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Inhibition of colon cancer growth and metastasis by NK4 gene repetitive delivery in mice

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Abstract

NK4, originally prepared as a competitive antagonist for hepatocyte growth factor (HGF), is a bifunctional molecule that acts as an HGF-antagonist and angiogenesis inhibitor. When the expression plasmid for NK4 gene was administered into mice by hydrodynamics-based delivery, the repetitive increase in the plasma NK4 protein level was achieved by repetitive administration of NK4 gene. Mice were subcutaneously implanted with colon cancer cells and weekly given with the NK4 plasmid. The repetitive delivery and expression of NK4 gene inhibited angiogenesis and invasiveness of colon cancer cells in subcutaneous tumor tissue and this was associated with suppression of primary tumor growth. By fifty days after tumor implantation, cancer cells naturally metastasized to the liver, whereas NK4 gene expression potently inhibited liver metastasis. Inhibition of the HGF-Met receptor pathway and tumor angiogenesis by NK4 gene expression has potential therapeutic value toward inhibition of invasion, growth, and metastasis of colon cancer.

Keywords: Met; Colon cancer; HGF; Liver metastasis; NK4; Tumor angiogenesis

Colon cancer is one of the most common cancers in the world, with a high propensity to metastasize; 30–40% of patients have metastatic disease at the initial diagnosis. The most common sites of metastasis from colon cancer are the regional lymph nodes, the liver, the lung, and the peritoneum, in which the liver being the most frequent site of metastases [1,2]. Because the majority of deaths with colon cancer are due to metastatic disease, inhibitions of growth and metastasis of colon cancer are expected to become effective treatment, leading to prolongation of the life span. Among them, anti-angiogenesis strategy has been growing and standard for treatment of patients with colon cancer [3,4]. Inhibition of tumor angiogenesis is likely to decrease frequency of metastasis, however, inhibition of dissociation, spreading, and invasion of cancer cells is a way to directly inhibit metastatic behavior of cancer cells.

Hepatocyte growth factor (HGF), originally identified and cloned as a mitogenic protein for hepatocytes [5,6], is closely involved in invasion and metastasis of a variety of cancer cells, including colon cancer [7–10]. Previous studies demonstrated that NK4, a specific antagonist against HGF, competitively inhibited Met receptor activation and this was associated with inhibition of biological events driven by HGF-Met receptor coupling, including the invasion of colon cancer cells [11–14]. NK4 is composed of the NH_2-terminal hairpin domain and four-kringle domains in the α-chain of HGF [11]. NK4 binds to the Met receptor, but does not induce tyrosine phosphorylation of Met [11]. Furthermore, NK4 is an angiogenesis inhibitor as well as an HGF-antagonist, and that the anti-angiogenic action of NK4 is independent of its activity as HGF-antagonist [15]. The bifunctional properties of NK4 to act as an

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angiogenesis inhibitor and as an HGF-antagonist raises the possibility that NK4 may be unique therapeutic strategy for treatment of metastatic colon cancer.

Gene therapy has been considered to expand the horizon of anti-angiogenesis therapy by virtue of its ability to locally and/or systemically deliver polypeptide angiogenesis inhibitors for a certain period [16,17]. Taking together with potential therapeutic value of NK4, the present study was performed to evaluate the therapeutic potential of the hydrodynamics-based gene therapy of NK4 for the treatment of invasive and metastatic colon cancer in mice.

Materials and methods

Recombinant materials and measurement of NK4. Recombinant human HGF and NK4 were respectively purified from culture media of CHO cells, which stably secrete human HGF and NK4 [6,18]. Concentration of NK4 in the plasma was determined using enzyme-linked immunosorbent assay (ELISA) kit for human HGF (B-Bridge International Inc., CA, USA). Because this ELISA system does not cross-react with mouse HGF, human NK4 can be specifically detectable in mouse tissues and plasma. Recombinant human NK4 was used for determination of the standard curve.

