Hepatocyte Growth Factor and Inducible Nitric Oxide Synthase Are Involved in Multidrug Resistance–Induced Angiogenesis in Hepatocellular Carcinoma Cell Lines

Nadia Lasagna,1 Ornella Fantappie,1 Michela Solazzo,1 Lucia Morbidelli,2 Serena Marchetti,1 Greta Cipriani,1 Marina Ziche,1 and Roberto Mazzanti1

1Department of Internal Medicine, Postgraduate School in Oncology, DENOTh, University of Florence, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy and 2Department of Molecular Biology, University of Siena, Siena, Italy

Abstract
Based on literature, it is possible to hypothesize that multidrug resistance (MDR) and angiogenic phenotypes are linked to each other in human liver cancer cells. Our goal is to assess whether MDR cells trigger angiogenesis and to study the possible molecular mechanisms involved. Conditioned medium from parental drug-sensitive P5 cells (P5-CM) and MDR-positive P1(0.5) cells [P1(0.5)-CM] stimulated human umbilical vein endothelial cells (HUVEC) survival, proliferation, migration, and microtubular structure formation, but P1(0.5)-CM had a significantly greater effect than P5-CM. Cell implants were done in the rabbit avascular cornea to measure angiogenesis in vivo: P1(0.5) cells induced an important neovascular response in rabbit cornea after 1 week, whereas P5 cells had no effect. P1(0.5) and P5 cells produced vascular endothelial growth factor, but only P1(0.5) secreted hepatocyte growth factor (HGF) into the medium, and small interfering RNA specific for MDR1 clearly reduced HGF production in P1(0.5) cells. The transcription factor Ets-1 and the HGF receptor c-Met were up-regulated in P1(0.5) cells and in HUVEC cultured in P1(0.5)-CM. Inducible nitric oxide synthase (iNOS) seemed to play a major role in the proangiogenic effect of P1(0.5), and its inhibition by 1400W blunted the capacity of P1(0.5) cells to stimulate HUVEC proliferation, migration, and Ets-1 expression. In conclusion, these data show that development of MDR and angiogenic phenotypes are linked to each other in MDR cells. HGF production, Ets-1 and c-Met up-regulation, and iNOS expression can be part of the molecular mechanisms that enhance the angiogenic activity of the MDR-positive hepatocellular carcinoma cell line. (Cancer Res 2006; 66(5): 2673-82)

Introduction
Hepatocellular carcinoma (HCC) is a hypervascular solid cancer characterized by a high degree of drug resistance (1). The multidrug resistance (MDR) phenotype, an intrinsic or acquired cross-resistance to a variety of structurally and functionally unrelated drugs, is almost constantly expressed in HCC and represents one of the major problems for cancer eradication by limiting the efficacy of chemotherapy (2). The hallmark of the MDR phenotype in cancer cells is the overexpression of the energy-dependent efflux pump P-glycoprotein (P-gp; ref. 2), encoded by the MDR1 gene, which is commonly overexpressed in HCC (3, 4). P-gp binds different amphipathic molecules, including chemotherapeutic agents that are exported out of the cell by ATP hydrolysis (5).

Tumor growth depends on angiogenesis, and the “angiogenic switch” of preexisting vessels is required to allow tumor progression, growth, and propagation to supply nutrients and oxygen (6–8). Angiogenesis is a complex multistep process, characterized by endothelial cell spreading, migration, and proliferation, modulated by several activators and inhibitors produced from the tumor microenvironment. Several factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and angiopoietin-1, have been proven to induce angiogenesis by promoting the migration and proliferation of endothelial cells (9–12).

HGF is a multifunctional factor that acts as a potent mitogen and morphogen for a variety of cell types, including endothelial, epithelial, liver cells, and others (13–15). HGF facilitates tumor cell motility by binding to the c-Met receptor expressed on various malignant cells, including HCC cells (16, 17), and binds to receptors expressed on endothelial cells, thus stimulating angiogenesis (15, 18).

A family of transcription factors known as ETS, which regulates the expression of numerous genes and integrins, is also involved in angiogenesis (19–22). Ets-1 is induced in endothelial cells in response to angiogenic growth factors, including basic FGF and VEGF (23). In addition, Ets-1 modulates the coordinated transactivating genes that are stimulated by the HGF, located upstream of the angiogenesis cascade (10).

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) products (NO and prostaglandins, respectively) also play an important role in angiogenesis, regulating several biological processes crucial for tumor growth, such as neovascularization (24, 25). With regard to this point, we showed that the induction of the MDR phenotype is associated with the expression of COX-2 and iNOS in human HCC cell lines in vitro (26). COXs are enzymes (COX-1 is constitutively expressed and COX-2 is inducible) that synthesize prostaglandins and thromboxanes from arachidonic acid (27). COX-2 is an immediate-early gene that is associated with different cellular functions and overexpressed in several human tumors. NOS catalyzes the enzymatic production of NO: endothelial and neuronal NOS are constitutively expressed in several cells of the organism, whereas the expression of the inducible isofrom (iNOS) occurs after specific stimuli and is implicated in various pathologic processes (28). Prostaglandins and NO, produced to a great extent by MDR-positive HCC cells,
play an important role in tumor growth (29) and in angiogenesis in vivo (30, 31) and in vitro (32). Accordingly, we hypothesized that MDR and angiogenic phenotypes could be linked to each other in human liver cancer cells. Because angiogenesis is correlated with the patient's prognosis, it is possible that both phenotypes are correlated with the aggressivity of the cancer.

