Expression of the c-Met Proto-Oncogene and Its Possible Involvement in Liver Invasion in Adult T-cell Leukemia

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ABSTRACT

c-Met is a tyrosine kinase receptor for hepatocyte growth factor and suggested to be involved in oncogenesis or metastatic phenotypes in many malignancies. Adult T-cell leukemia (ATL) is a neoplasia characterized by massive invasion of the leukemic cells into various organs. Recently, we have reported frequent hepatic involvement and the relationship between liver invasion and the poor prognosis in ATL. In the present study, we investigated the expression of c-Met in ATL cells and its relation to liver dysfunction. In three of four human T-cell lymphotrophic virus-I-positive T-cell lines, c-Met was expressed both at mRNA and protein levels, whereas it was not expressed in human T-cell lymphoma virus-I-negative T-cell lines. The expressed c-Met should be functional, because hepatocyte growth factor could induce the autrophosphorylation of c-Met. Although the viral-transactivating protein Tax has been shown to be involved in the deregulated expression of cellular genes, Tax mRNA was not detected in c-Met mRNA-expressed cell lines. From freshly isolated peripheral blood mononuclear cells, the expression of c-Met mRNA was detected in 10 of 16 ATL patients but not from healthy individuals. Finally, serum transaminase levels were significantly increased in c-Met-positive ATL cases, and all of the infiltrated c-Met-positive cells into liver were shown to be multilobularly nucleated phenotype. Taken together, these data suggest for the first time that c-Met is involved in the liver invasive phenotype of ATL.

INTRODUCTION

ATL is a mature helper T cell-derived neoplasia caused by HTLV-I (1, 2). One of the frequent manifestations of ATL is invasion of leukemic cells into various tissues. In general, T-cell infiltration into tissue depends on a cascade of rapid and selective adhesive interactions with endothelium. A step in this cascade requires a triggering to activate T-cell integrins. Thus, several studies have focused on the mechanism of ATL cell invasion from its expression of integrins and associated molecules (3, 4). Involvement of other molecules such as chemokine receptors CCR7/EBI1, heparin sulfate, and leukotactic factor activity-I has also been reported in ATL (5–10). Hence, various molecules required for adhesive interactions and migrations seem to be involved in the invasion step of ATL cells.

HGF, also known as scatter factor, was identified as a chemoattractant for a variety of cells. HGF is produced by cells of mesenchymal origin, including liver, but not by epithelial origin and has a pleiotropic function, such as liver regeneration. It also has mitogenic, morphogenic, and motogenic effects on epithelial cells, as well as endothelial cells (11). The receptor for HGF is encoded by met proto-oncogene (c-Met). The c-Met protein belongs to a tyrosine kinase cell surface receptor and consists of an extracellular α- and a transmembrane β chain. The β chain contains the tyrosine kinase domain as well as the site for tyrosine autophosphorylation. Ligation of HGF causes the autophosphorylation of c-Met, followed by a variety of signaling cascade (12). Although normal HGF/c-Met signaling is involved in many aspects of embryogenesis, abnormal HGF/c-Met signaling has been implicated in both tumor development and progression (13). In particular, HGF/c-Met signaling has been shown to play a significant role in promoting tumor cell invasion and metastasis (14). Furthermore, HGF and/or c-Met

1 The abbreviations used are: ATL, adult T-cell leukemia; HTLV-I, human T-cell lymphoma virus-I; PBMC, peripheral blood mononuclear cell; FBS, fetal bovine serum; GPT, glutamic pyruvic transaminase; GOT, glutamic oxaloacetic transaminase; MoAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; HGF, hepatocyte growth factor.
expression/overexpression has been documented in a wide variety of human tumors (15–20).

c-Met is predominantly expressed in epithelial cells but has been detected in various hematopoietic cells as well (21–29). Furthermore, lymphoid malignancies, such as multiple myeloma and several B cell lymphomas, were found to express c-Met, suggesting that c-Met is involved in the pathogenesis of these diseases (30–34). Regarding T cells, it was reported that HGF could trigger a subset of T-cell adhesion and migration; however, the expression of c-Met was not detectable by surface staining, PCR, or antiphosphotyrosine staining (35). On the other hand, thymic lymphoid cells were found to express c-Met and respond to HGF to generate mature T cells expressing antigen receptors (36).

