 83 (Dahlman et al, unpublished results), C 643 (Heldin et al, 1988), SW 1736 and KAT-4 (Ain and Taylor, 1994) were routinely grown in Eagle’s minimal essential medium (HTh 7, HTh 74, C 643 and SW 1736) or in RPMI 1640 medium (HTh 83 and KAT-4). As a control for Met expression we used a hepatic carcinoma cell line, Hep G2, cultured in RPMI 1640 medium. The B-cell line U2889, used as a control in the Southern blot analysis, was cultured in RPMI 1640 medium. JJN 3 (Börset et al, 1996), a myeloma cell
Northern blot analysis

Total RNA was extracted from cells using a LiCl/urea method (Auffray and Rougeon, 1980), and mRNA was enriched by poly-A+ selection. Total or poly-A+ RNA (15 or 10 μg per lane respectively) was size-fractionated in a 0.8% agarose gel under denaturing conditions and transferred to a nitrocellulose filter. The filters were hybridized as described before (Heldin and Westermark, 1988), using a 32P-labelled (MegaPrime kit; Amersham Pharmacia Biotech, Uppsala, Sweden) human Met (Rong et al., 1992) or a 0.7 kb human HGF-β subunit cDNA fragment (pKK233-DEβS; Nakamura et al., 1989). The hybridization with the HGF probe was performed using QuikHyb solution (Stratagene, La Jolla, CA, USA). After a wash in 2× saline sodium citrate (SSC) (1× SSC consists of 0.15 M sodium chloride and 0.125 M sodium citrate, pH 7.0) and 0.5% sodium dodecyl sulphate (SDS) for 30 min at 37°C, and a subsequent wash in 0.1× SSC and 0.1% SDS at 60°C for 25 min. Filters were autoradiographed at –70°C using Kodak XAR-5 films and intensifying screens. In order to check for loading differences the filters were hybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH; pGAP) probe (Tso et al., 1985).

Southern blot analysis

Chromosomal DNA was extracted from the cell lines by using proteinase K (100 μg ml–1) in 10 mM Tris–HCl, 1 mM EDTA, 1% SDS and 0.1 M sodium chloride (NaCl) for 20 h at 37°C, followed by a phenol extraction. Chromosomal DNA was precipitated in ethanol after extraction twice with isobutanol/phenol/chloroform. The hybridization with the probe was performed using QuickHyb solution (Stratagene, La Jolla, CA, USA). After a wash in 2× saline sodium citrate (SSC) containing 0.15 M sodium chloride and 0.125 M sodium citrate, pH 7.0 and 0.5% sodium dodecyl sulphate (SDS) for 30 min at 37°C, the filters were hybridized for 1 h at 4°C and subsequently washed four times in 0.1× SSC and 0.1% SDS at 37°C for 30 min. Filters were then autoradiographed at –70°C using Kodak XAR-5 films and intensifying screens. As a control for equal loading, the filter was stripped and rehybridized with a 3 kb human HGF-β subunit cDNA fragment (pPAI3; obtained from Dr. Sawdey, Scripps Research Institute, CA, USA).

Western blot analysis

Cells were incubated with or without HGF (rhHGF; R&D Systems, Oxford, UK), 10 ng ml–1, for 6 min at 37°C, washed with phosphate-buffered saline (PBS) containing 1 mM Na2VO4, and lysed in a buffer consisting of 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA and 0.5% NP-40, with protease and phosphatase inhibitors (35 ng ml–1 phenylmethylsulphonyl fluoride (PMSF), 1.4 μg ml–1 aprotinin, 1 μM Na2VO4, 10 mM NaF, 1 mM ZnCl2 and 50 μM Na3VO4). After a 30 min incubation on ice the cell lysates were centrifuged for 15 min at 20 000 g at 4°C. The clear supernatants were collected and used in the experiments. The protein concentrations were determined using a commercial kit (Pierce Chemical Co., Rockford, IL, USA). Following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions, an immunoblot analysis was performed using anti-Met (C-28) or anti-Syp 2 antibodies (C-18, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), with a secondary horse-radish peroxidase conjugated anti-rabbit-IgG (Amersham Pharmacia Biotech, Uppsala, Sweden). Detection of signals was done with a commercial kit for chemoluminescense (Pierce Chemical Co., Rockford, IL, USA).

Glycoproteinase F digestion

Total cell lysate (20 μg per sample) from each cell line was diluted to 100 μl with a buffer consisting of 0.25 M Na2HPO4, pH 7.5, 10 mM EDTA, 10 mM β-mercaptoethanol (β-ME) and 0.5% (w/v) n-octylglucoside, boiled 3 min and digested with 2 U glycoproteinase F (USB, Cleveland, OH, USA) for 24 h at 37°C. Samples were analysed in a Western blot using the anti-Met antibody.