The hypothesis that MDR and angiogenic phenotypes are linked to each other at least in HCC is also supported by the observation that angiogenesis in the liver may precede the occurrence of HCC for a long time (33). In particular, chronic hepatitis C virus (HCV) infection, one of the main risk factors for the development of HCC, is characterized by active angiogenesis of the liver during chronic hepatitis, which is significantly higher than that observed during matched chronic hepatitis B virus infection or other chronic inflammatory liver diseases (34). In addition, Rahman et al. showed a positive correlation between COX-2 and iNOS expression with hepatic angiogenesis (evaluated through microvessel density) in HCV-positive HCC, suggesting that both enzymes may be pathogenic factors in HCV-positive HCC due to their role in tumor angiogenesis (35). Accordingly, we showed recently that liver angiogenesis, estimated by the microvessel density in liver biopsies of patients affected by HCV-related liver cirrhosis, can be predictive of the occurrence of HCC.3

Based on these observations, the aim of this research was to study, in vitro and in vivo, the angiogenic activities of drug-sensitive and MDR HCC cell lines that constitutively express iNOS and COX-2 and that serve as a good experimental model. We also tried to show that the relationship between MDR and angiogenic phenotypes involves specific molecular aspects of these cell lines. Once they are well characterized, new pathways to cancer therapy may result.

Materials and Methods

Cell culture. Experiments were done on two cellular clones of a human HCC cell line (PLC/PRF/5; ref. 36). A MDR cell subclone, P1(0.5), was developed by a series of prolonged exposure to increasing concentrations of doxorubicin (Pharmacia & Upjohn, Milan, Italy) from parental drug-resistant 518HCC (PLC/PRF/5; ref. 36). A MDR cell subclone, P1(0.5), was developed by a series of prolonged exposure to increasing concentrations of doxorubicin (Pharmacia & Upjohn, Milan, Italy) from parental drug-resistant PLC/PRF/5 (PLC/PRF/5; ref. 36). MDR phenotype and are 100 times more resistant to doxorubicin than P5 (37).

Preparation of HCC-CM. P5 and P1(0.5) cells were grown in DMEM-10% FBS until 60% to 70% confluent. The culture medium was then removed, and cell layers were washed and incubated with DMEM-1% FBS (unconditioned control medium). After 1, 2, 3, and 6 days of culture in conditioned medium, the cells were washed twice with PBS, trypsinized, and counted using the trypan blue exclusion method.

Apoptosis assay. HUVEC (12,500 per well) were seeded in EGM onto coverslips in a 24-well plate. After 24 hours at 37°C, the medium was replaced with 500 μL P5-CM, P1(0.5)-CM, and P1(0.5)-CM treated with neutralizing anti-HGF and DMEM-1% FBS (unconditioned control medium). After 1, 2, 3, and 6 days of culture in conditioned medium, the cells were washed twice with PBS, trypsinized, and counted using the trypan blue exclusion method.

Evaluation of endothelial cell survival. HUVEC at the fourth passage were plated in 60-mm Petri dishes (125,000 per dish) and cultivated in EGM for 24 hours. After washing, the medium was replaced with 3 mL P5-CM, P1(0.5)-CM, and P1(0.5)-CM treated with neutralizing anti-HGF and DMEM-1% FBS (unconditioned control medium). After 1, 2, 3, and 6 days of culture in conditioned medium, the cells were washed twice with PBS, trypsinized, and counted using the trypan blue exclusion method.

Cell proliferation assay. Cell proliferation was determined by the [3H]thymidine incorporation assay. Briefly, 10^5 cells per well were seeded in 24-well plates in EGM. After 24 hours, the cells were serum starved in DMEM-1% FBS for 16 hours and then cultivated in P5-CM, P1(0.5)-CM, or DMEM-1% FBS for 24 hours. During the last 4 hours, the cells were pulsed with 1 μCi [methyl-3H]thymidine (Perkin-Elmer Italia, Milan, Italy) per well. The radioactivity was then removed and DNA was precipitated with cold 40% trichloroacetic acid solution and extracted with 1 mL of 0.2 mol/L NaOH. Recovered radioactivity was measured in a β-counter (model 1900 TR; Packard Tri-Carb, Zurich, Switzerland).