Recently, we have reported frequent hepatic involvement and the relationship between liver invasion and poor prognosis in ATL (37). To clarify this issue, we investigated the expression of c-Met on ATL cells and found for the first time that c-Met is expressed in HTLV-I-positive T-cell lines. The expression of c-Met in the ATL cell line was functional, because we detected the autophosphorylation of c-Met in response to HGF. In addition, the expression of c-Met mRNA is documented in PBMCs freshly isolated from ATL patients. Furthermore, its expression is correlated with the liver dysfunction of ATL patients. Thus, we propose the possible involvement of HGF/c-Met signaling pathway in the invasion into tissues, particularly liver, in ATL.

MATERIALS AND METHODS

Cell Lines. Jurkat and MOLT4 are HTLV-I-negative T-cell lines. HUT102, MT-4, ST-1, and KK-1 are HTLV-I-positive T-cell lines (38, 39). ST-1 and KK-1 are cell lines derived from ATL patients (37). All of these cell lines are grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin G (50 units/ml), and streptomycin (50 μg/ml) in a humidified incubator containing 5% CO2 in air. HeLa cells were maintained in Dulbecco’s medium supplemented with 10% FBS.

Patient Samples. The diagnosis of ATL was based on clinical features, hematological findings, and serum anti-HTLV-I antibodies. Monoclonal HTLV-I provirus integration into the genomic DNA of leukemic cells was confirmed by Southern blot hybridization in all cases (data not shown). Mononuclear cells from patients with ATL were obtained by density gradient separation from the peripheral blood, before chemotherapy, after informed consent. Serum transaminase levels at the sampling point for each case were analyzed.

RT-PCR. Total RNA was extracted by using RNeasy mini kit (Qiagen, Hilden, Germany), according to the protocol provided by the manufacturer. First-strand cDNA was synthesized by using RT-PCR kit (Stratagene, La Jolla, CA) with oligodeoxythymidylic acid primers. Thereafter, cDNA was amplified for 30 cycles for c-Met, HGF, and Tax and for 23 cycles for β-actin, respectively. The oligo nucleotide primers used were as follows: for c-Met, sense 5’-ACT CCCCTCTGAAAACCAAGCC-3’, antisense 5’-GGCTTTACACTTCCGGCATTTAC-3’; for HGF, sense 5’-ACTGGCTTTTATTAGGCCTGACTCT-3’, antisense 5’-TGTTCTCC- TGGTACACTGCTTCTTT-3’, for Tax, sense 5’-ATCCCGT- GGAGACTCCCTCAA-3’, antisense 5’-AACACGTTAGACTGGG- TATCC-3’ (40); for β-actin, sense 5’-AAGAGGGCCATCCCT- CACCCT-3’, antisense 5’-TACATCGCTGGGTGTGAA-3’. Product sizes were 536, 505, 145, and 218 bp, respectively. Cycling conditions were as follows: denaturing at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. The PCR products were fractionated on 2% agarose gel and visualized by ethidium bromide staining.

Detection of c-Met Protein on the Cell Surface by Flow Cytometric Analysis. The expression of c-Met on the cell surface was analyzed by flow cytometry. Briefly, 3 × 105 cells were washed twice with PBS containing 2% FBS (FBS/PBS). Cells were incubated at 4°C with a mouse antihuman c-Met MoAb (Do-24; Upstate Biotechnology, Lake Placid, NY) for 60 min. After being washed twice with FBS/PBS, cells were incubated with FITC-labeled antimouse IgG MoAb (PharMingen), washed twice with FBS/PBS, suspended in FBS/PBS, and analyzed by FACScan using CellQuest software (Becton Dickinson).