Immunoprecipitation of tyrosine phosphorylated proteins

Cell lysates were prepared as described above. Tyrosine phosphorylated proteins were immunoprecipitated from 0.5 to 1 mg of total lysate (0.5 mg for the experiment in Figure 4 and 1.0 mg for experiment in Figure 5) using agarose-conjugated anti-phenotyposphotyrosine (PY) antibodies (PY-20-agarose; Transduction Laboratories, Lexington, KY, USA). Lysates were incubated for 1.5 h at 4°C, and subsequently washed four times in lysis buffer. The samples were boiled in sample buffer containing β-ME, and separated on a 7.5% polyacrylamide gel. Immunoblots with the anti-Met antibody were done as previously described.

Co-immunoprecipitation of Met and Shc proteins

Total cell lysates (1.2 mg per sample) were incubated with anti-Shc antibodies (1 μg ml–1; PG-797 from Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for 1 h at 4°C. After another 45 min incubation with GammaBind Plus Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). Detection of signals was done with a commercial kit for chemoluminescense (Pierce Chemical Co., Rockford, IL, USA). Following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions, an immunoblot analysis was performed using anti-Met (C-28) or anti-Syp 2 antibodies (C-18, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), with a secondary horse-radish peroxidase conjugated anti-rabbit-IgG (Amersham Pharmacia Biotech, Uppsala, Sweden). Detection of signals was done with a commercial kit for chemoluminescense (Pierce Chemical Co., Rockford, IL, USA).

Figure 1. Expression of c-met mRNA in human anaplastic thyroid carcinoma cell lines. Ten micrograms of poly A+ ·RNA were separated in a 0.8% agarose gel, transferred to a nitrocellulose filter and hybridized with a human Met cDNA probe as described in Materials and Methods.
Biotech, Uppsala, Sweden) the precipitates were washed three times in lysis buffer, boiled in SDS-PAGE sample buffer with β-ME, and Western blots were performed as described above using the anti-Met antibody.

**RT-PCR and sequencing**

Total RNA was extracted from the HTh 83 and KAT-4 cells as described above. The RNA was converted into cDNA using the forward polymerase chain reaction (PCR) primer as template, utilizing a commercial kit (GeneAmp, Perkin-Elmer, NJ, USA). The cDNA was amplified by PCR, 30 cycles, using two primers spanning exon 18 and 19 of the MET gene: GGCAAGAAAATTCACTGTGC (forward) and TCCCAAGAGGGACGCCCAA (reverse). Following PCR, the reaction products were purified using a commercial kit (Qiaquick PCR-purification kit, Qiagen, Hilden, Germany) and ligated into the pGEM-T cloning vector (Promega, Madison, WI, USA). The Met fragments were sequenced on an ABI 377 automated sequencer, using the forward and reverse primers described above as sequencing primers.

**RESULTS**

**Expression of Met in human anaplastic thyroid carcinoma cell lines**

Northern blot analysis of poly-A+-RNA extracted from the anaplastic thyroid carcinoma cells revealed c-met transcripts in all six cell lines (Figure 1). The size of the mRNA found was approximately 7 kb, i.e. of the same size as in AG1518 human foreskin fibroblasts and in the hepatic carcinoma cell line Hep G2. The amount of Met mRNA observed differed between cell lines; however, there was a fairly good correlation between the mRNA level and the amount of Met protein detected in immunoblotting experiments (Figure 2A); showing a high or very high expression of Met in HTh 83, C 643, SW 1736 and KAT-4 cell lines, compared to the Met protein level in primary cultures of non-neoplastic human thyroid follicle cells (Figure 2B).

The size of the Met protein varied in the different cell lines. This appeared to be due to differences in glycosylation since treatment with glycoproteinase F resulted in deglycosylated Met proteins of similar size (Figure 3).

The high expression of Met observed was not a result of gene amplification since Southern blot analysis of DNA extracted from the cell lines did not show any signs of increased MET gene copy number (Figure 4). As a DNA loading control the filter was hybridized with a plasminogen activator inhibitor-1 (PAI-1) cDNA probe with similar results (data not shown).

**Constitutive tyrosine phosphorylation of Met**

The expressed Met proteins seemed functionally active since stimulation with HGF induced tyrosine phosphorylation of the receptors, determined by immunoprecipitation with anti-PY antibodies.
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(Figure 5). However, in four of the cell lines, HTh 83, C 643, SW 1736 and KAT-4 cells, we found phosphorylated Met also in unstimulated cells. This finding suggests a constitutive phosphorylation and maybe also activation of Met. As seen in Figure 5, in KAT-4 cells there was virtually no difference between the amounts of phosphorylated Met precipitated in unstimulated compared to the HGF-stimulated cells.