Cell culture and scattering assay. For measurement of biological activity of NK4 expressed using pCAGGS-NK4 plasmid, COS-7 cells were transfected with pCAGGS-NK4 or pCAGGS-empty, and cultured for 48 h. The culture media were collected and used for the cell scattering assay using MDCK cells. MDCK cells were plated at 250 cells/cm^2 in DMEM containing 10% fetal bovine serum onto six-well plates and cultured for 3 days. After culture medium was changed, the conditioned medium obtained from cultures of COS-7 cells transfected with pCAGGS-empty or pCAGGS-NK4 was added at 50:50 (% in vol/vol) to the culture of MDCK cells in the presence or absence of 110 pM HGF, and the cells were cultured for 24 h.

Experimental models for cancer growth and metastasis. Expression plasmid for human NK4 (pCAGGS-NK4) was constructed as described elsewhere [14]. For hydrodynamics-based gene delivery, plasmid DNA (pCAGGS-empty or pCAGGS-NK4) in saline was injected into the tail vein at 80 μl/g body weight within 5 s [19]. Seven-week-old male C57BL/6 mice (Japan SLC Inc., Hamamatsu, Japan) were subcutaneously implanted with 1×10^6 MC-38 cells. Five days after tumor implantation, pCAGGS-empty or pCAGGS-NK4 plasmid solution was injected into the tail vein as described above, and this procedure was repeated with an interval of 7 days. For histopathological analysis, mice were analyzed on day 28. The size of tumors was measured using a dialcaliper, and the volume of tumors was determined using the formula $\text{width}^2 \times \text{length} \times 0.5$. Six mice were used for each experimental group.

For analysis of metastasis, mice were killed on day 50 after tumor implantation and the number of macroscopically visible metastatic nodules (larger than 1 mm in diameter) on the surface of livers was measured. Ten mice were used in each experimental group.

Histopathological analysis. For conventional histological analysis, tissues fixed in formalin were embedded in paraffin, and tissue sections were stained with hematoxylin and eosin. Apoptotic cell death was determined by in situ detection of DNA fragmentation using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL assay) as described elsewhere [12,14,15]. For blood vessel staining, tissues were fixed in 70% ethanol and embedded in paraffin. Tissue sections were subjected to immunostaining for von Willebrand factor (wWF) (diluted 1:100; Dako, Glostrup, Denmark) as described by Kuba et al. [15]. The number of blood vessels was counted under a light microscope at 100× magnification using at least 20 randomly selected fields per each section. For detection of proliferating cells, tissue sections fixed in 10% formalin solution were subjected to immunostaining for PCNA as described elsewhere [12,14,15].

To semiquantitate the invasiveness of the subcutaneously implanted cancer, we defined the invasion score based on histological observations as follows: score 0, invasion was undetectable; score 1, invasive regions were less than 50% in periphery of each metastasis; score 2, invasion regions were 60–80% in periphery of each metastasis; score 3, invasion was extensive, and normal muscle tissue and tumor regions could not be distinguished [18].

Statistical analysis. For statistical analyses, we used unpaired Student's t test (two-tailed) unless otherwise mentioned. Differences were considered to be statistically significant at $p < 0.05$.

Results

Inhibition of HGF-induced cell scattering by NK4 expressed in COS-7 cells

We first tested whether NK4 expressed in mammalian cells using pCAGGS-NK4 plasmid would inhibit biological response driven by HGF-Met association. COS-7 cells were transfected with pCAGGS-NK4 plasmid and biological activity of NK4 in the conditioned medium was examined in cell scattering assay using MDCK cells (Fig. 1A). When 110 pM HGF was added to monolayer culture of MDCK cells, HGF induced dissociation and scattering of the cells (Fig. 1A(a) and (b)), however, recombinant NK4 inhibited MDCK cell scattering in the presence of HGF (Fig. 1A(f)). The addition of the conditioned medium from COS-7 cells transfected with pCAGGS-NK4 inhibited cell scattering induced by HGF (Fig. 1A(d)). In contrast, the conditioned medium from COS-7 cells transfected with empty plasmid did not inhibit MDCK cell scattering (Fig. 1A(c)). The addition of the conditioned medium from COS-7 cells transfected with pCAGGS-NK4 had no effect on the scattering of MDCK cells (Fig. 1A(e)). Thus NK4 expressed using pCAGGS-NK4 antagonizes biological activity of HGF.

