HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor

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Abstract We prepared a specific antagonist for hepatocyte growth factor (HGF) and designated it HGF/NK4. HGF/NK4 is composed of N-terminal 447 amino acids of the α-chain of HGF, thus contains the N-terminal hairpin domain and subsequent four kringle domains. HGF/NK4 competitively inhibited the specific binding of HGF to the receptor. Importantly, HGF/NK4 neither stimulated DNA synthesis of primary cultured rat hepatocytes (mitogenesis) nor induced cell scattering (motogenesis) and branching tubulogenesis (morphogenesis) of MDCK renal epithelial cells, however, HGF/NK4 almost completely inhibited the mitogenic, motogenic, and morphogenic activities of HGF. HGF/NK4 also suppressed tyrosine phosphorylation of the c-Met/HGF receptor induced by HGF. Apparently this is the first documentation of a specific antagonist which abrogates the mitogenic, motogenic, and morphogenic activities of HGF.

Key words: Hepatocyte growth factor; HGF-antagonist; HGF/NK4

1. Introduction

Hepatocyte growth factor (HGF) was originally isolated and cloned as a potent mitogen for mature hepatocytes [1–6], but subsequent studies revealed that HGF is a pleiotrophic factor which predominantly targets a wide variety of epithelial and endothelial cells [7,8]. Several lines of studies revealed multipotent features of HGF. Scatter factor was found to be the same molecule as HGF [9], and HGF stimulates the motility of various epithelial cells. HGF was also found to be a fibroblast-derived epithelial morphogen which induces branching tubular morphogenesis [10]. HGF is expressed in mesenchymal tissues, while c-Met/HGF receptor is expressed in epithelial tissues during development of various organs [11]. Together with its mitogenic, motogenic (enhancement of cell motility), and morphogenic activities, HGF has been considered to be a mediator in epithelial-mesenchymal interactions, which supports organogenesis of various organs (reviewed in [7,8]). In neoplastic tissues, HGF functions in tumor invasion as a mediator in tumor-stromal interactions [12,13] and HGF is likely to be one factor which stimulates neovascularization in tumors [14,15]. Particular physiological functions of HGF have been extensively studied as an organotrophic factor for regeneration of various organs [7,8,16,17].

HGF is biosynthesized as a single-chain precursor of 728 amino acid residues and is proteolytically processed to a heterodimer molecule composed of a 69 kD α-chain and a 34 kD β-chain [5]. The α-chain contains four kringle domains and the β-chain the serine protease-like domain [5]. Studies on structure-function relationships indicated that the N-terminal hairpin loop, the first and second kringle domains of HGF are essential for high-affinity binding to the c-Met/HGF receptor and thus essential for eliciting HGF activity [18–22]. Indeed, HGF/NK2, a variant form of HGF composed of the N-terminal hairpin domain and the first and second kringle domains is produced by alternative splicing as a naturally occurring variant [23,24]. Interestingly, HGF/NK2 can bind the c-Met/HGF receptor and exert motogenic activity, but it has no mitogenic activity, thus a small variant antagonizes the mitogenic activity of HGF. On the other hand, the β-chain of HGF alone fails to bind to the receptor, however the β-chain seems essential for the mitogenic activity of HGF [18,21].

We have now prepared an antagonistic molecule for HGF which we termed 'HGF/NK4'. Notably, this HGF-antagonist, HGF/NK4 antagonizes the mitogenic, motogenic, and morphogenic activities of HGF. We describe herein the first report on preparation of this HGF-antagonist.

2. Materials and methods

2.1. Materials

Human recombinant HGF was purified from the conditioned medium of CHO cells transfected with human HGF cDNA [5,25]. The purity of HGF exceeds 98%, as determined by SDS-PAGE and protein staining. Anti-phosphotyrosine monoclonal antibody (PY-20) was obtained from Chemicon International, Inc. (Temecula, CA) and anti-c-Met polyclonal antibody (C-12) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Pyroglutamate aminopeptidase was obtained from TAKARA Co. Ltd. (Otsu, Japan). Recombinant HGF/NK2 was purified from the conditioned medium of COS-7 cells transfected with cDNA corresponding to the sequence of human HGF/NK2, as described elsewhere [22,23].