To study if the observed tyrosine phosphorylation of Met was due to endogenous production and autocrine stimulation by HGF, the HGF expression in the cell lines was investigated. Northern blot analysis of total RNA extracted from the cell lines showed expression of HGF mRNA only in the HTh 74 cells, and not in the cell lines with constitutively phosphorylated Met proteins (Figure 6). Furthermore, incubation of cells with suramin (200 µg ml⁻¹) for 4 h prior to lysis showed the receptors to be tyrosine phosphorylated even in the presence of suramin (Figure 7; only results from HTh 83 and KAT-4 are shown, however, similar results were obtained with the C 643 and SW 1736 cells). Since suramin blocked Met activation by 10 ng ml⁻¹ of HGF added to the Hep G2 cells (Figure 7), the current findings suggest a ligand-independent phosphorylation of the receptor in the carcinomas.

Changes or differences in the phosphatase activity might contribute to the constitutive activation of Met found. Therefore, we performed Western blot analysis of the phosphatase functionally coupled to Met, Syp 2, and also compared the phosphatase activity in the different cell lines. Syp 2 protein was found in equal amounts in all cell lines, and we could not detect any differences in the phosphatase activity (data not shown). Moreover, PCR-amplification and nucleotide sequencing of exon 17–19 of the kinase domain of the MET gene, known to contain mutations in renal carcinomas (Jeffers et al, 1997; Schmidt et al, 1997), did not show any nucleotide alterations in HTh 83 and KAT-4 cells (data not shown). To investigate the possibility of covalent dimerization of receptors due to aberrant di-sulphide bond formation between cysteine residues in Met, we performed Western blot analysis under non-reducing conditions of immunoprecipitated Met. Covalent dimerization would result in a Met species of approximately 380 kDa; however, this was not detected in HTh 83 or KAT-4 (data not shown).
Co-immunoprecipitation of Met and Shc proteins

To demonstrate a functional coupling to intracellular signalling proteins of the constitutively phosphorylated receptors, we performed co-immunoprecipitation experiments with anti-Shc antibodies. As seen in Figure 8, binding of Met to Shc was detected in lysates from both unstimulated and HGF stimulated cells in the HTh 83 and KAT-4 cell lines. However, in the control cells Hep G2, co-precipitated Met was only seen after HGF stimulation (Figure 8). Furthermore, we could also precipitate Met in unstimulated KAT-4 cells using GST-fusion proteins with SH2-domains containing activated proteins of the constitutively phosphorylated receptors, as judged by Western blot (data not shown). Thus, it seems as if the constitutively phosphorylated receptors in the carcinoma cells are functionally active in signal transduction.

DISCUSSION

Overexpression of Met has been observed in many different kinds of tumours, and there appears to be a link between Met activation, neoplastic transformation and malignant behaviour of tumour cells (Rong et al, 1992, 1994; Scotlandi et al, 1996). Anaplastic (undifferentiated) thyroid carcinoma of the giant cell type is one of the most aggressive and malignant tumours found in humans. The tumour usually kills the patient within a year from diagnosis (Carcangiu et al, 1985). In vivo, normal thyroid tissue barely expresses Met protein but many thyroid carcinomas have a 100-fold increase in Met expression (Di Renzo et al, 1991, 1992, 1995b). Interestingly, none of the anaplastic thyroid carcinomas studied (n = 5) by Di Renzo et al (1995b) had an elevated Met expression, although, in the same study three out of four poorly differentiated thyroid carcinomas overexpressed Met. However, in the present study we found Met expression in all human anaplastic thyroid cancer cell lines studied. Interestingly, in four out of six cell lines the receptors were found to be tyrosine phosphorylated even in unstimulated cells, suggesting a constitutive activation of the receptors. This notion was strengthened by the finding of an association of Met to the adaptor protein Shc in unstimulated cells, indicating a functionally active receptor. Thus, our current data clearly show that some anaplastic thyroid carcinomas have the capacity to overexpress a constitutively activated Met protein.

Since it is known from other cell types that growth in vitro up-regulates Met expression (de Juan et al, 1994), we compared the expression level in the anaplastic thyroid carcinoma cells to the level found in primary cultures of normal human thyroid follicle cells. The expression of Met was severalfold higher in some of the thyroid carcinoma cell lines, compared to control cultures of normal thyrocytes, clearly showing an overexpression of Met.