Transgene expression after repeated plasmid delivery

Previous studies demonstrated that the hydrodynamics-based gene transfection achieved an efficient expression of NK4 genes predominantly in the liver but much lesser in the kidney and spleen, and the plasma NK4 level was increased for a certain period [14]. We here examined a possibility that plasma NK4 level could be maintained for a longer period by repeated administration of expression plasmid than a single administration. Mice were injected with 5 μg of pCAGGS-NK4 or pCAGGS-empty plasmid per animal on day 0, day 7, and day 14, and the NK4 plasma levels were measured (Fig. 1B). The plasma NK4 reached 49.5 ng/ml 24 h after delivery of the pCAGGS-NK4 and decreased to 15.4 ng/ml on day 3. Following the second and third injection, the plasma NK4 level again reached 72.9 and 125.1 ng/ml on day 8 and 15, respectively. Thus, the plasma NK4 level was repetitively increased following administration of the expression plasmid, and it was kept over 8.2 ng/ml during 3 weeks post-administration.

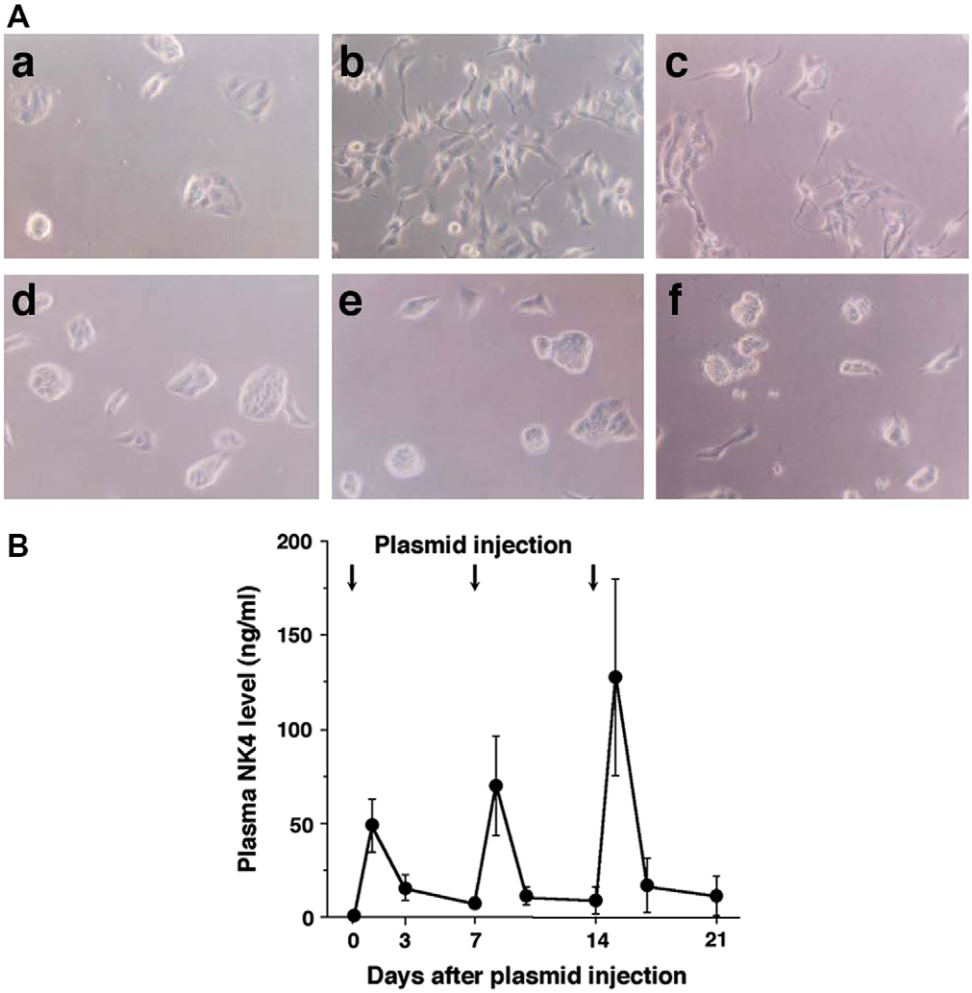

Fig. 1. Inhibition of HGF-dependent cell scattering by NK4 gene expression and change in the plasma NK4 level following repetitive administration of expression plasmid for NK4. (A) Inhibition of HGF-dependent cell scattering by NK4 secreted from COS-7 cells. The conditioned medium (CM) obtained from culture of COS-7 cells transfected with pCAGGS-empty or pCAGGS-NK4 was added to the culture of MDCK cells. MDCK cells were cultured for 24 h in the absence (a) or presence of 110 pM HGF (b), 110 pM HGF plus CM from COS-7 transfected with pCAGGS-empty (c), 110 pM HGF plus CM from COS-7 transfected with pCAGGS-NK4 (d), CM from COS-7 transfected with pCAGGS-NK4 (e), or 110 pM HGF plus 110 nM recombinant NK4 (f). (B) Change in the plasma NK4 level following repetitive administration of expression plasmid for NK4. Five microgram of pCAGGS-NK4 was administered intravenously into mice on day 0, 7, and 14. NK4 level in the plasma was measured by ELISA. Each value represents the mean ± SE ($n = 6$ in each group). Arrows indicate the time of plasmid administration.

Suppression of tumor angiogenesis and growth by NK4 gene expression