2.2. Preparation of HGF/NK4 and N-terminal amino acid sequencing

HGF was digested with pancreatic elastase (Sigma, St. Louis, MO) in 0.2 M Tris/HCl buffer pH 8.8 for 20 min at 37°C with an enzyme/substrate ratio 1/100. The digested material was applied onto μBondapack C4 reverse-phase HPLC column (Nihon Waters Ltd., Tokyo) and eluted with a gradient of acetonitrile containing 0.05% trifluoroacetic acid. Eluted fractions were evaporated by vacuum centrifugation and dissolved in 0.1 M phosphate buffer (pH 7.3) containing 0.05% CHAPS (Sigma) and 1 M NaCl. The N-terminal amino acid sequence of the purified digested polypeptides was determined using an automated protein sequencer model 492 (Applied Biosystem Inc., Foster City, CA).

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HGF, hepatocyte growth factor; HPLC, high performance liquid chromatography

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2.3. HGF receptor assay

Hepatic plasma membranes were prepared from adult rat livers and HGF and HGF/NK4 were respectively radioiodinated by the chloramine-T method, as described elsewhere [26]. The specific activities of ^{125}I-HGF and ^{125}I-HGF/NK4 were 30–186 μCi/μg protein. The binding assay was carried out by incubating ^{125}I-HGF and ^{125}I-HGF/NK4 with plasma membranes at 12°C for 1 h, as described elsewhere [26]. The standard assay mixture contained various concentrations of ^{125}I-HGF and ^{125}I-HGF/NK4, with or without 100 times excess molar ratio of unlabeled HGF or HGF/NK4 and 50 μg of plasma membrane. All binding experiments were performed in quadruplicate. The competitive binding assay was carried out by adding 60 pM ^{125}I-HGF and various concentrations of cold HGF or HGF/NK4, simultaneously, to rat plasma membranes.

2.4. Cell culture and assay for mitogenic activity in hepatocytes

MDCK clone 3B cells (a kind gift from Dr. R. Montesano) were cultured in DMEM containing 10% FCS. For cell scattering assay, MDCK cells were at a density of 1×10^4 cells/ml with or without HGF and/or HGF/NK4, and the culture was run for 20 h. For three-dimensional culture in collagen gels, MDCK cells were suspended in ice-cold 0.2% collagen solution at a density of 10^4 cells/ml. After the collagen solution gelled, culture medium containing HGF and/or HGF/NK4 was added and cells were cultured for 6 h. Mitogenic activity of HGF, HGF/NK4 and HGF/NK2 was determined by measuring DNA synthesis of adult rat hepatocytes in primary culture, as described elsewhere [5,18].

2.5. Detection of receptor tyrosine autophosphorylation

Subconfluent A549 cells were cultured in serum-free DMEM supplemented with 0.2% bovine serum albumin for 20 h. Serum-starved cells were treated with HGF and/or HGF/NK4, and washed with PBS containing 1 mM Na_3VO_4. Cells were frozen on dry-ice and scraped off in lysis buffer composed of 20 mM Tris/HCl (pH 7.4), 10 mM EDTA, 150 mM NaCl, 2 mM Na_3VO_4, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100 and extracted for 2 h at 4°C. Tyrosine phosphorylation was detected following immunoprecipitation of c-Met/HGF receptor and subsequent Western blotting, using an ECL enhanced chemiluminescence method (Amersham, Little Chalfont, UK) as described elsewhere [18–20].

