Agonist monoclonal antibodies against HGF receptor protect cardiac muscle cells from apoptosis

Stefano Pietronave,1 Giancarlo Forte,2 Deborah Locarno,1 Simone Merlin,1 Andrea Zamperone,1 Giuseppina Nicotra,3 Ciro Isidoro,4,5 Paolo Di Nardo,2* and Maria Prat1,5*

Laboratories of 1Histology and 4Molecular Pathology, Department of Medical Sciences and 3Laboratory of Pathologic Anatomy, Department of Clinical and Experimental Medicine, Università del Piemonte Orientale “A. Avogadro,” Novara; 2Laboratory of Molecular and Cellular Cardiology, Department of Internal Medicine, Università “Tor Vergata” di Roma, Rome; and 5Biotecnologie per la Ricerca Applicata (BRMA), Novara, Italy

Submitted 23 December 2008; accepted in final form 28 December 2009

Hepatocyte growth factor (HGF), a pleiotropic cytokine with mitogenic, motogenic, morphogenic, and antiapoptotic effects in various cell types, is a cardioprotective growth factor that can counteract the loss of cardiomyocytes usually observed in cardiac diseases. HGF is a quite unstable molecule in its biologically active heterodimeric form. Since all HGF-induced biological responses are mediated by its high-affinity tyrosine kinase receptor (Met/HGF-R) encoded by the Met gene, we asked whether a monoclonal antibody (MAb) that displays receptor full agonist activity could protect cardiac muscle cell lines from hydrogen peroxide-induced apoptosis. We report that the MAb efficiently inhibited hydrogen peroxide-induced cell shrinkage, DNA fragmentation, annexin V positivity, mitochondrial translocation of bax, and caspase activation. The MAb was thus able to counteract apoptosis evaluated by both morphological and biochemical criteria. The agonist activity of the MAb was mediated by Met/HGF-R, since a Met/HGF-R-specific short hairpin RNA (shRNA) inhibited both activation of transduction pathways and motility triggered by MAb DO-24. The protective antiapoptotic effect of MAb DO-24 was dependent on activation of the ras-MAPK Erk1/2 and phosphatidylinositol 3-kinase (PI3-kinase)-Akt transduction pathways, since it was abrogated by treatments with their specific pharmacological inhibitors, PD-98059 and wortmannin. Moreover, the MAb induced a motogenic, but not mitogenic, response in these cells, mimicking in all aspects the natural ligand HGF but displaying a significant higher stability than HGF in culture. This MAb may thus counteract the loss of cardiomyocytes usually observed in cardiac diseases. HGF is a quite unstable molecule in its biologically active heterodimeric form. Since all HGF-induced biological responses are mediated by its high-affinity tyrosine kinase receptor (Met/HGF-R) encoded by the Met gene, we asked whether a monoclonal antibody (MAb) that displays receptor full agonist activity could protect cardiac muscle cell lines from hydrogen peroxide-induced apoptosis. We report that the MAb efficiently inhibited hydrogen peroxide-induced cell shrinkage, DNA fragmentation, annexin V positivity, mitochondrial translocation of bax, and caspase activation. The MAb was thus able to counteract apoptosis evaluated by both morphological and biochemical criteria. The agonist activity of the MAb was mediated by Met/HGF-R, since a Met/HGF-R-specific short hairpin RNA (shRNA) inhibited both activation of transduction pathways and motility triggered by MAb DO-24. The protective antiapoptotic effect of MAb DO-24 was dependent on activation of the ras-MAPK Erk1/2 and phosphatidylinositol 3-kinase (PI3-kinase)-Akt transduction pathways, since it was abrogated by treatments with their specific pharmacological inhibitors, PD-98059 and wortmannin. Moreover, the MAb induced a motogenic, but not mitogenic, response in these cells, mimicking in all aspects the natural ligand HGF but displaying a significant higher stability than HGF in culture. This MAb may thus be a valuable substitute for HGF, being more easily available in a quite unstable molecule in its biologically active heterodimeric form. Since all HGF-induced biological responses are mediated by its high-affinity tyrosine kinase receptor (Met/HGF-R) encoded by the Met gene, we asked whether a monoclonal antibody (MAb) that displays receptor full agonist activity could protect cardiac muscle cell lines from hydrogen peroxide-induced apoptosis. We report that the MAb efficiently inhibited hydrogen peroxide-induced cell shrinkage, DNA fragmentation, annexin V positivity, mitochondrial translocation of bax, and caspase activation. The MAb was thus able to counteract apoptosis evaluated by both morphological and biochemical criteria. The agonist activity of the MAb was mediated by Met/HGF-R, since a Met/HGF-R-specific short hairpin RNA (shRNA) inhibited both activation of transduction pathways and motility triggered by MAb DO-24. The protective antiapoptotic effect of MAb DO-24 was dependent on activation of the ras-MAPK Erk1/2 and phosphatidylinositol 3-kinase (PI3-kinase)-Akt transduction pathways, since it was abrogated by treatments with their specific pharmacological inhibitors, PD-98059 and wortmannin. Moreover, the MAb induced a motogenic, but not mitogenic, response in these cells, mimicking in all aspects the natural ligand HGF but displaying a significant higher stability than HGF in culture. This MAb may thus be a valuable substitute for HGF, being more easily available in a biologically active, highly stable, and purified form.