We next examined whether NK4 would inhibit tumor growth in vivo. MC-38 colon cancer cells were subcutaneously implanted into C57/BL6 mice, and pCAGGS-NK4 or pCAGGS-empty was repeatedly administered with an interval of 7 days (Fig. 2). Tumor volume increased rapidly following two weeks after implantation, however, the increase in tumor volume was inhibited by the expression of NK4. The volume of subcutaneous primary tumor in NK4 treated mice was inhibited to 43% on day 28 as compared to control mice given empty plasmid ($n = 6$, $p < 0.05$) (Fig. 2).

Because tumor growth is regulated by a counter-balance between proliferation and death of cancer cells, proliferating and apoptotic cancer cells were detected by immunohistochemistry for PCNA and TUNEL, respectively. The population of PCNA-positive cells in tumor tissues had no significant difference between values in empty vector and pCAGGS-NK4 vector (Fig. 3A–C). On the other hand, the population of TUNEL-positive cancer cells was $1.9 \pm 0.1\%$ in control mice injected with empty plasmid, whereas it increased to $4.6 \pm 0.4\%$ in mice injected with pCAGGS-NK4 plasmid (Fig. 3D–F). The result indicates that growth suppression by NK4 gene expression might be due to the increase in apoptotic cancer cells rather than the inhibition of proliferation of cancer cells. When we analyzed blood vessels in tumor tissues by immunostaining for von Willebrand factor, the size of blood vessels in mice injected with pCAGGS-NK4 plasmid appeared much smaller than that of control mice injected with the empty plasmid (Fig. 3G and H). Moreover, the blood vessel density in mice injected with pCAGGS-NK4 plasmid

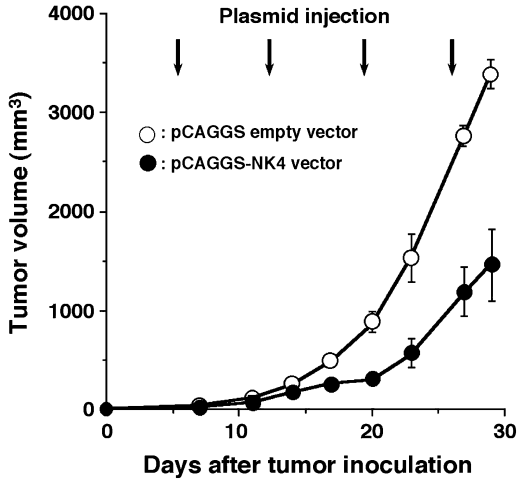

Fig. 2. Suppression of primary tumor growth by NK4 gene delivery and expression. MC-38 colon cancer cells (1×10^6 cells) were subcutaneously implanted into mice. pCAGGS-empty or pCAGGS-NK4 (5 μg each mouse) was administered on day 5, 12, 19, and 26 after tumor implantation. Each value represents the mean ± SE ($n = 6$ in each group). Arrows indicate the time of plasmid administration.

decreased to 33.3 % of that of control mice ($p < 0.01$) (Fig. 3I). Thus, NK4 gene expression inhibited tumor angiogenesis, thereby suppressing angiogenesis-dependent tumor growth.