Migration assay. HUVEC migration was assayed with a 48-well microchemotaxis chamber (Neuroprobe, Biotar, Milan, Italy). The upper and lower wells were separated by a polycyrylpropylene-free polycarbonate filter (Bioramp), 8-μm pore, coated with type I collagen (100 μg/mL) and fibronectin (10 μg/mL; Sigma-Aldrich, Milan, Italy). DMEM-1% FBS and culture medium conditioned from HCC cells were placed in the lower wells, and cell suspension (50 μL; 12.5 × 10^5 cells) was added to the upper well. The chamber was incubated at 37°C for 4 hours and the filter was then removed and fixed in methanol. Cells migrated to the lower surface of the filter were stained with Diff-Quik (Biotar) and counted using a light microscope at ×40 magnification in six random fields per well. The results are expressed as number of migrated cells per square millimeter.

Measurement of tube formation by HUVEC. HUVEC were incubated with Cytodex 3 microcarrier beads (Sigma-Aldrich) at a concentration of 400 HUVEC per bead in 1 mL EGM. Beads with cells were shaken gently every 30 minutes for 3 hours at 37°C and 5% CO2 and after 12 to 16 hours were resuspended at a concentration of 150 cell-coated beads/mL in 2.5 mg/mL fibrinogen (Sigma-Aldrich) with 200 units/mL aprotinin (Sigma-Aldrich) at pH 7.4. Thrombin (0.19 units/mL) Sigma-Aldrich, Milan, Italy) were added to 300 μL fibrinogen/bead solution in each well of a 48-well tissue culture plate. Fibrinogen/bead solution was allowed to clot for 5 to 10 minutes at 37°C and 5% CO2. Three microliters of EGM (positive control), DMEM-1% FBS (negative control), and HCC-CM were added to each well and equilibrated with the fibrin clot for 1 hour at 37°C and 5% CO2. 300 μL were added after 1 hour and every other day. Bead assays were monitored for 4 days: high-resolution images of beads were captured on a Nikon Eclipse TE300 microscope at ×20 magnification, and tube formation was quantitated daily by vessel count at ×10 magnification.

Angiogenesis in vivo: rabbit cornea assay. Corneal assays were done in New Zealand albino rabbits (Charles River, Calco, Como, Italy) as described (39) in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law No. 86/609). Rabbis received 3 × 10^5 wild-type P5 cells in the left cornea and an equal number of P1(0.5) cells in the right cornea. Subsequent daily observation of the implants was made with a slit lamp stereomicroscope without anesthesia. The potency of angiogenic activity was evaluated based on the number and growth rate of newly formed capillaries, and an angiogenic score was calculated [vessel density × distance from limbus] as described (39).

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3 R. Mazzanti et al. Angiogenesis as a prognostic factor for HCC development in hepatitis C virus liver cirrhosis, unpublished data.
**Western blot analysis.** After washing the cells with PBS, cell monolayers were lysed in ice-cold buffer [0.09% NaCl, 20 mmol/L Tris-HCl (pH 7.6), 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 0.01% leupeptin] and centrifuged at 4°C for 10 minutes at 10,000 × g. The protein content of the supernatants was determined by using a BCA protein assay kit with bovine serum albumin (BSA) as standard (Pierce, Rockford, IL). The samples were boiled and subjected to 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes. Blots were blocked with 5% BSA blocker solution (Pierce) in PBS containing 0.1% Tween 20 and incubated overnight at 4°C with the primary antibodies: monoclonal anti- caspase-3 (E-8), polyclonal anti-eti-1, and polyclonal anti-c-Met were purchased from Santa Cruz Biotecnology (Santa Cruz, CA), monoclonal anti-P-gp (C219) was purchased from Calbiochem (San Diego, CA), and polyclonal anti-p-Met[Y1230/1234/1235] was purchased from Biosource Europe S.A. (Nivelles, Belgium). Anti-p-actin mouse monoclonal antibody (Sigma-Aldrich) was used as a control of protein loading. Blots were further incubated with secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature and finally incubated with SuperSignal West Pico Chemiluminescent for 5 minutes and exposed to CL-Xposure (Pierce). Densitometric analysis of the protein bands was evaluated by the software Scion Image.

**Determination of HGF and VEGF production.** The ELISA method was used to measure secreted HGF and VEGF in the culture medium by P5 and P1(0.5) cells. Cells (6 × 105) per well were seeded in 24-well plates and after 24 hours were washed with serum-free medium and then incubated with 1 mL DMEM-1% FBS for 3, 6, and 8 days at 37°C. The concentration of HGF and VEGF in culture medium was assessed using the Human HGF Immunoassay (Quantikine, R&D Systems, Minneapolis, MN) and the Human VEGF ELISA kit (Biosource Europe) according to the manufacturer's protocol. At 3, 6, and 8 days of culture, the cells were counted and the number of P5 and P1(0.5) cells was similar.