**APOTOPSIS IS A CONSERVED MECHANISM of cell removal that has an important role in tissue homeostasis and therefore is involved in various physiological and pathological conditions (18, 58). In the heart, physiological apoptosis is observed during ontogenesis in the postnatal shaping of ventricles (22). In adults, cardiomyocyte apoptosis is generally observed in response to a variety of cardiac stresses including ischemia-reperfusion injury, heart failure, diabetic cardiomyopathy, cardiac arrhythmias, congestive heart failure, myocarditis, and anthracycline-induced cardiomyopathy (11, 18). Oxidative stress, the result of an imbalance of the cellular redox state, plays a crucial role in the pathogenesis of myocardial ischemia-reperfusion injury (25, 34). In this case, apoptosis is executed mainly through the intrinsic pathway, where extra- and intracellular death stimuli are transmitted to mitochondria. Proapoptotic molecules such as bax are translocated there from the cytosol, with the consequence of release and activation of caspases and assembly of the apoptosome (11). Counteracting cardiomyocyte apoptosis may be envisaged as an innovative strategy in preserving heart function.**

The hepatocyte growth factor (HGF) receptor (Met/HGF-R) is a transmembrane tyrosine kinase, encoded by the Met protooncogene, that mediates several biological responses after stimulation by its cognate ligand (5, 7, 43, 57). In particular, its activation promotes cell motility, proliferation, morphogenesis, and protection from apoptosis, which may be present alone or in combination depending on cell type and environmental conditions (10, 16). While HGF is synthesized by cells of mesenchymal origin, the receptor is expressed on a broad range of tissues, including epithelial, endothelial, hemopoietic, muscle, and neuronal cells (5, 7). This ligand-receptor pair has a role during embryogenesis and organogenesis (6, 7, 51, 60, 62) as well as in the homeostasis of adult tissues (5, 7). Under normal conditions, the HGF signaling pathway is generally silent but can be resumed if an organ is injured, promoting protection from apoptosis and possibly wound healing and organ regeneration (33, 35, 36, 48).

Much evidence suggests that HGF and its cognate receptor are involved in myocardium homeostasis: 1) while both of them are expressed transiently and normally only during embryonic development of the myocardium (49), they are reexpressed when this tissue is injured by experimentally induced infarction (36, 41, 59) or in genetically determined degenerative cardiomyopathies (14, 37); 2) HGF plasma levels in infarcted patients and rodents that undergo experimental infarction are increased (32, 41); 3) endogenous and exogenous HGF display cardioprotective activity in experimental infarction and genetic degenerative cardiomyopathies; in all these cases endogenous and exogenous HGF display cardioprotective activity in experimental infarction and genetic degenerative cardiomyopathies; and finally, 5) HGF can act as a differentiating factor inducing the expression of cardio-specific markers in mesenchymal stem cells (16) and as a mobilizing factor for progenitor cardiac cells (30).

*P. Di Nardo and M. Prat contributed equally to this work.

Address for reprint requests and other correspondence: M. Prat, Dept. of Medical Sciences, Università del Piemonte Orientale “A. Avogadro,” Via Solaroli 17, 28100 Novara, Italy (e-mail: mprat@med.unipmn.it).

http://www.ajpheart.org 0363-6135/10 $8.00 Copyright © 2010 the American Physiological Society
The different biological responses are the consequence of the binding of HGF to its receptor, an event that induces receptor dimerization and activation (phosphorylation on tyrosine residues), which is then followed by the activation of multiple signal pathways (5, 43, 57). The phosphatidylinositol 3-kinase (PI3-kinase)-Akt and ras-MAPK Erk1/2 pathways are generally activated and involved in the antiapoptotic effect of HGF in different cell systems including cardiomyocytes (26, 36, 58, 63).

Unfortunately, the use of HGF in vitro and in vivo experimentation encounters several limitations. This growth factor is difficult to purify in its heterodimeric biologically active form and is quite unstable (40). To circumvent these limitations and since all HGF-induced biological responses are mediated by its specific high-affinity tyrosine kinase receptor Met/HGF-R, we asked whether a monoclonal antibody (MAb) that displays receptor full agonist activity could protect cardiac muscle cell lines from hydrogen peroxide-induced apoptosis. MAbs are highly stable molecules (46, 65), and it has been reported that the MAb DO-24, produced against the extracellular domain of the human receptor in our laboratory (44), could activate the receptor and trigger the several biological responses elicited by HGF also in species other than humans, being broadly cross-reactive among species (45).

We report that the MAb efficiently protects cardiac muscle cell lines from apoptosis, as evaluated by morphological (cell shrinkage, DNA fragmentation) and biochemical (annexin V positivity, mitochondrial translocation of bax, caspase activation) criteria. The biochemical and biological responses evoked by MAb DO-24 were Met/HGF-R dependent, since they were impaired in cells transduced with a lentiviral vector carrying a Met/HGF-R-specific short hairpin RNA (shRNA). The protective antiapoptotic effect of MAb DO-24 is dependent on the activation of the ras-MAPK Erk1/2 and PI3-kinase-Akt transduction pathways, since it is abrogated by treatments with specific pharmacological inhibitors. Moreover, the MAb induces a motogenic, but not mitogenic, response in these cells, mimicking in all aspects the natural ligand HGF. The MAb was found to be remarkably more stable than HGF in tissue culture. The DO-24 MAb can thus be a valuable substitute of the natural ligand HGF, with the added advantage of being more stable than HGF in tissue culture.