It is also notable that histological observation of tumor tissues indicated that NK4 gene therapy suppressed invasive behavior of the cancer cells (Fig. 3J–L). In control tumor tissue, the cancer cells invaded surrounding tissues, particularly into muscle tissue (Fig. 3J). In contrast, invasion of the tumor cells was suppressed in mice injected with pCAGGS-NK4 plasmid (Fig. 3K). Histological estimation of tumor invasion indicated that the invasion score in pCAGGS-empty plasmid-injected mice reached 2.8 ± 0.1, whereas in pCAGGS-NK4 plasmid-injected mice it was inhibited to 1.6 ± 0.2 (Fig. 3L; $p < 0.01$). Because both in vitro and in vivo inhibition of Met receptor activation/tyrosine phosphorylation in MC-38 cancer cells by NK4 was closely associated with inhibition of invasion and spreading of the cancer cells [14], the inhibition of cancer cell invasion in the subcutaneous tumor tissue by NK4 gene therapy may be attributable to the biological activity

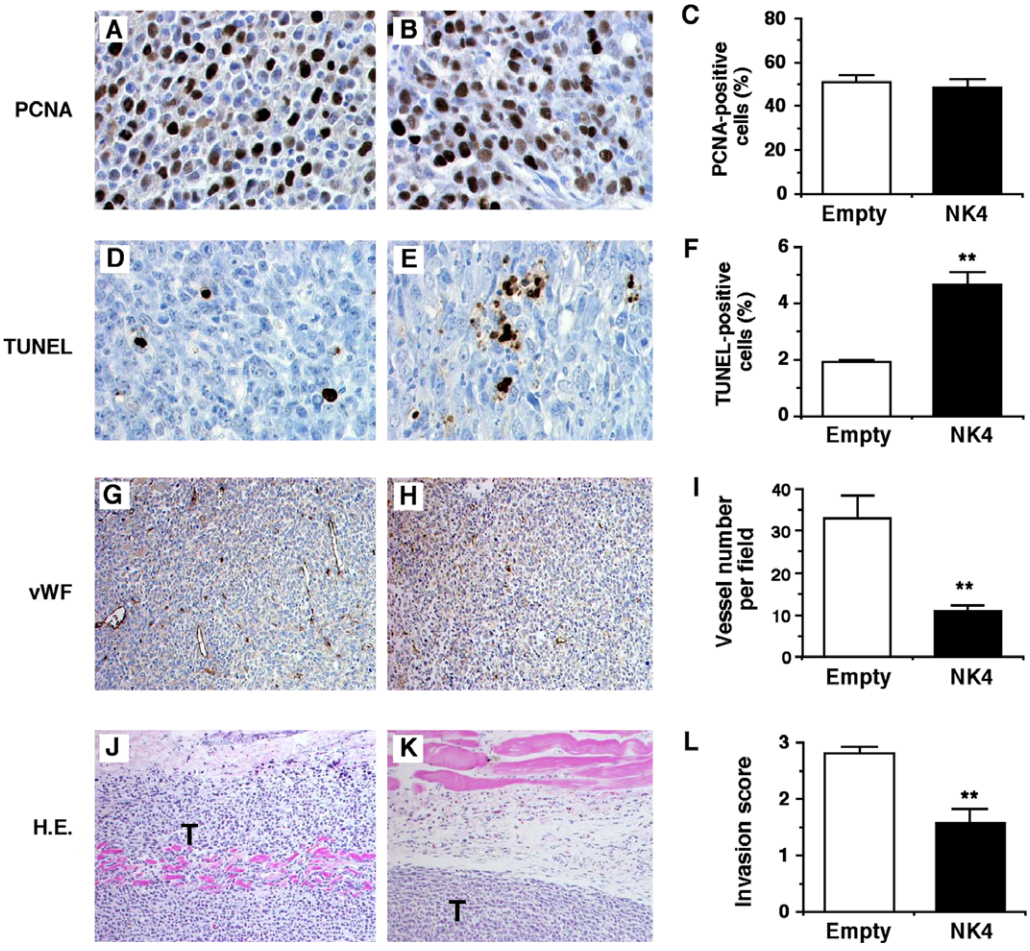

Fig. 3. Histopathological changes in primary tumor in mice administered with pCAGGS-empty or pCAGGS-NK4 plasmid. (A,B,C) Distribution and change in PCNA-positive proliferating cancer cells. (D,E,F) Distribution and change in TUNEL-positive apoptotic cancer cells. (G,H,I) Distribution and change in the blood vessel density. (J,K) Typical histological appearances of tumor tissues (T indicates tumor regions). (L) Invasive potential of colon cancer in primary tumor, evaluated by histological scoring (described in Materials and methods). MC-38 cells were subcutaneously implanted into mice, and pCAGGS-empty (A,D,G,J) or pCAGGS-NK4 (B,E,H,K) was administered on day 5, 12, 19, and 26. Tumors were resected on day 28. Each value represents the mean ± SE ($n = 6$ in each group). $^{**}p < 0.01$.

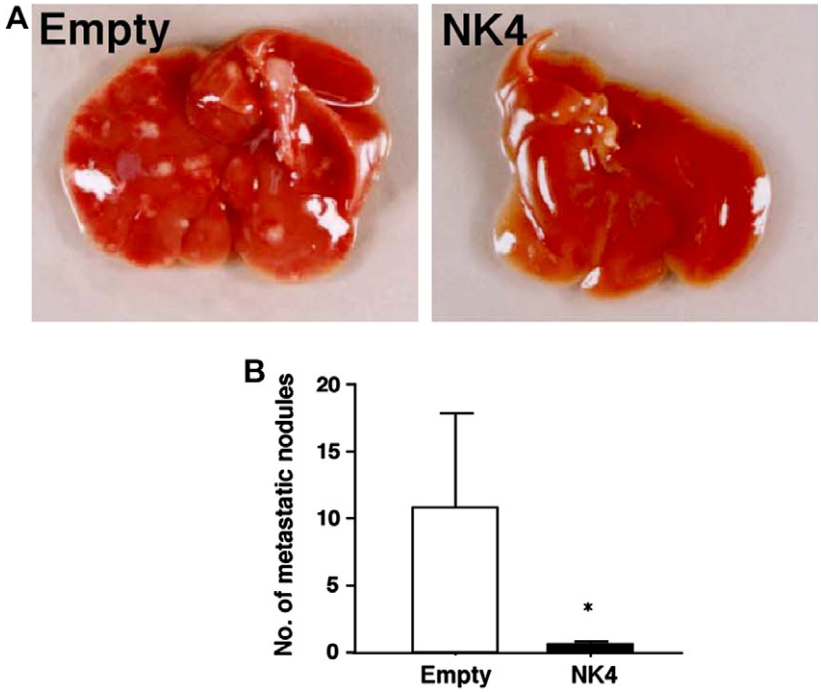