3. Results

3.1. Preparation and structural analysis of HGF/NK4

To generate an antagonistic molecule for HGF, highly purified recombinant HGF was digested with elastase and the digested material was subjected to reverse-phase HPLC. The elution profile showed that the digested material separated to three distinct peaks (Fig. 1A). SDS-PAGE and the following protein staining indicated that a fragment of the first peak has a M_r of 50 kD, under non-reducing conditions and 67 kD under reducing conditions (Fig. 1B, HGF/NK4). Subsequent analysis revealed that this fragment, designated as HGF/NK4 is an antagonistic molecule for HGF (see below). On the other hand, the second peak corresponded to an undigested heterodimeric HGF composed of 69 kD of α-chain and 34/32 kD β-chain, in which the two distinct bands of β-chain were attributed to differences in glycosylation. When the third peak was subjected to SDS-PAGE, it showed 33/31 kD under non-reducing conditions, and 34/32 kD under reducing conditions (data not shown). Therefore, SDS-PAGE analysis suggested that HGF was digested mainly into two fragments: the one (HGF/NK4) is slightly smaller than the α-chain but has most parts of the α-chain. The other fragment may be composed of the entire β-chain and C-terminal small peptide of the α-chain, in which these are linked by a disulfide bridge, because the cysteine residue involved in the disulfide bridge to the β-chain is 487Cys.

To determine the N-terminal amino acid sequence of HGF/NK4, the purified material was subjected to N-terminal amino acid sequence analysis, but this approach was unsuccessful due to modification of the N-terminal amino acid. Since the N-terminal amino acid of the HGF α-chain is pyroglutamate [27], HGF/NK4 was treated with pyroglutamate aminopeptidase and then subjected to N-terminal amino acid sequencing. The N-terminal amino acid sequence probed to be RKRRNTIHEF which completely coincides with the 2nd to 11th N-terminal amino acids of the α-chain. Therefore, the N-terminal amino acid of HGF/NK4 is glutamine modified to pyroglutamate. Thus, the N-terminal structure of HGF/NK4 is the same as undigested HGF.

Next to determine the C-terminal end of HGF/NK4, we analyzed N-terminal amino acid sequences of the remaining fragment, assumed to be composed of the β-chain and the C-terminal small peptide of the α-chain. In this case, we considered that the N-terminal amino acid analysis would produce mixed sequences: one derived from the C-terminal small peptide of the α-chain, and the other from the β-chain. As expected, N-terminal amino acid analysis of the other fragment revealed paired amino acid residues: V/N, V/L, N/D, G/H, I/P, P/V, T/I, R/S, T/-, N/A. The N-terminal amino acid se-

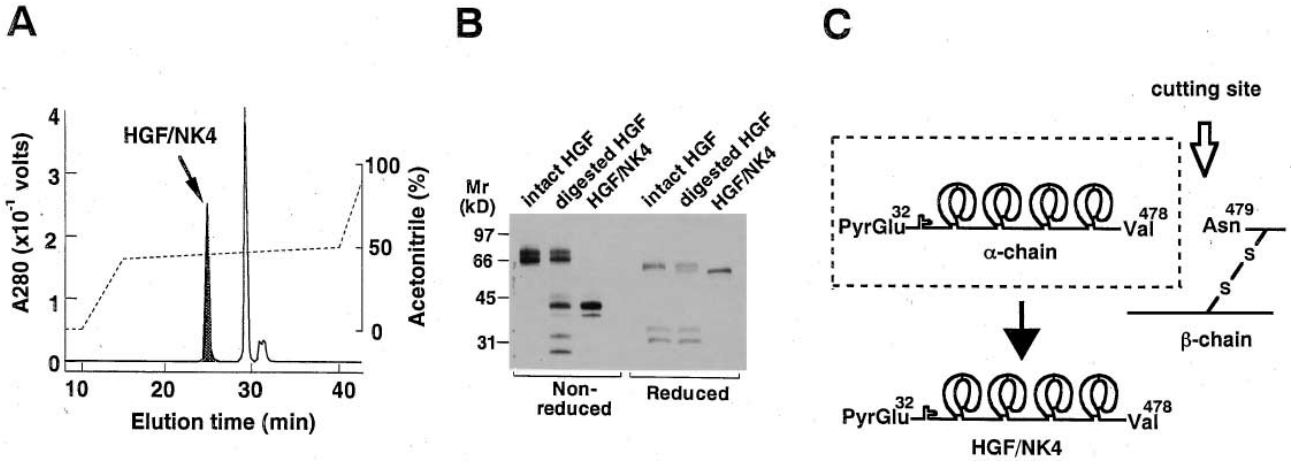

Fig. 1. Elution profile of HGF digested with elastase in C4 reverse-phase HPLC (A), analysis of polypeptide composition under SDS-PAGE (B), and schematic structure of HGF/NK4 as determined by amino acid sequence analysis (C). Three distinct peaks separated by reverse-phase HPLC were respectively subjected to SDS-PAGE under non-reducing or reducing conditions. Proteins were visualized by silver staining.