METHODS

Materials. All reagents were from Sigma-Aldrich (St. Louis, MO), except where otherwise stated. HGF was purchased from Peprotech (Rocky Hill, NJ). The hybridomas producing MAb DO-24 directed against the extracellular domain of the human Met/HGF-R (44, 45) and the MAb BD-31 (47) were previously obtained by M. Prat in her laboratory. The antibodies were purified from ascitic fluid by ammonium sulfate precipitation and affinity chromatography on protein A-Sepharose 4B. Annexin V-FITC was from Alexis (Lausen, Switzerland). MitoTracker-CMXRos was from Molecules Probes (Invitrogen, Carlsbad, CA). For terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) was used. Antibodies against the following antigens were used: phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY), mouse Met/HGF-R and Erk1/2 (sc-8057 and sc-154, Santa Cruz Biotechnology, Santa Cruz, CA), phospho (p)-Erk 1/2, Akt, and p-Akt (nos. 9106, 9272, and 4051, Cell Signaling Technology, Danvers, MA). Protein A-Sepharose and protein G-Sepharose, secondary antibodies, either horseradish peroxidase conjugated or fluorescein labeled, and polyvinylidene difluoride (PVDF) membranes were from Amersham (Amersham Biosciences, Otelfingen, Switzerland). The enhanced chemiluminescence (ECL) kit was from Pierce (Thermo Fisher Scientific, Rockford, IL). Caspase-9 activity was assayed by fluorometry (MBL International, Woburn, MA).

Cell cultures. The rat embryonic ventricular myoblast cell line H9c2 (2), Madin-Darby canine kidney (MDCK) cells, and NIH-3T3 fibroblasts (a line checked for not producing HGF) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 1,500 mg/l NaHCO3, supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM penicillin, and 50 μg/ml streptomycin. HL-5 cells, a clone of HL-1 cells (8, 9) were cultured on fibronectin-coated plates in Claycomb medium, 10% FCS, and antibiotics. Cells were passaged regularly and subcultured when at ~90% confluence before experimental procedures. For experiments involving immunostaining, cells were plated on glass coverslips in 24-well plates at 6 × 104 per well 1 day before the experiment. For immunohistochemistry and Western blot experiments, cells were plated on 10-cm petri dishes.

Cell transduction with shRNA. H9c2 cells were transduced with the third-generation lentivector mouse pCCLsin.PPT.hPGK.GFP.Wpre containing the green fluorescent protein (GFP) sequence under the control of the ubiquitous promoter hPGK (15) and a shRNA specific for Met/HGF-R (sequence 5’_GUCUAUGAGAAGGCGAUU_3’) (55) (H9c2-GFP-M3 cells), previously reported to downregulate Met expression and HGF-dependent effects in other cell types (42). As control, cells were transduced with the lentiviral vector pCCLsin.PPT.hPGK.GFP.Wpre (H9c2-GFP cells). Lentiviral vectors were produced as previously described (15). Vector titers were evaluated by transducing cells with serial dilutions of vectors, followed by cytofluorimetric analysis for GFP expression. Efficient transduction (85–95%) was demonstrated by cytofluorimetric analysis and in cells transduced by the shRNA Met/HGF-R expression was reduced by 70%.

Cell stimulation, immunoprecipitation, and Western blotting analysis. Subconfluent quiescent cells were incubated for 12 min at 37°C in the absence or presence of 0.50 nM HGF or 20 nM DO-24 for 30 min. Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in detergent-insoluble membrane (DIM) buffer (in mM: 50 PIPES pH 7.4, 300 succharose, 100 NaCl, 5 EGTA, 5 MgCl2, 100 ZnCl2) containing 1% Triton X-100, 1 mM orthovanadate, and a cocktail of protease inhibitors. Cell lysates were centrifuged at 13,000 rpm and 4°C for 15 min and incubated 2 h with anti-Met/HGF-R MAbs (DO-24 and DN-30) and protein G-Sepharose, as already described (3). Bound proteins were washed several times with ice-cold DIM buffer, eluted, and denatured by heating for 5 min at 95°C in reducing Laemmli buffer; proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in two identical gels. The proteins were then transferred onto PVDF filters, which were blocked with methanol for 5 min, then rinsed in water, and probed either with MAbs against phosphotyrosine or with commercial (sc-8057, Santa Cruz) MAbs against mouse-Met/HGF-R diluted in Tris-buffered saline (TBS)-5% BSA, for 2 h at 22°C. After extensive washing, immunocomplexes were detected with horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence. Similarly, total cell extracts (20 μg protein) were separated onto SDS-PAGE, transferred onto PVDF, and probed with antibodies against Erk 1/2, p-Erk 1/2, Akt, and p-Akt.

Apoptosis induction. To trigger apoptosis, cells were first incubated in culture medium containing H2O2 for 15 min; then the medium was changed, and cells were incubated for further increasing periods of time (19). HGF or MAbs were added with the fresh medium after H2O2 withdrawal. The Erk1/2 inhibitor PD-98059 and the PI3-kinase inhibitor wortmannin (WM) were added either alone or in combination 30 min before and during transient H2O2 treatment and then along the next 6 h of incubation. All experiments were performed in

AJP-Heart Circ Physiol • VOL 298 • APRIL 2010 • www.ajpheart.org
compared with Student's t-test. The level of significance was $P < 0.05$.