Fig. 4. Inhibition of liver metastasis of colon cancer by NK4 gene expression. Typical appearances of the livers (A) and the number of the hepatic surface metastases (B) in mice administered with pCAGGS-empty (Empty) or pCAGGS-NK4 (NK4). MC-38 cells were subcutaneously implanted into mice, and pCAGGS-empty or pCAGGS-NK4 was administered on day 5, 12, 19, and 26. On day 50, mice were autopsied and the number of metastases (larger than 1 mm in diameter) on the surface of livers was measured. Each value represents the mean ± SE ($n = 10$ in each group). $^{*}p < 0.05$.

of NK4 to inhibit functional association between HGF and the Met receptor.

Inhibition of liver metastasis by NK4 gene expression

Subcutaneous MC-38 cancer cells naturally metastasized to the liver 7 weeks after tumor implantation. We therefore analyzed the effect of repetitive gene expression of NK4 on liver metastasis in the subcutaneous implantation model. MC-38 cells (1×10^6 cells) were implanted subcutaneously, pCAGGS-empty and pCAGGS-NK4 plasmids were repeatedly delivered four times on day 5, 12, 19, and 26, and the mice were analyzed on day 50 (Fig. 4). In control mice injected with the empty plasmid, 7 among 10 mice displayed the metastatic nodules in the surface of liver, whereas 3 among 10 displayed surface metastasis in the liver in mice injected with pCAGGS-NK4 (Fig. 4A). The mean number of the surface metastases in the liver reached 11.1 ± 7.1 in control mice injected with pCAGGS-empty, however, it was suppressed to 1.1 ± 0.2 in mice injected with pCAGGS-NK4 (Fig. 4B). In addition to the liver metastasis, 30% of control mice injected with empty plasmid displayed peritoneal metastasis in control mice, whereas no mice displayed peritoneal metastasis in mice subjected to NK4 gene therapy. Thus repetitive delivery and expression of NK4 gene potently inhibited cancer metastasis naturally spread from the primary subcutaneous tissue.