Detection of c-Met Protein by Western Blot Analysis. Cellular lysates were fractionated by 10% SDS-PAGE gel, electrophoretically transferred to polyvinylidine difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA), and then analyzed for immunoreactivity with a mouse antihuman c-Met polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated sheep antimouse IgG (Amersham Life Science, Inc., Arlington Heights, IL) with an enhanced chemiluminescence detection system (Amersham Life Science, Inc.).

Immunohistochemistry. Using a formalin-fixed, paraffin-embedded section of liver from an autopsy specimen, we performed immunohistochemical analysis. The antibody used was the same as flow cytometric analysis at a dilution of 1:500. The protein was visualized using the DAKO LSAB kit (DAKO A/S, Glostrup, Denmark) that uses a biotinylated second antibody complexed with horseradish peroxidase and a diaminobenzidine-based stain.

Statistical Analysis. Statistical significance was determined by Student’s t test, Fisher’s exact probability test, and the Mann–Whitney test. Differences were considered to be statistically significant at P < 0.05.

RESULTS

Expression of c-Met and HGF in HTLV-I-positive T-cell Lines. The expression of c-Met mRNA in T-cell lines was determined by RT-PCR using c-Met-specific primers. In four HTLV-I-positive T-cell lines, HUT102, MT-4, and KK-1 expressed c-Met mRNA, whereas ST-1 did not (Fig. 1A, Lanes 3–6). In HTLV-I-negative T-cell lines Jurkat and MOLT4, the expression could not be detected (Fig. 1A, Lanes 1 and 2). Western blot analyses showed the same expression pattern of c-Met protein as that of mRNA (Fig. 1B). In KK-1, which is an ATL patients-derived cell line, c-Met protein is definitely detected, whereas the expression of the viral transactivating protein Tax could hardly be detected (Fig. 1B, Lane 6), suggesting the existence of the Tax-independent mechanism of c-Met expression in ATL cells. The cell surface expression of c-Met protein was confirmed in HTLV-I-positive T-cell lines by flow cytometric analyses (Fig. 1C). These data indicate that c-Met is
expressed in some of the HTLV-I-positive T-cell lines, including one established from an ATL patient. In other lymphoid malignancies, such as multiple myeloma or primary effusion lymphoma, coexpression of c-Met and HGF was reported, and an auto-stimulatory loop of HGF/c-Met signaling has been suggested (30, 31, 34, 41). Thus, we examined the expression of HGF mRNA in T-cell lines. HGF mRNA was also expressed in some of the HTLV-I-positive cell lines (Fig. 1A). The expression level of HGF was strong in HUT102 but weak in ST-1 and KK-1. Interestingly, coexpression of HGF and c-Met was demonstrated in HUT102 and KK-1, suggesting possible auto-stimulatory loop in these cells.

**Autophosphorylation of c-Met Was Induced in HTLV-I-positive T-cell Lines by HGF Treatment.** To examine whether c-Met expressed in HTLV-I-positive T-cell lines is functional, we examined the autophosphorylation of c-Met in response to HGF treatment. When KK-1, a c-Met-positive cell line, was incubated with HGF, several signals representing tyrosine phosphorylation were newly detected (Fig. 2A, Lanes 1 and 2). One of these signals was Mr 140,000, which corresponds to the autophosphorylation of c-Met. In ST-1, a c-Met-negative cell line, no difference could be seen with or without HGF treatment, suggesting that no signal could be transduced into ATL cells without c-Met expression (Fig. 2A, Lanes 3 and 4). To further confirm the autophosphorylation of c-Met in response to HGF treatment, c-Met was immunoprecipitated with c-Met-specific antibody, and Western blotting was performed using c-Met or phosphotyrosine-specific antibodies (Fig. 2B). A signal of Mr 140,000 representing autophosphorylation of c-Met was detected in HeLa, a positive control cell line, and KK-1, only when they were incubated in the presence of HGF (Fig. 2B, Lanes 1–4). In KK-1, the autophosphorylation level of HGF was strong in HUT102 but weak in ST-1 and KK-1. Interestingly, coexpression of HGF and c-Met was demonstrated in HUT102 and KK-1, suggesting possible auto-stimulatory loop in these cells.