**Annexin V.** For cytofluorometer assessment of cell death, cells were collected, washed in PBS, and incubated for 15 min at room temperature with 2 μl of annexin V-FITC (100 nM) and 5 μl of propidium iodide (PI, 50 μg/ml) dissolved in 93 μl of buffer (in mM: 10 HEPES-NaOH, pH 7.4, 140 NaCl, 2.5 CaCl$_2$). At least 10,000 cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 488-nm argon laser. All experiments were performed in triplicate and repeated at least three times. Data are given as means ± SD. Couples of conditions were compared with Student’s t-test. The level of significance was $P < 0.05$.

**Mitochondrial staining and confocal microscope imaging.** Mitochondria were identified by staining with MitoTracker red probe (Invitrogen). H9c2 cells cultured on coverslips were fixed with 4% paraformaldehyde-2% sucrose in PBS and then permeabilized in 0.5% Triton X-100 in PBS. After blocking in 5% BSA, cells were stained with the bax antibody diluted (1:100) in PBS, 0.1% Triton X-100, 4% BSA-FCS for 1 h, followed by the secondary FITC-labeled antibody, as previously described (50). The coverslips were then mounted in Mowiol (1% in PBS). Images were captured with the Leica DMIRE2 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with Leica Confocal Software v. 2.6.1. For each experimental condition three coverslips were prepared. At least 4 fields in each coverslip were examined by two independent investigators. Representative images of selected fields are shown. Data were reproduced in at least three independent experiments.

4',6-Diamidino-2-phenylindole and TUNEL staining. Apoptotic cells were revealed by in situ TUNEL assay performed with the In Situ Cell Death Detection Fluorescent Kit according to manufacturer’s instructions (56). Cell nuclei were labeled with the DNA-labeling fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 50 μg/ml) in PBS, 0.1% Triton X-100, 4% FCS. Cells were visualized under a Leica DMI 600B fluorescence microscope equipped with a UV lamp for DAPI detection. Twenty consecutive fields for each sample and in any case not less than 150 cells were scored in a double-blind manner, and the ratio of TUNEL-positive figures versus total figure (DAPI-positive figures) in the untreated samples was given a value of 1, to which the values similarly obtained in the treated samples were referred. The assay was repeated three times in triplicate. Data are given as means ± SD. Couples of conditions were compared with Student’s t-test. The level of significance was $P < 0.05$.

**Casparase-9 activity assay.** After 15-min transient H$_2$O$_2$ treatment and a further 6-h treatment with medium containing Mab or HGF or not, H9c2 cells were scraped off the plates in ice-cold PBS. The cells from each 6-cm plate were then lysed in 50 μl of ice-cold cell lysis buffer from the kit for 10 min and then ruptured by 20 passages through a 26-gauge needle. The lysate was centrifuged at 12,000 g for 10 min at 4°C. For the assay, 50 μl of the cytosolic preparation (supernatant) was reacted into 50 μl of the kit assay buffer containing freshly added 10 mM DTT with 5 μl of the fluorescent substrate acetyl-(Leu-Glu-His-Asp)-7-amino-4-trifluoromethylcoumarin (LEHD-AFC, 1 mM) in each well of a 96-well dark plate. The reaction was evaluated after excitation at 400 nm and fluorescence emission at 525 nm in a Fluoro-Count reader (Packard, Bioscience, now PerkinElmer, Waltham, MA) at 0 min and every 30 min for 90 min. The values after subtraction of blanks were used to calculate the ratios of H$_2$O$_2$ to control and HGF-R agonists-H$_2$O$_2$ to control, and the values were normalized to the protein concentration.

**Proliferation assay.** For the cell growth assay, 2.5 × 10$^4$ cells were plated in 96-well plates in medium containing 10% or 2% FCS with or without HGF or DO-24 Mab. Fresh medium with or without stimulating factors was replenished every 2 days. Cell number was estimated by a colorimetric assay after staining with 0.1% crystal violet. Briefly, the cells were fixed for 20 min at room temperature with 2.5% glutaraldehyde and then stained with crystal violet. The stained cells were solubilized with acetic acid and read at 595 nm in a Microplate Reader (model 3550, Bio-Rad). A calibration curve was set up with a known number of cells. Proportionality between absorbance and cell count exists up to 8 × 10$^4$ cells.

**Migration assay.** For wound healing assay, 2.5 × 10$^5$ cells were grown in 24-well Costar plates, allowed to reach confluence, and further incubated in medium containing 0.2% FCS for 18 h. The monolayers were then wounded with a plastic pipette as described previously (3). After wounding, cells were washed with PBS and incubated for 24 h in medium containing 0.2% FCS, with or without HGF or Mab DO-24 at different concentrations, fixed with 11% glutaraldehyde, and stained with 0.1% crystal violet in 20% methanol, as previously described (3). Images of cell samples were taken with a digital camera.

Migration was quantified by calculating the area of wound at time points $t_0$ (time of wound) and $t_2$ (after 24 h). Normalization was obtained by the formula $\frac{\text{area}(t_0)}{\text{area}(t_2)}$. At least triplicates were analyzed when quantification was performed. Data are reported as means ± SD. Couples of conditions were compared with Student’s t-test.

For migration on gelatin, cells (3 × 10$^5$/50 μl) were seeded in the upper chamber of a modified Boyden chamber (Neuroprobe). The undersurface of a PVDF filter (8-μm pores, Nucleopore) was coated with 0.1% gelatin. The lower chamber was filled with medium-0.1% FCS with or without HGF, Mab DO-24, or 10% FCS, as control, and incubated for 24 h. Filters were then removed and, after cells on the upper surface were washed away, were stained with Diff-Quik (Baxter Diagnostic). Cells migrated on the lower surface of the filter were counted in a double-blind manner at the inverted microscope with a high-power oil-immersion objective (Zeiss). The experiment was performed three times with six replicates. Data are reported as means ± SD. Couples of conditions were compared with Student’s t-test. The level of significance was $P < 0.005$.