Discussion

Since HGF activates intracellular and extracellular events that lead to dissociation and invasion of cancer cells, the HGF-Met system is closely involved in acquisition of invasive and metastatic potentials [7–10]. HGF decreases cadherin-mediated adhesiveness of cells [20,21], and stimulates proteolytic breakdown of the extracellular matrix, through enhancing matrix metalloproteinase and urokinase-type plasminogen activator-dependent proteolytic network [7–11,22,23]. Based on these backgrounds, the inhibition of HGF-Met system has been considered to be a therapeutic strategy, particularly to inhibit cancer invasion and metastasis. Several lines of distinct approaches for inhibiting the HGF-Met pathway have been demonstrated in experimental models, including small molecules that inhibit the tyrosine kinase activity of the Met, ribozyme, siRNA, neutralizing antibodies, soluble Met receptor, and NK4 [12,14,15,18,24–27].

NK4 gene expression in hydrodynamics-based delivery of pCAGGS-NK4 plasmid was the highest in the liver and much lesser expression of NK4 was seen in other organs such as the kidney and spleen [14]. In our previous report, we showed that the single injection of pCAGGS-NK4 plasmid inhibited intrahepatic invasive growth and liver metastasis in intrahepatic or intrasplenic inoculation of MC-38 cancer cells [14]. Thus, the previous report did not address effects of NK4 gene expression on entire processes of tumor metastasis from the dissociation in primary

site to growth of metastases in distant organs. We here demonstrated that repetitive hydrodynamics-based delivery and expression of NK4 gene inhibited invasive growth of primary tumor and subsequent liver metastasis even when the colon cancer cells were implanted subcutaneously. Therefore, considerable significance of the present study seems to be that 1) NK4 gene expression inhibited colon cancer metastasis, wherein metastatic process includes dissociation and invasion of cancer cells from the primary subcutaneous tumor tissues, the entry into the blood flow and extravasation, hepatic colonization, and subsequent growth in the liver, and 2) systemically delivered NK4 via blood circulation inhibited subcutaneous tumor invasion, angiogenesis and growth.

NK4 is a bifunctional molecule, acting as HGF-antagonist and angiogenesis inhibitor [11,15,27]. NK4 inhibits angiogenic response induced by basic fibroblast growth factor and vascular endothelial cell growth factor, as well as by HGF [15]. We cannot discriminate contribution of each biological activity of NK4 to its inhibitory effect on liver metastasis of cancer cells spread from the primary subcutaneous site. However, it is highly likely that both activities as HGF-antagonist and angiogenesis inhibitor are involved in the anti-metastasis, because NK4 gene therapy suppressed dissociation and invasion of the cancer cells in subcutaneous tissue (Fig. 3), and the specific inhibition of tumor angiogenesis was associated with the decrease in tumor metastasis [28]. On the other hand, the inhibition of tumor angiogenesis is associated with an increase in hypoxic regions, whereas the presence of hypoxic regions within tumors increases risk of invasion and metastasis [29]. Pennacchietti et al. demonstrated that hypoxia induced the transcriptional activation of the Met receptor gene and the subsequent amplification of HGF-Met signaling, thereby promoting the invasiveness of cancer cells [29]. Therefore, a connection between hypoxia and the Met receptor explains why hypoxia is often correlated with the acquisition of invasive and metastatic behavior. Perhaps, simultaneous inhibition of tumor angiogenesis and the HGF-Met pathway may be a way for augmenting the effectiveness of anti-angiogenesis therapy of cancers.

Inhibition of tumor invasion and metastasis awaits the development of new strategies in cancer treatment and prevention. The inhibition of tumor angiogenesis is also a key therapeutic strategy that holds promise for the advancement of cancer treatment. Based on the notion that HGF is a mediator in tumor-stromal interactions that confer invasive and metastatic characteristics in tumor cells, NK4 could offer a new therapeutic option toward the inhibition of colon cancer metastasis and growth.

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