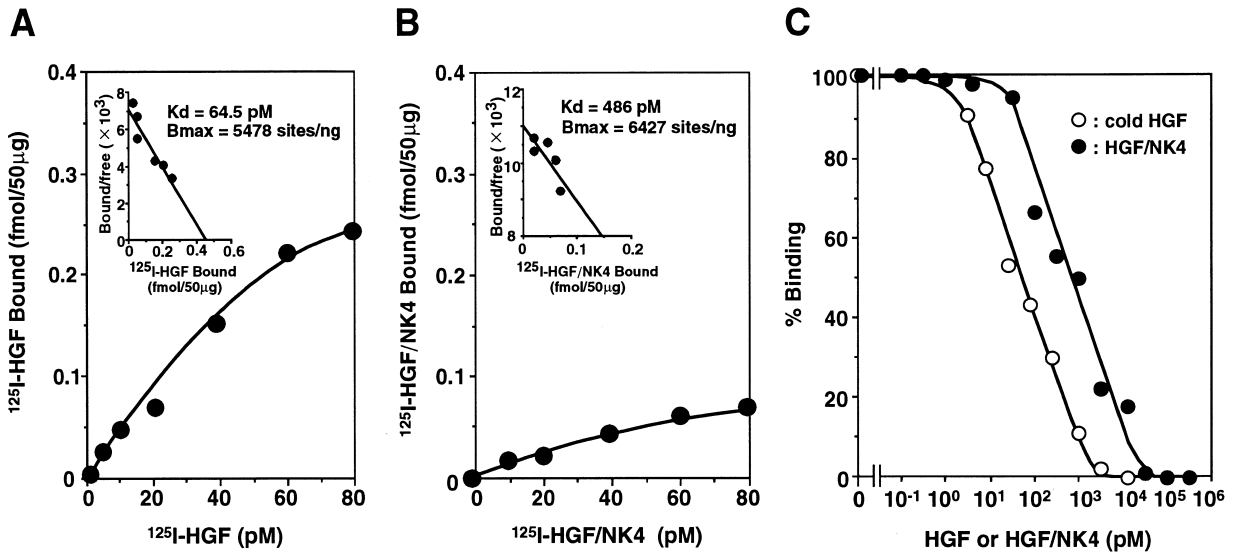

Fig. 2. Concentration-dependent binding of radiolabeled HGF and HGF/NK4 to plasma membranes from rat liver (A, B), and replacement curve of ^{125}I-HGF binding to rat liver plasma membranes by unlabeled HGF or HGF/NK4 (C). Inset graphs in A and B show Scatchard plots of each binding.

quence of the β-chain is VVNGIPTRTN, therefore, the remainder should be NLDHPVIS-A. This sequence corresponds to 479Asn-488Ala. These results indicated that the C-terminal end of HGF/NK4 is 478Val and that the remaining is composed of the C-terminal 16 amino acids of the α- and β-chains which are linked by a disulfide bridge between 487Cys and 604Cys (Fig. 1C). Thus, HGF/NK4 is composed of a hairpin domain and K1–K4 domains (Fig. 1C).

3.2. Receptor analysis

To determine if HGF/NK4 can bind to the cell surface receptor, concentration-dependent binding of radiolabeled HGF and HGF/NK4 was analyzed, using plasma membranes prepared from rat liver (Fig. 2A, B). Scatchard analysis of concentration-dependent binding of ^{125}I-HGF up to 80 pM resulted in a rectilinear plot (inset of Fig. 2A). The K_d value and the number of HGF receptors were calculated to be 64.5 pM and 5478 sites/ng, respectively. Likewise, concentration-dependent binding of HGF/NK4 and its Scatchard plot indicated that the K_d value and the number of HGF/NK4 receptors are 486 pM and 6427 sites/ng, respectively (Fig. 2B).