**Small interfering RNA transfection.** Positive control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) small interfering RNA (siRNA), siCONTROL nontargeting siRNA, and a pool of four different dsiRNA oligonucleotides directed against MDR1 (siGENOME SMARTpool, human ABCR1) were purchased from Dharmacon (Lafayette, CO). P1(0.5) cells were seeded in 60-mm dishes to reach 30% to 50% confluency after 24 hours of incubation and transfected with a total of 100 nmol/L siRNA using LipofectAMINE 2000 (Invitrogen Life Technologies, Milan, Italy) in antibiotic-free medium according to the manufacturer's instructions. Seventy-two hours after transfection, total proteins and RNA were extracted for reverse transcription-PCR and Western blot analysis.

**Neutralization of HGF in conditioned medium.** HGF produced by P1(0.5) cells was neutralized in P1(0.5)-CM with 1 µg/mL monoclonal mouse anti-human HGF/SF antibody, clone 24G12.111 (Sigma-Aldrich). Before the addition to HUVEC, the P1(0.5)-CM was incubated with neutralizing antibody for 1 hour at 22°C.

**Statistical analysis.** The significance of the differences between groups was determined by ANOVA followed by the Bonferroni post hoc test or Dunnnett’s multiple comparison test using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). P < 0.05 was considered significant.

**Results**

**P1(0.5)-CM enhances HUVEC survival and inhibits HUVEC apoptosis.** To study the *in vitro* angiogenic activity of the MDR-positive HCC cell line, we first determined the effect of culture medium conditioned from drug-sensitive and drug-resistant cell lines [P5-CM and P1(0.5)-CM, respectively] on endothelial cell survival and apoptosis. HUVEC were incubated with DMEM-1% FBS, P5-CM, or P1(0.5)-CM for 1, 2, 3, and 6 days. Both P5-CM and P1(0.5)-CM determined significant increases in endothelial cell survival compared with the unconditioned control medium. However, HUVEC survival was markedly increased when cultured in P1(0.5)-CM, with differences especially evident after 2, 3, and 6 days compared with controls (Fig. 1A). HUVEC were incubated with DMEM-1% FBS, P5-CM, or P1(0.5)-CM for 24 hours to evaluate the effect of these media on cellular apoptosis (Fig. 1B). Hoechst staining nuclear fragmentation analysis clearly showed that both P5-CM and P1(0.5)-CM protected endothelial cells from apoptosis induced by the unconditioned control medium, but the effect of P1(0.5)-CM was greater (Fig. 1B, i and ii). Moreover, in the same experimental conditions, we evaluated the expression of caspase-3 precursor protein (procaspase-3) by Western blot analysis (Fig. 1B, iii). Evident cleavage of caspase-3 was observed in HUVEC cultured in DMEM-1% FBS and P5-CM and it was not observed in HUVEC cultured in P1(0.5)-CM.

**P1(0.5)-CM stimulates HUVEC proliferation and migration.** The effect of conditioned medium on HUVEC proliferation was determined by measurement of [3H]thymidine incorporation into the DNA of endothelial cells cultured in the conditioned medium for 24 hours. These experiments showed that the addition of P5-CM or P1(0.5)-CM to quiescent HUVEC led to a significant increase in [3H]thymidine incorporation, P1(0.5)-CM stimulating HUVEC proliferation to a greater extent than the parental P5-CM (Fig. 1C, i).

The ability of P5-CM and P1(0.5)-CM to affect endothelial cell chemotaxis was assayed by measuring the capacity of HUVEC to cross a polycarbonate filter in a Boyden chamber. Both conditioned medium increased migration of endothelial cells (compared with the unconditioned medium); again, the maximal effect was observed by the use of P1(0.5)-CM (Fig. 1C, ii).

**Ets-1 and c-Met overexpression in HUVEC cultured in P1(0.5)-CM.** Ets-1 is one of the major regulators of the angiogenic switch in endothelial cells (40). We analyzed the expression of Ets-1 protein in HUVEC cultured in P5-CM or P1(0.5)-CM for 24 hours. In agreement with what has been observed in cell proliferation and migration assays, P1(0.5)-CM caused the up-regulation of Ets-1. The effect of P5-CM was clearly minor in comparison with that of P1(0.5)-CM (Fig. 1D). Endothelial cells express the c-Met receptor and HGF acts as an angiogenic growth factor (13, 41); the expression of c-Met receptor in HUVEC cultured in P5-CM or P1(0.5)-CM was modulated according to what was observed for Ets-1 (Fig. 1D).

**P1(0.5)-CM stimulates tubule formation by HUVEC.** To complete the study of the *in vitro* angiogenic activity of P5 and P1(0.5) cells, we tested the effect of these cells on the induction of microtubular structure formation (Fig. 2A). Results of microcarrier bead assays showed that HUVEC formation of tubular structures invading fibrin gel was significantly increased on the addition of medium conditioned by P1(0.5)-CM for 3 days (Fig. 2B).