**Evaluation of biological activity of HGF- and DO-24 Mab-containing solution.** In preliminary experiments the scatter activity of solutions containing known concentrations of HGF and Mab DO-24 was titrated in serial experiments on MDCK reference cells after overnight incubation (45). One unit of scatter activity was defined as the highest dilution (the lowest concentration) of HGF or Mab per milliliter that clearly dissociated >70% of small colonies of MDCK cells. To evaluate the stability, HGF and DO-24 Mab were incubated on monolayers of cells expressing (H9c2, MDCK) or not (MDCK cells). To evaluate the stability, HGF and DO-24 Mab were titrated in serial dilution experiments on MDCK reference cells after overnight incubation (45). One unit of scatter activity was defined as the highest dilution (the lowest concentration) of HGF or Mab per milliliter that clearly dissociated >70% of small colonies of MDCK cells. To evaluate the stability, HGF and DO-24 Mab were incubated on monolayers of cells expressing (H9c2, MDCK) or not (MDCK cells).

Statistics. All experiments were performed in triplicate, except where otherwise stated, and reproduced at least three times. Data are given as means ± SD. Couples of conditions were compared with Student’s t-test. The level of significance was $P < 0.05$ or 0.005 as stated in Figs. 2–7.

**RESULTS**

Agonist anti-Met/HEGF-R monoclonal antibody DO-24 activates Met/HEGF-R and its downstream pathways in rat and mouse cardiomyocytes. It was shown previously that the Mab DO-24 reacts with the Met/HEGF-R expressed by mouse bone marrow-derived mesenchymal stem cells (16) and is able to trigger the various biological effects elicited by the natural
ligand HGF on human and canine cells (3, 45). In all the experiments reported here rat H9c2 cardiomyoblasts or mouse HL-5 cardiomyocytes were used. These cells represent good models of cardiomyoblasts to work with in vitro, and, on the other hand, it cannot be excluded that procedures to isolate primary cardiomyoblasts—ideally the best cell type for these studies—represent a stress for the cells and thus could activate various cellular responses, possibly increasing the expression of Met/HGF-R, as reported in the case of induced infarction in vivo (36). We thus checked whether MAb DO-24 could behave as an agonist and stimulate Met/HGF-R phosphorylation when incubated with quiescent H9c2 cardiomyoblasts or HL-5 cardiomyocytes for 12 min. HGF was used as control. Both ligands induced Met/HGF-R phosphorylation, as shown in Western blots performed with anti-phosphotyrosine MAb on immunoprecipitates prepared from cell extracts with anti-Met/HGF-R antibodies (Fig. 1A). The same band was recognized also by a commercial anti-Met/HGF-R antibody. Also, the main transducers Erk1/2 and Akt were found activated upon cell stimulation by DO-24 or HGF (Fig. 1B). To strengthen the idea that these responses were specifically dependent on Met/HGF-R activation by MAb DO-24, H9c2 cells were transduced with a lentiviral vector carrying the M3 interfering RNA (shRNA) specific for Met/HGF-R sequence previously reported to downregulate Met/HGF-R expression and HGF-dependent effects in other cell types (42). Indeed, in these cells Akt and MAPK Erk1/2 phosphorylation were severely impaired (Fig. 1C). In control cells transduced with a lentiviral vector carrying GFP no such inhibition was observed.

From these data we can conclude that MAb DO-24 reacts with the Met/HGF-R expressed by the two rodent cardiomyoblast cell lines and is able to specifically activate both Met/HGF-R and two of the main signaling pathways. Moreover, these biochemical responses elicited by DO-24 are specifically silenced by an interfering shRNA specific for the Met/HGF-R.

**Agonist anti-Met/HGF-R monoclonal antibody DO-24 protects against intrinsic apoptotic pathway.** Oxidative stress was performed in H9c2 cells with the protocol of Han and colleagues (19) with slight modifications. This protocol was chosen since it better mirrors the frequently encountered situation of acute myocardial ischemia-reperfusion of a transient exposure to injuring agent/situation. Preliminary experiments were performed to determine H2O2 concentrations required to induce apoptosis. Cells were exposed to H2O2 for a short-term treatment (15 min) and then incubated for a further 24 h in complete medium. Thereafter, apoptosis and necrosis were analyzed in flow cytometry by labeling with fluorescein-conjugated annexin V and PI, respectively. As shown in Fig. 2A, left, the percentage of apoptotic cells increased along with the concentration of H2O2, while necrosis was significantly enhanced only when concentrations of H2O2 as high as 400 μM were used. Changes in cell morphology were observed after the treatment. When 200 μM H2O2 was used, cell shrinkage, membrane blebbings, and some apoptotic bodies, typical markers of apoptosis, were observed. At 400 μM H2O2, a marked increase in necrotic figures and, moreover, disruption of cell integrity were observed, thus confirming progression toward cell death (Fig. 2A, right). Parallel experiments performed on HL-5 cells gave similar results (data not shown). A dose-response curve of the activity of the two Met/HGF-R agonists on H9c2 cells was done. Maximal antiapoptotic activity of DO-24 MAb was at 20 nM, while maximal antiapoptotic activity of HGF was at 0.5 nM (Fig. 2B).