To determine if HGF/NK4 has the same binding site as HGF, competitive binding analysis was carried out using ^{125}I-HGF (Fig. 2C). Liver plasma membranes were incubated in the presence of ^{125}I-HGF alone, or ^{125}I-HGF plus various concentrations of cold HGF or HGF/NK4. Addition of cold HGF competitively inhibited the specific binding of ^{125}I-HGF to the plasma membrane, and 50% inhibition by cold HGF was seen with 60 pM HGF: the dose approximately equimolar to that of ^{125}I-HGF. Likewise, addition of cold HGF/NK4

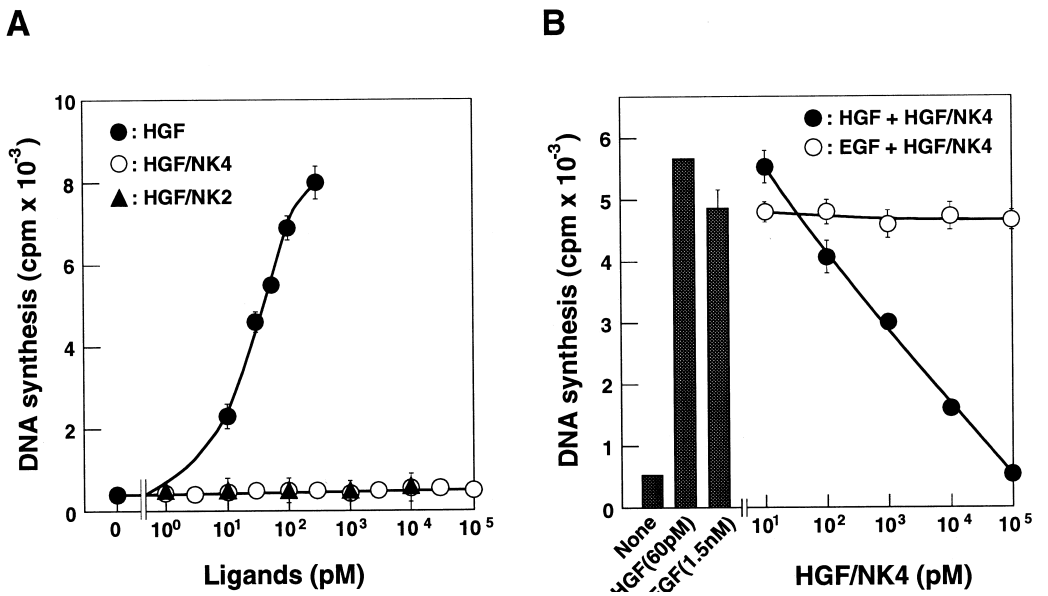

Fig. 3. Antagonistic effect of HGF/NK4 on mitogenic activity of HGF. Mitogenic activity was determined by measuring DNA synthesis of rat hepatocytes in primary culture. A: DNA synthesis of hepatocytes in the presence of HGF, HGF/NK4 or HGF/NK2. B: Effects of HGF/NK4 on DNA synthesis of hepatocytes in the presence of 60 pM HGF or 1.5 nM EGF.