**P1(0.5) cells induce angiogenesis in vivo.** To evaluate the angiogenic activity of P5 and P1(0.5) cells *in vivo*, the avascular rabbit cornea assay was done. Cells (density of 3 × 10^5 in 5 µL) were implanted under anesthesia in the micropocket surgically produced in the cornea of albino rabbits. The angiogenic output was evaluated over time by stereomicroscopic observation. No inflammatory response was observed after cell implantation. As shown in Fig. 3, whereas parental P5 cells were devoid of any angiogenic activity, P1(0.5) drug-resistant cells produced an evident neovascular response after 1 week.

**HGF production by P1(0.5) cell clone.** These experiments were done to assess whether HGF and/or VEGF production and secretion could explain what was observed in previous experiments.

**Determination of HGF production.** We analyzed HGF production from cultured P5 and P1(0.5) cells because of its importance as an angiogenic factor (10). HGF was not detected in P5-CM at 3, 6,
and 8 days of culture. On the contrary, P1(0.5) cells produced HGF and its production remained evident up to 8 days after the beginning of incubation (Fig. 4A). The marked difference in HGF concentrations also characterized P5-CM and P1(0.5)-CM derived from P5 and P1(0.5) cells cultured for 4 days in DMEM-1% FBS as described in Materials and Methods, which were used for cell survival, proliferation, and migration assays on HUVEC: P5-CM did not contain HGF at all, whereas P1(0.5)-CM contained 1.9 ng/mL HGF.
To study the role of iNOS and COX-2 in inducing HGF production that characterized only the P1(0.5) subclone, these cells were cultivated for 4 days in DMEM-1% FBS containing 10 μmol/L celecoxib or 10 μmol/L 1400W; these treatments did not significantly modify the production of HGF in P1(0.5) cells (data not shown).

**Determination of VEGF production.** P5 and P1(0.5) cells constitutively secreted VEGF into the culture medium and the concentration of soluble VEGF was similar in P5 and P1(0.5) cell culture medium at 3 days (118.0 ± 2.10 versus 114.60 ± 15.30), 6 days of culture (545.85 ± 94.00 versus 515.40 ± 55.30), and 8 days of culture (670.25 ± 63.25 versus 786.80 ± 114.50; data not shown). This observation was validated also in P5-CM and P1(0.5)-CM that had been used for endothelial cell survival, proliferation, and migration assays. P5-CM and P1(0.5)-CM, derived from P5 and P1(0.5) cells cultured for 4 days in DMEM-1% FBS, as described in Materials and Methods, contained similar quantities of VEGF (2.5 and 1.8 ng/mL, respectively).

**Inhibition of MDR1 expression by RNA interference decreases HGF production in P1(0.5) cells.** The effect of inhibiting the expression of MDR1 by siRNA on HGF production and Ets-1 expression was tested. The expression of P-gp was decreased by MDR1-targeted siRNAs (Fig. 4B, i) and siRNA targeting GAPDH strongly reduced GAPDH transcript levels (data not shown). The inhibition of P-gp expression was not observed using the nonspecific control siRNA or the mock transfection control (data not shown). MDR1-targeted siRNAs decreased HGF production of P1(0.5) cells (Fig. 4B, ii), whereas it had no significant effect on Ets-1 expression (data not shown).

**Ets-1 and c-Met overexpression in P1(0.5) cells.** Ets-1 has a crucial role in hepatocarcinogenesis and HCC progression (42), and the overexpression of Ets-1 and c-Met contribute to the increase of invasiveness and metastasis of HCC (43). Thus, we analyzed the expression of these proteins in P5 and P1 (0.5) cells (Fig. 4C): Ets-1 and c-Met were clearly up-regulated in P1(0.5) cells and, on the contrary, weakly expressed in P5 cells at basal conditions. Moreover, to test the hypothesis of an autocrine loop for HGF/c-Met in P1(0.5) cells, we evaluated the phosphorylation status of c-Met. Total proteins were extracted from P5 and P1 (0.5) cells that had conditioned the medium used for previous experiments: Western blot analysis showed a major intensity of the band of phospho-c-Met in P1(0.5) cells compared with P5 cells (Fig. 4C).

**Inhibitory effect of iNOS selective inhibitor on HUVEC proliferation and migration induced by P1(0.5)-CM.** To study the role of COX-2 and iNOS expression in determining the angiogenic capacity of P1(0.5) cells, the effect of conditioned medium from P1(0.5) cells, cultured in the presence of 10 μmol/L celecoxib or 10 μmol/L 1400W, was tested on HUVEC proliferation and migration. Results of these experiments showed that iNOS activity inhibition significantly reduced the stimulatory effect induced by P1(0.5)-CM on DNA synthesis (Fig. 5A) and migration (Fig. 5B) of HUVEC. Inhibition of COX-2 activity with celecoxib had a lower effect (Fig. 5A and B).

To support our observation on proliferation and migration assays, we analyzed the Ets-1 expression in HUVEC cultured in the same experimental conditions. Western blot analysis showed that Ets-1 expression significantly decreased in HUVEC cultured in P1(0.5)-CM plus 1400W, whereas P1(0.5)-CM plus celecoxib had a minor effect (Fig. 5C).