On the basis of these results, 200 μM H2O2 was used to induce apoptosis in both cell lines in the following experiments, in which 20 nM DO-24 MAb or 0.5 nM HGF was added to cell cultures after induction of the transient oxidative stress. Both Met/HGF-R ligands effectively prevented cell death. In particular, DO-24 MAb and HGF decreased apoptosis by 36.5% and 33.5% in HL-5 cells and 44.2% and 35% in H9c2 cells, respectively, compared with the controls (Fig. 2C, left). A MAb directed against an unrelated molecule (BD-31; Ref. 47) was not able to protect cells from H2O2-induced apoptosis. Higher MAb concentrations did not increase the level of protection from apoptosis. Moreover, flow cytometric analysis performed on H9c2 cells indicated that 10.2±0.3 on July 7, 2017 http://ajpheart.physiology.org/ Downloaded from
Apoptosis proceeds through sequential steps, and bax up-regulation and/or translocation to mitochondria is one of the early markers of this phenomenon (11). bax belongs to the bcl-2 protein family, and when in excess over the antiapoptotic bcl-2 proteins, it translocates from the cytosol to the mitochondrial membrane, where it promotes the formation of pores. As a consequence the intrinsic apoptotic pathway is activated, with the release of proapoptotic factors and activation of the final effector caspases (11). The localization of bax in cells induced to apoptosis by H2O2 treatment was analyzed by immunofluorescence after 3 h. Whereas untreated cells displayed a slight cytosolic bax labeling, many of the treated cells displayed a stronger and punctuate bax labeling pattern, which colocalized with mitochondria, visualized by labeling with MitoTracker (Fig. 3A). Incubation with MAb DO-24 or HGF significantly reverted this effect (Fig. 3A). Finally, caspase-9 activation was measured 6 h after H2O2 withdrawal. H2O2 significantly increased the active form of this enzyme, while MAb DO-24 and HGF reverted this effect (Fig. 3B). Altogether, these data indicate that MAb DO-24, as well as HGF, protected H9c2 cells from H2O2-induced apoptosis by inhibiting the intrinsic apoptotic pathway, interfering with the mitochondrial translocation of bax and caspase-9 activation.

Agonist anti-Met/HGF-R antibody DO-24 protects against apoptosis induced by oxidative stress through activation of ras/Erk1/2 and PI3-kinase/Akt pathways. Erk1/2 and Akt kinases are multifunctional signal transducers for cell proliferation and survival in response to growth factor stimulation (58, 63). The possibility that these transducers could be involved in the antiapoptotic effect induced by MAb DO-24 was thus investigated. DNA fragmentation, as detected in a TUNEL assay (TUNEL-DAPI costaining) was chosen as the marker of
apoptosis for cells exposed to short-term \( \text{H}_2\text{O}_2 \) treatment and incubated for further 6 h after \( \text{H}_2\text{O}_2 \) withdrawal. The treatment with \( \text{H}_2\text{O}_2 \) induced DNA fragmentation, and incubation with the DO-24 MAb or HGF significantly reverted this effect (Fig. 4A). Preincubation with WM or PD-98059 significantly interfered with the protective effect of HGF-R ligands, decreasing it to about half. These data indicate that the protective effects of the MAb depend on the activation of MAPK Erk1/2 and Akt.

Agonist anti-Met/HGF-R monoclonal antibody DO-24 does not induce proliferation in H9c2 cells. HGF-R mediates different biological responses, which may differ in a cell type- and agonist-dependent fashion (3, 10, 45). For this reason, the agonist MAb was also tested for its ability to induce mitogenic and motogenic responses on H9c2 cells. MAb DO-24 did not induce a proliferative response, in accordance with the ineffectiveness of the natural ligand HGF in this assay (Fig. 5). Cells were induced to proliferate in control experiments, in which 10% FCS was added.

Fig. 4. Agonist anti-Met/HGF-R MAb DO-24 protects against apoptosis in a ras/Erk1/2 and phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway-dependent way [terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay]. A: H9c2 cells underwent transient oxidative stress and further incubation in complete medium in the presence of 20 nM MAb DO-24 or 0.5 nM HGF. After 3 h cells were stained red with MitoTracker to localize mitochondria (left) and after paraformaldehyde fixation and permeabilization were stained green with rabbit anti-bax antibodies followed by goat FITC-labeled anti-rabbit Ig antibodies (center) Micrographs were merged (right). On \( \text{H}_2\text{O}_2 \) treatment bax is translocated to mitochondria, but DO-24 or HGF interferes with this translocation. Images are representative of cell population appearance in 3 independent experiments. B: caspase activation was measured 6 h after \( \text{H}_2\text{O}_2 \) withdrawal in a fluorometric assay. Treatments with MAb DO-24 and HGF reverted the caspase activation induced by oxidative stress. All these experiments were repeated at least 3 times in triplicate. Couples of conditions were compared with Student’s \( t \)-test (* \( P < 0.05 \)).
Agonist anti-Met/HGF-R monoclonal antibody DO-24 activates motility in H9c2 cells. The motogenic activity of MAB DO-24 was tested on H9c2 cardiomyoblasts in a wound healing assay performed on confluent cell monolayers that, after induction of the wound, were incubated with HGF or MAB DO-24 for 24 h. Both stimuli induced wound repair (Fig. 6A). A dose-response curve experiment was also performed, which showed that 10 nM DO-24 and 0.25 nM HGF were the best concentrations to induce such a biological response (Fig. 6B). Cell migration was also assessed in a Boyden chamber test, with similar results (data not shown). This kind of assay was also used to test the effect of silencing Met/HGF-R on biological responses. Indeed, motility of H9c2 cells transduced with a Met/HGF-R-specific shRNA in response to DO-24 MAb stimulation was completely abrogated, while H9c2 cells transduced with GFP displayed a normal motogenic response to DO-24 MAb (Fig. 6C). Both cell lines migrated in response to 10% FCS. This experiment shows that this biological response evoked by the DO-24 MAb on H9c2 cardiomyoblasts is Met/HGF-R specific.