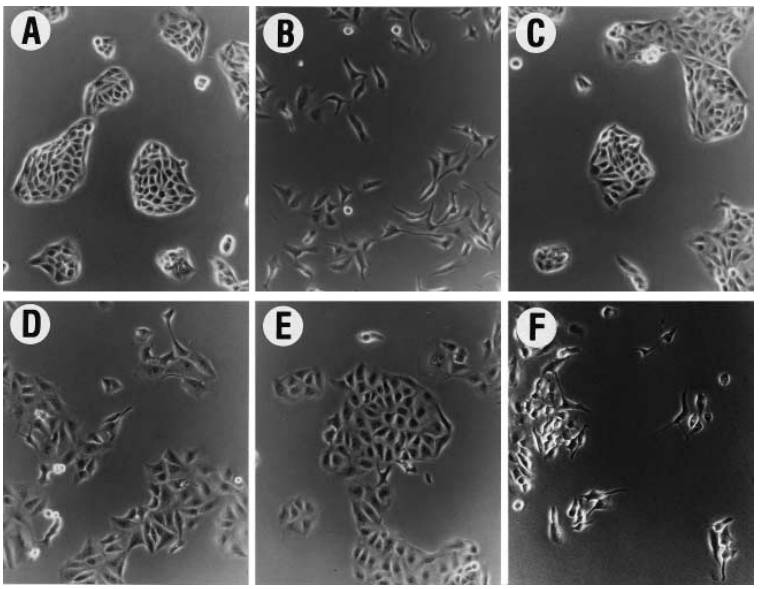

Fig. 4. Antagonistic effect of HGF/NK4 on the motogenic activity of HGF. Motogenic activity was measured by evaluating cell scattering in MDCK cells in monolayer culture. MDCK cells were cultured for 20 h in the absence (A) or presence of 22 pM HGF (B), 220 nM HGF/NK4 (C), 22 pM HGF plus 22 and 220 nM HGF/NK4 (D and E, respectively), or 220 pM HGF/NK2 (F).

also inhibited the binding of ^{125}I-HGF. Inhibitory effects were seen from 60 pM HGF/NK4 and HGF/NK4 almost completely inhibited the ^{125}I-HGF binding at 60 nM; a 1000-fold higher concentration than that of ^{125}I-HGF. The inhibition by 50% was seen with 600 pM HGF/NK4 and the concentration was 10-fold higher than that of HGF. Taken together, HGF/NK4 seems to competitively bind to c-Met/HGF receptor with 8–10-fold lower affinity than that of native HGF.

3.3. Antagonizing activities of HGF/NK4

To address whether HGF/NK4 would abrogate biological activities of HGF, we first examined the mitogenic activity of HGF/NK4, using rat hepatocytes in primary culture (Fig. 3). HGF dose-dependently stimulated DNA synthesis of hepatocytes (Fig. 3A), whereas HGF/NK4 as high as 100 nM had no stimulatory effect (Fig. 3A). Since HGF/NK2 is a competitive inhibitor for binding of HGF to the receptor, we also examined whether HGF/NK2 has mitogenic and morphogenic activities. HGF/NK2 as high as 10 nM had no stimulatory effect on DNA synthesis (Fig. 3A). Importantly, when HGF/NK4 was simultaneously added to cultures in the presence of HGF, HGF/NK4 dose-dependently inhibited the DNA synthesis stimulated by HGF, and almost completely inhibited at 60 nM, a 1000-fold higher concentration of HGF (Fig. 3B). However, the inhibitory effect of HGF/NK4 was not seen when DNA synthesis was stimulated with epidermal growth factor (EGF) (Fig. 3B). Thus, HGF/NK4 specifically abrogates the mitogenic activity of HGF.

We next examined the biological activity of HGF/NK4,

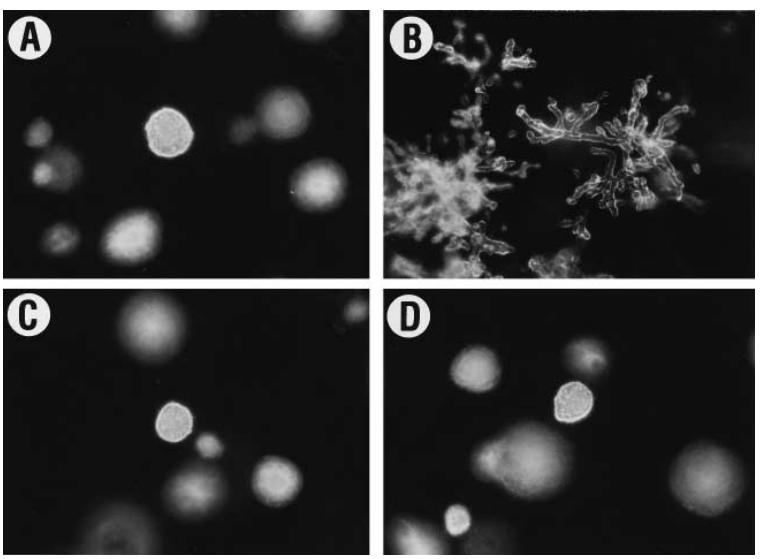

Fig. 5. Antagonistic effect of HGF/NK4 on morphogenic activity of HGF. Morphogenic activity was analyzed by evaluating the formation of branching tubulogenesis in MDCK cells grown in a collagen gel matrix in the absence (A) or presence of 55 pM HGF (B), 55 nM HGF/NK4 (C), or 55 pM HGF plus 55 nM HGF/NK4 (D).