The possibility that the inhibitory effect of P1(0.5)-CM could be caused by a direct effect of celecoxib or 1400W on endothelial cells, proliferative and migrating activities were ruled out by experiments where treatment of HUVEC with 10 μmol/L celecoxib or 10 μmol/L 1400W had no effect on [3H]thymidine incorporation, on migration, and on Ets-1 expression in HUVEC (data not shown). We further evaluated the effect of conditioned medium from P5 cells, cultured in the presence of celecoxib or 1400W, on HUVEC proliferation and migration, but, as expected, no difference was found with respect to the effect of P5-CM (data not shown).

**Effect of neutralizing anti-HGF antibody on the angiogenic activity of P1(0.5) cells.** To study the role of HGF in determining
the angiogenic activity of P1(0.5) cells, the effect of the neutralizing anti-HGF antibody added to P1(0.5)-CM was tested on HUVEC survival, apoptosis, and migration (Fig. 6). Neutralizing anti-HGF antibody strongly suppressed P1(0.5)-CM-induced HUVEC survival, determining a 50% reduction in the viable cell number at 3 days of culture and a 30% reduction at 6 days of culture (Fig. 6A). Moreover, neutralizing anti-HGF antibody inhibited P1(0.5)-CM-induced protection from apoptosis (Fig. 6B) and stimulation of migration (Fig. 6C).

Discussion

In a previous work, we showed that the development of the MDR phenotype was associated with phenotypic changes that make human HCC cells potentially angiogenic (26). We showed that P1(0.5) cells, which overexpress P-gp and express the MDR phenotype, constitutively express iNOS and COX-2. That data prompted us to test whether these molecular changes could determine an effective stimulation of HUVEC migration and proliferation. To answer these questions, we cultured umbilical endothelial cells in conditioned medium derived from HCC cell lines [P5 and P1(0.5)]. Using this model, we now show that both HCC cell lines stimulate the growth, proliferation, migration, and microtubular structure formation by HUVEC in vitro. However, the MDR-positive cell clone, P1(0.5), has a significantly greater effect in comparison with their parental drug-sensitive clone P5. Moreover, P1(0.5) cells are able to induce angiogenesis in vivo when implanted in the rabbit avascular cornea, whereas P5 cells do not induce any response. We think that these results, which to our knowledge are reported for the first time in the international literature, are an important experimental demonstration to support the hypothesis of a relationship between angiogenic and MDR phenotypes in HCC cell lines. P1(0.5) cells were much more capable than parental P5 cells in stimulating angiogenic activities of HUVEC in vitro and angiogenesis in vivo in the model of avascular rabbit cornea assay.

To clarify the molecular mechanisms that could explain this phenomenon, Ets-1 expression in P5 and P1(0.5) cells at basal conditions was assessed. Ets-1 is involved in cell transformation, invasion, and metastasis (43) and is up-regulated and involved in the overexpression of matrix metalloproteinase-7 in human HCC, possibly contributing to the progression of HCC (44). Ets-1 was up-regulated in P1(0.5) cells, confirming the potential angiogenic phenotype of MDR-positive HCC cells. We also considered the possibility that acquisition of the MDR phenotype could be associated with the production and/or secretion of a paracrine factor (or more factors) that stimulates angiogenesis in HCC. With regard to this point, VEGF is the most important known growth factor of angiogenesis and it may be hypothesized that the MDR-positive HCC cells secrete greater amounts of VEGF compared with parental cells. This possibility was ruled out as we observed that the VEGF concentration was similar in the culture medium obtained from P5 and P1(0.5) cells [the number of P5 and P1(0.5) cells that conditioned the culture medium was similar]. Because it has been shown that HGF, produced by nonparenchymal liver cells, is involved in the stimulation of endothelial cell proliferation and induction of tube-like morphogenesis, with a synergistic effect together with VEGF (45), experiments were done to assess whether HGF production and/or c-Met expression could explain the difference between the effect of P5-CM and P1(0.5)-CM. We showed that the MDR phenotype is associated with enhanced HGF secretion and c-Met expression, whose phosphorylation is also enhanced in MDR-positive cells. Parental drug-sensitive HCC cells do not produce HGF at all and weakly express c-Met. The phenomenon that P1(0.5) cells express significant amounts of c-Met and contemporarily secrete its ligand (HGF), whereas P5 express only small amount of HGF receptor supports the hypothesis that MDR-positive cells possess an autocrine loop to enhance their proliferative activity, whereas the non-MDR parental cells depend, in some manner, on HGF secreted by other types of cells.

Figure 3. P1(0.5) cells induce angiogenesis in vivo. A, neovascularization is reported as angiogenic score, calculated at each observation. Columns, mean of three implants; bars, SE. B, corneal response at days 3 and 7 after implantation of P5 (i and ii) and P1(0.5) (iii and iv) cells, respectively. Arrows, newly formed vessels in implants with P1(0.5) cells.
could be important in clinical setting, as it makes MDR-positive HCC cells much more aggressive compared with parental drug-sensitive HCC cells.