![Fig. 1](image1.png)  
**Fig. 1.** HGF and c-Met are expressed in HTLV-I-positive T-cell lines. In A, HGF and c-Met mRNA were detected by RT-PCR analysis in HTLV-I-positive T-cell lines. Lane 1, Jurkat RNA; Lane 2, MOLT4 RNA; Lane 3, HUT102 RNA; Lane 4, MT-4 RNA; Lane 5, ST-1 RNA; Lane 6, KK-1 RNA. Top, c-Met; middle, HGF; bottom, β-actin. In B, c-Met protein is expressed in HTLV-I-positive T-cell lines. The expression of c-Met and Tax protein in human T-cell lines was analyzed by immunoblotting using anti-c-Met antibody or anti-Tax antibody. The same blots were reprobed with antiactin antibody for loading standard. In C, c-Met protein is expressed on the cell surface of HTLV-I-positive T-cell lines. T-cell lines were incubated with anti-c-Met MoAb or isotype control antibody, followed by staining with FITC-conjugated antimouse immunoglobulin antibody. Histograms were shown for anti-c-Met MoAb (solid lines) and isotype control antibody (dotted line).
Expression of c-Met mRNA in Clinical Samples from ATL Patients. To examine the expression of c-Met and HGF mRNA in primary ATL cells, RT-PCR analyses were performed using PBMCs freshly isolated from ATL patients. We could observe a definite band of c-Met in 10 of 16 samples from ATL patients, whereas no band could be detected in PBMCs from healthy donors (Fig. 3 and data not shown). As the number of atypical cells in each patient does not reflect the result of the c-Met mRNA amplification (Table 1), we regarded that the failure of the detection of c-Met mRNA was not caused by a lower input of tumor-derived RNA. As reported previously, the expression of Tax could not be detected in primary ATL cells, suggesting the existence of the Tax-independent mechanism in c-Met expression. On the other hand, the expression of HGF could not be detected in PBMCs from ATL patients.

Expression of c-Met Correlates with Liver Dysfunction in ATL. To evaluate the correlation between the expression of c-Met and liver dysfunction, serum transaminase level was examined in ATL patients. Interestingly, in 10 of c-Met-positive cases, 8 cases showed increased levels of serum transaminase, whereas only 1 of 6 c-Met-negative cases showed increased transaminase levels (Fisher’s exact probability test, P < 0.05). The levels of serum GOT and GPT were higher in c-Met-positive patients (Fig. 4), and there was a statistically significant difference in serum transaminase levels between the c-Met-positive and -negative patients (Mann-Whitney test: for GOT, P < 0.02; for GPT, P < 0.05).

As liver transaminase is a nonspecific marker of liver cell damage, liver autopsy specimens from a patient suffering with liver dysfunction (GOT, 94; GPT, 46) were analyzed. The patient died of leukemic crisis, associated with massive hepatosplenomegaly as a result of tumor progression. Immunostaining revealed most but not all infiltrated atypical lymphocytes were c-Met-positive cells (Fig. 5). On the other hand, all c-Met-positive lymphocytes showed multilobularly nucleated phenotype. Hepatocytes were stained weak but homogenous with anti-c-Met antibody as described previously (17). Similar pathological findings were seen in another autopsy case of ATL associated with liver dysfunction (data not shown).