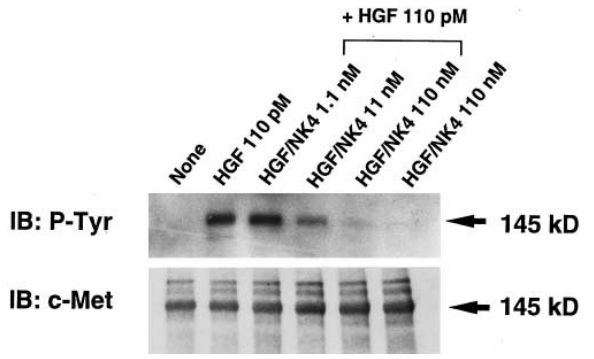

Fig. 6. Inhibitory effect of HGF/NK4 on tyrosine autophosphorylation of the c-Met/HGF receptor induced by HGF. HGF (110 pM) and/or HGF/NK4 were added to the cultures of A549 cells and cells were extracted 10 min later. The c-Met/HGF receptor was immunoprecipitated with anti-c-Met polyclonal antibody and immunoprecipitated materials were subjected to SDS-PAGE, under reducing conditions. Proteins were electroblotted onto PVDF membrane and probed with anti-c-Met polyclonal antibody or anti-phosphotyrosine monoclonal antibody. IB, immunoblotting.

using MDCK renal epithelial cells (Fig. 4). In control cultures, MDCK cells formed tight colonies (Fig. 4A), whereas the addition of 22 pM HGF enhanced the motility of MDCK cells, and the MDCK cells scattered (Fig. 4B). In contrast, HGF/NK4 did not induce any scattering of the cells, and the cell-cell contact was well maintained (Fig. 4C). Moreover, when HGF/NK4 was simultaneously added in the presence of 22 pM HGF, HGF/NK4 inhibited scattering of the cells as induced by HGF and maximal inhibition was seen with 22 nM HGF/NK4 (Fig. 4D and E). On the other hand, HGF/NK2 at 220 pM induced cell scattering (Fig. 4F), which is consistent with previous reports [18].

To determine if HGF/NK4 would antagonize the morphogenic activity of HGF, MDCK cells were grown in a collagen gel matrix in the presence of HGF and/or HGF/NK4 (Fig. 5). In control cultures without HGF, MDCK cells grew in the form of spherical cysts (Fig. 5A). As previously noted, HGF at 55 pM induced branching tubulogenesis in MDCK cells (Fig. 5B), whereas HGF/NK4 as high as 55 nM, did not induce branching tubulogenesis (Fig. 5C). HGF/NK4 completely abrogated the HGF-induced branching tubulogenesis, and in this case MDCK cells grew as spherical cysts (Fig. 5D). HGF/NK2 did not induce branching tubulogenesis (data not shown).

Since HGF has growth inhibitory activity for several types of tumor cells, we also examined whether HGF/NK4 would antagonize the tumor inhibitory activity of HGF using HepG2 human hepatoma cells. HGF inhibited proliferation of HepG2 cells, whereas it was almost completely abrogated by addition of HGF/NK4 (not shown). Thus, HGF/NK4 antagonized the mitogenic, motogenic, morphogenic, and tumor inhibitory activities of HGF.

3.4. Inhibition of the HGF-induced Met tyrosine phosphorylation by HGF/NK4

We then asked whether the antagonistic effect of HGF/NK4 on biological activities of HGF could be attributed to the inhibition of tyrosine autophosphorylation of Met induced by HGF. A549 cells were serum-starved and tyrosine phosphorylation of Met upon addition of HGF and/or HGF/NK4 was detected after immunoprecipitation and the following immunoblotting (Fig. 6). Addition of 110 pM HGF induced tyrosine autophosphorylation of Met, however, the addition of a higher concentration of HGF/NK4 (110 nM) scarcely induced tyrosine phosphorylation of Met (Fig. 6). Simultaneous addition of HGF/NK4 dose-dependently inhibited tyrosine autophosphorylation of Met in the presence of HGF and almost complete inhibition of this phosphorylation was seen with 110 nM HGF/NK4 (Fig. 6). Therefore, HGF/NK4 suppressed tyrosine phosphorylation of the c-Met/HGF receptor and this inhibitory effect is attributed to its antagonistic effect on the biological activities of HGF.