In vitro stability of DO-24 MAb and HGF. A major limit to the use of HGF in in vitro and in vivo experiments is its instability once it is processed to its heterodimeric biologically active form by proteolytic cleavage (40). We thus compared the biological activity of HGF and MAB DO-24 maintained in tissue culture on cell monolayers for different periods of time up to 5 days. The biological activity was evaluated as scatter activity, i.e., dissociation of epithelial colonies, on MDCK monolayers.
cells, the standard cell line used for this kind of assay (45, 61). After quantification of scatter activity of HGF and DO-24, tissue culture media containing 10 scatter units/ml, corresponding to 1.2 nM HGF or 5 nM DO-24, respectively, were incubated on cells expressing (MDCK and H9c2) or not expressing (NIH-3T3) the Met/HGF-R and then taken at different times and tested for their scatter activity. Scatter activity was detected in samples containing HGF only until day 1, but not on days 3 and 5, while scatter activity was present at the same initial levels in samples of DO-24 until day 5, the last day on which supernatants were collected, when cells in monolayers were still alive (Fig. 7). No differences were observed between supernatants taken from cells expressing or not expressing the Met/HGF-R. The different cell lines on which the DO-24 MAb or HGF were incubated did not release any scatter activity (control in Fig. 7A). These data are in line with what we know from our experience: we can keep the MAb for 6 mo at least at 4°C and a 0.5–1 mg/ml concentration with no loss of activity, while we must use HGF stored at 4°C within 1 wk. Also, the in vivo data reported by Ohashi et al. (40) are in line with what we have observed in vitro.

**DISCUSSION**

The present investigation demonstrates that a MAb (DO-24) directed against the ectodomain of the Met/HGF-R can protect two different rodent cardiomyoblast cell lines from hydrogen peroxide-induced apoptosis. This antibody operates by activating the Met/HGF-R and interfering with the intrinsic apoptotic pathway, in a MAPK Erk1/2- and Akt-dependent manner. Furthermore, in these cells, the antibody recapitulates the activities of the natural ligand HGF, since it also promotes cell migration, but not proliferation, mimicking in all aspects the natural ligand. The effects of MAb DO-24 are specifically mediated by the Met/HGF-R, since transduction with a lentiviral vector of a Met/HGF-R-specific shRNA abrogates the activation of the signaling pathways as well as the motogenic response. The MAb is significantly more stable than HGF.

Apoptosis is a major cause of cardiomyocyte loss in both ischemic and nonischemic heart diseases. Indeed, it plays an important role in infarction, especially in the reperfusion phase that follows the ischemic event (13, 25, 35). Apoptosis also concurs with pathogenic mechanisms in inherited cardiomyopathies, myocarditis, transplant rejection, and heart failure (11, 18). Apoptosis can be elicited by various stimuli (11), and the factors counteracting it are a field of active investigation (21, 24, 36). HGF is one of the factors shown to display antiapoptotic and cardioprotective activity (36, 59). The HGF/Met/HGF-R axis is normally silent in the adult heart, but it is resumed when the organ undergoes injury, and this is considered an adaptive response to preserve or maintain homeostasis in vivo and has been reported also for other organs such as liver, kidney, lung, skin, and intestine and peripheral and central nervous systems (see Refs. 5, 7, 33, 35, 36). In the case of cardiac diseases HGF displays hypertrophic and antiapoptotic activities on cardiomyocytes, besides being an antibacterial and proangiogenic factor, as shown in animal models of experimentally induced ischemic cardiomyopathy (36, 54) or of hereditary cardiomyopathy (14, 37). One limit to the in vitro and in vivo use of HGF is its short half-life, which in vivo is measurable in minutes (40).

Agonist MAbs directed against cell surface receptors can be considered efficient substitutes for the natural ligand and can even be superior to it in some aspects (46, 65). In particular, we report here that, besides mimicking all the activities evoked by the natural ligand HGF, MAB DO-24 has a significantly higher stability compared with HGF. The antibody displayed antiapoptotic activity on cells transiently exposed to oxidative stress by hydrogen peroxide and doxorubicin (not shown). The MAb was able to interfere with the intrinsic apoptotic pathway, as assessed in the different assays performed to follow some of its more crucial events, i.e., bax translocation to mitochondria, caspase-9 activation, DNA fragmentation, and acquisition of annexin V positivity. DO-24 also mimicked the natural ligand biochemically, since it stimulated the phosphorylation and activation of the Met/HGF-R as well as of MAPK Erk1/2 and Akt. The antiapoptotic effect was dependent on the activation of these two pathways, since their pharmacological inhibition abolished the protective effect observed. Both pathways have been shown to be involved in the antiapoptotic signaling by HGF in cardiomyoblasts (26, 36, 59) as well as in other cell types (58, 63). Notably, in cardiomyoblasts these same pathways are activated in response to treatment with other factors...
displaying antiapoptotic activity (4, 17, 21, 24, 29, 66). It is noteworthy that the PI3-kinase/Akt pathway has recently been reported to exert its antiapoptotic activity by phosphorylating bax, which upon this modification is inhibited in its mitochondrial translocation (28, 31, 50).