DISCUSSION

In ATL, coetaneous involvement and hypercalcemia are frequently demonstrated. In addition, hepatosplenomegaly is often experienced, and this is one of the diagnostic criterion of ATL (42). Importantly, invasive characters of ATL cells into liver and consequent impaired general conditions seemed to be factors affecting poor prognosis (37). Cell migrations are a critical step in invasion of leukocytes. It has been reported that ATL cells adhere to endothelial cells through an adhesion cascade similar to normal leukocytes; thus, it is reasonable that...
several molecules found in usual pathological process have been required to infiltrate ATL. HGF has been proposed to be a physiologically relevant trigger in T-cell migration, although the expression of c-Met was not demonstrated (35). Indeed, c-Met expression on cells of T-cell origin has not been clearly recognized, although its expression has been documented in various tissue origins, including B cells. There was a report that the expression of c-Met was not detected in PBMCs but induced by reagents such as phorbol 12-myristate 13-acetate, Con A, and HGF in B cell-rich fractions (33). In this study, the expression of c-Met in ATL-derived cells was clearly detectable in PBMCs from ATL patients, and HTLV-I-infected T-cell lines, indicating that c-Met is expressed on cells of T-cell origin. In addition, the expressed c-Met was functional, because HGF could induce autophosphorylation of c-Met in the c-Met-positive ATL cell line KK-1. Although HGF was reported to induce cytoskeletal changes and migration of memory T cells on which c-Met was not detectable (35), this cytokine could not induce autophosphorylation and chemotaxis in the c-Met-negative ATL cell line ST-1. Thus, putative receptor(s) other than c-Met does not seem to be involved to mediate HGF signaling in ST-1. Alternatively, molecule(s) other than c-Met responsible for HGF signaling was concomitantly defective in this cell line.

It was reported that c-Met was expressed in Hodgkin’s Reed-Sternberg cells, and the expression of c-Met was strongly correlated with the presence of EBV (33). In addition, c-Met was expressed in human herpes virus-8-positive primary effusion lymphoma (34). Although the molecular mechanisms involved in the expression of c-Met in these virus-infected cells remain unknown, HTLV-I has the viral-transactivating protein Tax, which is involved in the deregulated expression of cellular genes; hence, Tax could induce the expression of c-Met in ATL cells. However, the expression of c-Met was not correlated with the expression of Tax in KK-1 (Fig. 1B, Lane 6). Similarly, c-Met mRNA could be definitely detected but not Tax mRNA in freshly isolated PBMCs (Fig. 3 and data not shown). Finally, we found that induced Tax in a Jurkat cell line, JPX9, could not induce c-Met but several Tax-induced genes (data not shown). Thus, the Tax-independent mechanism seems to operate the c-Met expression in ATL.

In some lymphoid malignancies, HGF and c-Met were coexpressed, and an autocrine mechanism is suggested to be involved in the pathogenesis (30, 31, 34). Concomitant expression of HGF and c-Met is also found in some ATL cell lines in our experiment. However, in primary ATL cells, the expression of HGF mRNA was not detected (Fig. 3). Although HGF is not expressed on primary ATL cells, HGF is induced in various cells by proinflammatory cytokines, such as interleukin-1 and tumor necrosis factor-α (43, 44), which were produced by ATL cells (45). Thus, HGF secreted in paracrine rather than autocrine mechanism seems to be involved in the pathogenesis of ATL. Furthermore, it was shown that HGF/c-Met signaling not only stimulates the growth of cells but also stimulates the release of chemokines in monocytes and other cell types (22, 46). Therefore, a part of stimulated ATL cells with HGF further augments the migratory activity of leukemic T cells through the liberation of chemokines. This probably accounts for the fact that not every invasive lymphocyte in liver expressed c-Met in our clinical specimens (Fig. 5). We speculate that monoclonally developed ATL cells become more aggressive phenotypes by the acquisition of c-Met expression on its surface.

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REFERENCES


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