Previous work by Van Belle et al. (46) showed that when submaximal concentrations of VEGF and HGF (1 ng/mL) are used VEGF or HGF taken singularly has no significant effect on endothelial cell proliferation and migration. On the contrary, a synergistic effect of the two growth factors is observed when they are used together. In our experiments, we showed that a concentration of 1.8 ng/mL VEGF and 1.9 ng/mL HGF was found in P1(0.5)-CM, whereas only VEGF at 2.5 ng/mL concentration was found in P5-CM. Thus, it is possible to hypothesize that the enhanced mitogenic and chemotactic response of endothelial cells cultured in P1(0.5)-CM is due to the synergistic effect of VEGF and HGF, whereas VEGF alone in P5-CM seems to have a minor effect.

In agreement with this observation, results of the present study show that the presence of HGF in P1(0.5)-CM is correlated with up-regulation of Ets-1 and c-Met in HUVEC cultured in P1(0.5)-CM for 24 hours. Molecular changes of Ets-1 and c-Met expression in endothelial cells seem to depend on the variation of HGF concentration in the medium of cultured HCC.

Tomita et al. showed that HGF up-regulates Ets-1 mRNA and angiogenic activity in human endothelial cells (10) and Ets-1 stimulates angiogenesis through the induction of VEGF and HGF (47). In other words, there is an autoloop up-regulating the HGF system (10). In our model, the overexpressed Ets-1 in P1(0.5) could stimulate HGF production, which can act on P1(0.5) cells themselves, possibly through c-Met activation, as also shown in our experiments, to enhance this pathway. Interestingly, modulation of P-gp expression by MDR1 siRNA resulted in a modulation of the HGF concentration in the conditioned medium of P1(0.5) cells, whereas Ets-1 expression was not changed. These observations suggest that Ets-1 regulate P-gp expression in MDR-positive cells (48), whereas P-gp is not involved in controlling Ets-1. The fact that HGF production and/or secretion decreased in MDR-positive cells transfected with siRNA specific for MDR1 could be explained by admitting an interference of these small molecules with a path-

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**Figure 4.** A, concentration of HGF in culture medium of P5 and P1(0.5) cells: culture medium was collected after 3, 6, and 8 days of culture and subjected to ELISA assay. B, transfection of MDR-1 siRNA determined a significant reduction in P-gp expression in P1(0.5) cells after 72 hours (i). Four days after transfection, cell culture supernatants were collected and HGF was measured by ELISA (ii). Columns, mean of three separate experiments in triplicate; bars, SE. *, P < 0.05 versus P1(0.5) cells. Comparison was made by one-way ANOVA followed by Bonferroni post hoc test. C, Western blot and densitometric analyses of Ets-1, c-Met, and p-Met[Y1230/1234/1235] expression in P5 and P1 (0.5) cells. Representative of three separate experiments.
for maintaining tumor growth and survival (51). In hepatocytes, Met directly binds to Fas and prevents Fas-induced apoptosis in a HGF-independent manner (52). It is important to consider that HGF is one of the most important humoral mediators of liver.

Many tumors constitutively express both HGF and Met to evade spatial and temporal regulatory mechanisms (49, 50). A high level of Met has been described in many carcinomas and it is required for maintaining tumor growth and survival (51). In hepatocytes, Met directly binds to Fas and prevents Fas-induced apoptosis in a HGF-independent manner (52). It is important to consider that HGF is one of the most important humoral mediators of liver.

way of HGF synthesis, but we cannot exclude other mechanisms, such as inhibition of its secretion.

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Figure 5. Proliferation (A), migration (B), and Ets-1 expression (C) in HUVEC cultured in P1(0.5)-CM. Effect of the exposure of P1(0.5) cells to celecoxib or 1400W. A, [3H]thymidine incorporation in HUVEC incubated for 24 hours with HCC-CM. Columns, mean of three separate experiments in quadruplicate; bars, SE. B, HUVEC migration induced by HCC-CM. Columns, mean of three experiments in triplicate; bars, SE. Control, DMEM-1% FBS; P1(0.5)-CM, conditioned medium from P1(0.5) cells; P1(0.5)-CM+CLX, conditioned medium from P1(0.5) cells treated with 10 μmol/L celecoxib for 4 days; P1(0.5)-CM+1400W, conditioned medium from P1(0.5) cells treated with 10 μmol/L 1400W for 4 days. *, P < 0.05 versus control; **, P < 0.05 versus P1(0.5)-CM. Comparison was made by one-way ANOVA followed by Bonferroni post hoc test. C, Western blot and densitometric analyses of Ets-1 expression in HUVEC cultured in P1(0.5) medium conditioned in the presence of celecoxib or 1400W. Representative of three separate experiments.