4. Discussion

Previous studies on the structure-function relationship in HGF molecule revealed that the N-terminal hairpin domain, the first kringle (K1), and the second kringle (K2) are responsible for binding to c-Met [19–22]. Small molecule consists of the N-terminal hairpin domain, K1, and K2 designated HGF/NK2 exists as a naturally biosynthesized variant [23,24], and importantly HGF/NK2 shows motogenic activity but lacks mitogenic activity [19]. HGF/NK2 capable of receptor binding is thus an antagonist for the mitogenic activity of HGF, yet retains selective agonistic activity in the induction of cell mobility. Lokker et al. subsequently demonstrated that HGF/NK1 which consists of the N-terminal hairpin domain and K1 and is smaller than HGF/NK2 which binds the c-Met/HGF receptor and exerts motogenic activity at higher concentrations [28]. Like HGF/NK2, HGF/NK1 lacks mitogenic activity, rather it antagonizes the mitogenic activity of HGF. However, an HGF-antagonist which abrogates mitogenic, motogenic, and morphogenic activities of HGF has heretofore never been identified. In the present study, we developed HGF/NK4 which consists of the N-terminal hairpin domain and four kringle domains following limited digestion of HGF. HGF/NK4 binds the c-Met/HGF receptor, but does not retain mitogenic, motogenic, and morphogenic activities. Importantly, HGF/NK4 antagonizes mitogenic, motogenic, morphogenic, and tumor inhibitory activities of HGF.

HGF/NK2 binds the c-Met/HGF receptor and retains motogenic activity. In contrast, HGF/NK4, which is larger than HGF/NK2 and consists of HGF/NK2 plus K3 and K4, also binds the c-Met/HGF receptor but does not induce cell scattering. We speculate that K3 and K4 in HGF/NK4 do not block the binding between HGF/NK2 and c-Met, but do suppress the scattering effect of HGF/NK2. On the other hand, using highly purified HGF/NK4, we obtained evidence that HGF/NK4 competitively inhibits both the binding of HGF to the receptor and HGF-induced tyrosine phosphorylation, but importantly it has no HGF-related biological activities. Our results raise a question on whether the entire α-chain of HGF might retain similar antagonistic activity for biological activities of HGF. Previous reports showed that recombinant α-chain binds to c-Met/HGF receptor and has no apparent mitogenic activity [18–21], but with regard to motogenic activity, the reports are controversial. One group suggested that the α-chain has a weak motogenic activity [19], but others found that it did not have a weak motogenic activity [18,21]. However, these previous studies were done using crude or partially purified preparation of the α-chain, and moreover, these studies neither examined multiple biological activities of the α-chain at higher concentrations nor examined antagonistic ac-

tivity of the α-chain for HGF. Whether the α-chain has a similar antagonistic activity as seen with HGF/NK4 remains to be addressed.

Recently, the relationship between tumor invasion and stromal-derived HGF have received much attention [12,13]. Most carcinoma cells (tumor cells originating from epithelial tissues and 90% of tumors are epithelial origin) express c-Met, and overexpression of c-Met is often noted in more malignant cancer cells [29–33]. HGF induces invasion of carcinoma cells in vitro [12,13,34] and functional coupling between HGF and Met enhances invasion and metastasis in certain tumor cells [35]. Moreover, HGF may also be involved in neovascularization in tumor tissues [14,15]. Based on all these observations, HGF/NK4, an antagonist of HGF may have therapeutic potential to prevent invasion and metastasis of various carcinoma cells. Ongoing studies have shown HGF/NK4 prevents tumor invasion and metastasis in vivo as well as in vitro.

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