In addition, DO-24 elicited a motogenic response in the cardiomyoblasts. It has already been reported that HGF promotes in vitro motility of bone marrow, umbilical cord blood stem cells (16, 38, 53), and cardiac progenitor cells (30) as well as recruitment of bone marrow-derived progenitor cells to the injured liver (27) and heart (64). On the other hand, neither HGF nor the MAb induced a proliferative response in H9c2 cells. A priori, among the many potentially possible responses, the biological responses elicited by HGF in peculiar cell types remain unpredictable. Human and mouse mesenchymal stem cells do not proliferate when challenged with HGF; in these cases, the ligand unexpectedly blocks proliferation (16, 38). In contrast, HGF induces the expression of cardiac-specific markers in mouse mesenchymal stem cells (16). Among the agonist activities exerted by this MAb, its previously reported in vivo proangiogenic effect is noteworthy (45). Indeed, the HGF cardioprotective effect is ascribed to its potent angiogenic action in vivo (1, 54) besides its direct action on cardiomyocytes. In line with the role of the HGF and its receptor in tissue oxygen homeostasis, it has been shown that hypoxia induces transcriptional activity of the HGF-R, which then amplifies HGF signaling (42). This can contribute to the resumed expression of HGF and its receptor in the heart after ischemic injury (36).

The biochemical and biological responses elicited by the MAb are specifically mediated by the Met/HGF-R. In fact, we have shown that upon inhibition of the Met/HGF-R expression obtained by interfering shRNA, activation of Akt and MAPK transduction pathways as well as cell migration induced by MAb DO-24 are impaired. The specificity of this MAb compares quite well with that observed for the natural ligand. However, in order to elicit both biochemical and biological responses, significantly higher doses of MAb are required. This may be explained by the very high affinity displayed by HGF for its receptor, which is lower than nanomolar (61), and by the fact that this antibody has been raised against the human receptor. MAb DO-24 binds to a conformational nonlinear epitope localized on the extracellular domain of the β-chain of Met/HGF-R (45), which is different from the HGF binding site. Interestingly, competition experiments have shown that another molecule, the internalin B molecule of Listeria monocytogenes, which activates both cell biological and biochemical responses, binds to the epitope identified by MAb DO-24 (52). While both HGF and internalin B require at least two separate contact sites with the receptor (4, 39) in order to promote biological effects, MAb DO-24 can activate all responses mediated by the Met/HGF-R by interacting with only a single receptor binding site. This fact can be explained by taking into account the mechanism of activation of Met/HGF-R, which, like most tyrosine kinase receptors, is activated by agonist-induced dimerization, which is easily elicited by the bivalent antibodies. Indeed only the bivalent, but not the monovalent Fab DO-24 MAb, is able to activate the receptor (45). Simple dimerization of the tyrosine kinase receptor, however, is not always enough, since in general only a few of the many antibodies binding to the receptor extracellular domains can fully activate them, suggesting that there are further requirements linked to the peculiar epitope recognized.

Relative to the natural HGF ligand, the MAb described here presents the major advantage of being significantly more stable. Indeed, when evaluated in the sensitive quantitative scatter assay, the biological activity of MAb DO-24 persisted unchanged after 5 days in tissue cultures at 37°C, while that of HGF was detectable only for 1 day and disappeared after 3 days. Moreover, HGF is a dimeric molecule, secreted as a monomeric precursor, which must undergo proteolytic cleavage to acquire biological activity (20). This complicates the process of its pharmacological preparation as a recombinant protein, and it makes the success of a gene therapy dependent on the activity of endogenous proteases. Once activated, as already discussed, the molecule is highly unstable (40). Besides binding to the high-affinity receptor on the cell surface, HGF binds with lower affinity to heparan sulfate of the extracellular matrix, which can cause its entrapment at the site of injection. By contrast, MAb are quite stable molecules, which can be easily produced in their active form in huge amounts and easily purified. Moreover, they bind to unique epitopes, which in the case of the DO-24 MAb differ from the HGF binding site and thus also from components of the extracellular matrix (45). Finally, conventional MAbs can be humanized, allowing therapeutic applications.

ACKNOWLEDGMENTS

We thank Gianluca Baldanzi for excellent discussions. We thank W. C. Claycomb for HL-5 cells, Riccardo Taulli for the shRNA-GFP-M3-containing plasmid, and Sara Sanpietro for helping in the chemotactic assay.

GRANTS

This work was supported by Universitá del Piemonte Orientale “A. Avogadro” Novara, Ricerca Finalizzata by Regione Piemonte, and Fondazione Cariplo. D. Locarno was supported by a PhD fellowship from Diasorin S.p.A.

DISCLOSURES

The authors have no financial conflicts of interest. S. Pietronave and M. Prat have a patent pending.

REFERENCES


65. Zarsky Y. Agonistic antibodies stimulate the kinase encoded by the neu protooncogene in living cells but the oncogenic mutant is constitutively active. *Proc Natl Acad Sci USA* 87: 2569–2573, 1990.