Constitutive activation of Met, as observed in the present study, has previously been reported from a gastric carcinoma cell line (GTL-16) (Ponzetto et al, 1991), and also in murine melanoma cells (B16-LS9) (Ruschiano et al, 1996). GTL-16 cells overexpress Met as a result of MET gene amplification (Ponzetto et al, 1991). So far, the exact mechanism behind the constitutive activation of Met in the GTL-16 cells has not been revealed; however, it is neither the result of rearrangements nor mutations of the MET gene (Ponzetto et al, 1991). A consistent finding in the present and previous studies is that a ligand-independent activation of Met is only observed in cell lines where Met is overexpressed (Ponzetto et al, 1991). Thus, it is possible that an overexpression leads to constitutive activation of the receptor proteins. Rusciano et al (1996) suggested that the ligand-independent activation observed in the B16-LS9 cells may be caused by the high number of receptors present on the cell surface, leading to spontaneous dimerization and phosphorylation. However, Ferracini et al (1995) reported very high expression of Met in the osteosarcoma cell line, U-2 OS, without any constitutive phosphorylation of the receptor.

Activation of the proto-oncogenes ras and ret is a common finding in thyroid carcinomas. Recently, Ivan et al (1997) demonstrated that infection of human thyrocytes with retroviruses containing activated ras or ret genes led to an up-regulation of the Met protein level. However, only one of the carcinoma cell lines in the present study, C 643, had activating ras mutations (Gly13Arg, in Ha-ras and Ala59Thr in Ki-ras; Ekwall et al, unpublished results) and none expressed activated ret (Alemi et al, unpublished results), suggesting that activation of ras or ret is not involved in the constitutive activation of Met.

Upon nucleotide sequencing of the Met kinase domain we were unable to detect any mutations in KAT-4 and HTh 83, showing that mutational activation of Met seems not to be present in our cell lines. Mutations in the kinase domain of Met in hereditary and sporadic papillary renal carcinomas were recently reported by Schmidt et al (1997). The mutations were located in the same region as activating mutations found in the Ret and Kit tyrosine kinase receptors, and were suggested to activate Met. The activating effect of the mutations was later demonstrated by Jeffers et al (1997). Neither did the HTh 83 or KAT-4 cell lines express a covalently dimerized receptor species, as judged by Western blot analysis under non-reducing conditions. This is the case in some of the mutated forms of Ret, where mutations in the cysteine-rich part of the extracellular domain yields a covalent association of receptors due to formation of di-sulphide bonds (Santoro et al, 1995).

Receptor phosphorylation and activation could be the result of an autocrine stimulation by HGF. Simultaneous expression of HGF and Met has been found in lung carcinoma cells (Tsao et al, 1993), in osteosarcoma cell lines (Ferracini et al, 1995), myeloma cell lines (Börset et al, 1996) and human breast carcinomas (Tuck et al, 1993), in osteosarcoma cell lines (Ferracini et al, 1995), myeloma cell lines (Börset et al, 1996) and human breast carcinomas (Tuck et al, 1993). In the thyroid carcinoma cell lines in the present study we also found HGF mRNA expression in one of the cell lines (HTh 74). However, in this cell line we have so far been unable to demonstrate the existence of an autocrine activation of Met. The
cell lines showing constitutive activation of Met did not express HGF mRNA. Moreover, autocrine activation of Met in the thyroid carcinoma cells does not seem likely since preincubation with suramin, which blocks the interaction between HGF and Met (Adams et al, 1991; Figure 6), had no effect on the phosphorylation pattern of the receptors, thus further strengthening the conclusion that ligand independent activation of the receptor occurred.

Differences in phosphatase activity, maybe of the protein phosphatase functionally coupled to Met (Villa-Moruzzi et al, 1993; Fixman et al, 1996), could result in an increased fraction of phosphorylated Met receptors. Increased phosphatase activity upon detachment of cells from the substratum leading to a decreased Met phosphorylation has been observed in murine melanoma cells (Rusciano et al, 1996). The cell lines in our study express equal amounts of the phosphatase Syt 2, and so far we have not been able to detect any differences in the activity of the cellular phosphatases. Thus, the constitutive activity of Met is not likely to be the result of impaired activity of the cellular phosphatases.

The role of the constitutive activation and overexpression of Met observed in the anaplastic thyroid carcinoma cells is not known. So far we have been unable to detect any differences between cells with and without constitutive Met activation with respect to growth characteristics or migration (data not shown). However, we speculate that constitutive activation of Met might have been of importance in early steps of malignant transformation, but at present stage, the contribution to the malignant phenotype is limited due to additional transformational events that these highly malignant tumour cells have undergone.

In summary, Met is expressed in all human anaplastic thyroid carcinoma cell lines studied. In four carcinomas with high expression levels of Met there was a ligand-independent activation of the receptors. The mechanism(s) behind the constitutive receptor phosphorylation and activation is not clear; however, receptor number seems to be an important factor. The importance of the overexpression and constitutive activation of Met for the malignant phenotype of the anaplastic thyroid carcinoma cells remains to be elucidated.

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