Figure 6. A, HGF neutralization in P1(0.5)-CM strongly reduced the effect of P1(0.5)-CM on HUVEC survival. HUVEC were cultured in P1(0.5)-CM treated with 1 μg/mL neutralizing anti-HGF antibody [P1(0.5)-CM+anti-HGF] for 3 and 6 days before cell count. Percentage of cell number at day 0. Points, mean of three separate experiments in triplicate; bars, SE. *, P < 0.05 versus control; **, P < 0.05 versus P1(0.5)-CM+anti-HGF. Comparison was made by two-way ANOVA followed by Bonferroni post hoc test. B, apoptosis of HUVEC cultured in DMEM-1% FBS, P1(0.5)-CM, and P1(0.5)-CM+anti-HGF for 24 hours. Apoptotic nuclei were visualized by Hoechst staining and identified by a fluorescence microscope. Apoptosis was expressed as a percentage of fragmented Hoechst-positive nuclei versus total Hoechst-positive nuclei of three experiments in triplicate. *, P < 0.05 versus control; **, P < 0.05 versus P1(0.5)-CM+anti-HGF. Comparison was made by one-way ANOVA followed by Bonferroni post hoc test. C, migration of HUVEC in response to DMEM-1% FBS, P1(0.5)-CM, and P1(0.5)-CM+anti-HGF. Columns, mean of three separate experiments in quadruplicate; bars, SE. *, P < 0.05 versus control; **, P < 0.05 versus P1(0.5)-CM+anti-HGF. Comparison was made by one-way ANOVA followed by Bonferroni post hoc test.
regeneration and is potentially related to the molecular mechanism of hepatocarcinogenesis (53). Xie et al. showed that, by transfecting the HGF CDNA into a weakly metastatic HCC cell line, it was possible to stimulate cell proliferation and mobility. The HGF autocrine system may increase the malignancy and metastatic potential of HCC (54). Findings of the present study not only support Xie et al.'s data but also show that acquiring of the MDR phenotype may result in a strengthening of the aggressiveness of cancer cells in terms of more intense angiogenesis.

In addition to the pathway that involves Ets-1 and HGF, it is important to consider the role that iNOS and COX-2, which are constitutively expressed in P1(0.5) but not in P5 cells (26), play in angiogenesis. By using specific inhibitors of COX-2 and iNOS activities, it has been possible to show, in vitro, the crucial role of iNOS in the proangiogenic effect of P1(0.5) cells on HUVEC. Exposure of P1(0.5) cells to 1400W, a specific inhibitor of iNOS activity, causes a significant decrease in the capacity of these cells to stimulate the migration and proliferation of HUVEC. Celecoxib, a specific inhibitor of COX-2 activity, caused a much minor effect. Direct exposure of HUVEC to 1400W and celecoxib was without effect, ruling out a possible direct effect of these two drugs on HUVEC proliferation and migration.

Furthermore, because NO stimulates endothelial cell differentiation to the angiogenic phenotype via the induction of the Ets-1 transcription factor (55), the expression of this protein was determined in HUVEC cultured in conditioned medium derived from P1(0.5) treated with celecoxib and 1400W. These experiments show that the marked increase in Ets-1 expression induced by P1(0.5)-CM was not significantly affected by celecoxib, whereas 1400W fully abolished the increase in Ets-1 expression. Because 1400W is a specific inhibitor of iNOS activity, these findings suggest that NO plays a crucial role in the regulation of Ets-1 expression. However, exposure of P1(0.5) cells to 1400W did not significantly change the HGF concentration in culture medium. This was unexpected and to us indicates that production of and/or secretion of HGF depends on several pathways in these cells and the inhibition of iNOS per se is not sufficient to block HGF production.

Trying to bridge these experimental findings to the clinical situation, we believe that our data are supported by findings obtained in patients with HCV-related liver cirrhosis who were monitored for the occurrence of HCC. In these patients, we were able to show that those with more intense liver angiogenesis, likely due to chronic HCV infection, were at higher risk for developing HCC compared with matched patients where liver angiogenesis did not occur or was much less pronounced. Because human HCC express the MDR phenotype almost constantly (1), it is possible to speculate that chronic HCV infection, development of MDR, angiogenic phenotype, and HCC occurrence are related to each other in patients with chronic HCV infection and that liver angiogenesis is an important predictive factor for the development of HCC.

Based on these observations, we conclude that the development of MDR and angiogenic phenotypes, through complex processes regulated by several factors, are linked to each other. Ets-1 overexpression, HGF production, c-Met expression and activation, and iNOS expression could be part of the molecular mechanisms responsible for the increased angiogenic activity of MDR-positive HCC cell line. Whether this phenomenon occurs in patients who develop HCC is not known; however, we do know that the occurrence of the MDR phenotype or a more intense tumor angiogenesis is related with the negative prognosis of cancer patients. If this is the case, better knowledge of the molecular mechanisms involved in producing these effects is essential, as it can lead to new therapeutic approaches in the future.

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Nadia Lasagna, Ornella Fantappiè, Michela Solazzo, et al.


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