NK4 Gene Therapy Combined with Cisplatin Inhibits Tumour Growth and Metastasis of Squamous Cell Carcinoma

GOICHI MATSUMOTO¹, YASUSHI OMI¹, USHAKU LEE¹, EIRO KUBOTA¹ and YASUHIKO TABATA²

¹Department of Oral and Maxillofacial Surgery, Kanagawa Dental College, Yokosuka, Kanagawa 238-8580, Japan; ²Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

Abstract. Background: NK4 inhibits vascularisation in tumour tissues, thereby arresting tumour growth. However, the antitumour efficacy of individual antiangiogenic molecules expressed in vivo is not sufficiently potent to induce regression in animal models. One of the strategies to overcome this disadvantage is to use chemotherapy.

Materials and Methods: This study evaluated the efficacy of combining NK4 gene therapy with cisplatin to treat experimental squamous cell carcinomas. For gene therapy, biodegradable cationised gelatin microspheres were used for the controlled release of NK4 plasmid DNA. Results: A combined regimen of antiangiogenic gene therapy and low-dose cisplatin led to a marked decrease in tumour volume and vascularity, and caused increased apoptosis compared to NK4 gene therapy alone. Moreover, combination treatment of NK4 gene therapy and low-dose cisplatin dramatically inhibited the formation of lung metastases. Conclusion: NK4 gene therapy combined with low-dose cisplatin may be an effective regimen for treating oral squamous cell carcinoma.

Oral squamous cell carcinomas grow rapidly, and they often recur, even after they have been treated aggressively with chemotherapy, radiation and/or surgery. The disease is characterized by a rapid invasion into bone and muscle and a high degree of neovascularity. Cancers of the oral and pharynx regions alone account for over 300,000 new cases worldwide and almost 200,000 deaths annually (1). The tongue in particular is the most common site for tumours of the oral cavity; the 5-year survival rates for patients treated for tongue carcinoma have not improved in the past 25 years and remain less than 50% (2). The poor prognosis for tongue carcinoma may reflect a limited understanding of the mechanisms of local and regional metastasis, accounting for the majority of deaths. Therefore, new treatment strategies need to be developed, especially those that capture the advantages of gene therapy. Several gene therapy strategies for head and neck squamous cell carcinoma are currently under investigation in clinical and preclinical settings (3, 4).

The neovascularisation process is induced by tumour cells, which normally stimulate endothelial cells to proliferate and differentiate (5). Turning on the neovascularisation process requires both the up-regulation of angiogenic stimulators and the down-regulation of angiogenic inhibitors in certain tumours (6). In contrast, several antiangiogenic factors suppress the growth of metastatic cells that have been released into the circulatory system from a primary tumour site. HGF, a potent stimulator of hepatocyte growth, stimulates the motility, invasiveness, proliferation and morphogenesis of the epithelium and may play a role in physiological and pathological processes such as embryogenesis, wound healing, organ regeneration, inflammation, and tumour invasion (7). It has been reported that HGF may play an important role in the progression of hypopharyngeal squamous cell carcinoma (8).

A specific HGF-antagonist termed NK4 has been prepared and binds to the c-Met/HGF receptor, without inducing tyrosine phosphorylation of c-Met. It has been reported that NK4 may inhibit the angiogenic responses induced by HGF, bFGF and VEGF (9). The blockade of HGF/c-Met coupling by NK4 may suppress the invasion, motility and subsequent intravasation of tumour cells, leading to the inhibition of lung metastases.

To increase the biological activity of the expressing plasmid DNA, it is necessary to increase its transfection efficiency to cells in vivo. As plasmid DNA is a large and negatively charged molecule, it cannot pass into the interior of cells even if it attaches to the negatively charged cell membrane. Gelatin has been used extensively in industrial, pharmaceutical and medical applications because of the ease...
with which chemical modification of a physicochemical nature takes place. For example, positively charged, cationised gelatin can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. These advantages have stimulated researchers to produce a new gene delivery system using cationised gelatin hydrogel as a carrier. This system allows controlled biodegradation of the local delivery agent and nuclease protect plasmid DNA from rapid degradation (10-12).

In view of the potential applicability of NK4 in clinical therapy, this study investigated the efficacy of combining NK4 antiangiogenic gene therapy with low-dose chemotherapy using cisplatin (CDDP), the latter therapy being one of the effective treatments for suppressing tumour growth and lung metastasis in cases of oral squamous cell carcinoma.

Materials and Methods

Tumour cell line and cell cultures. NR-S1 tumours are cutaneous mouse squamous cell carcinomas that spontaneously arise in the C3H/He mouse strain; these tumour cells do not metastasise (13). This study used cells that spontaneously metastasised to the lung from among a population of poorly metastatic NR-S1 tumour cells in vivo. NR-S1 cells were inoculated subcutaneously into C3H/He mice. After six weeks, the mice were killed and their lungs were resected. The metastatic nodules of resected lungs were cut into small fragments in vitro. Small fragments of metastatic nodules were cultured in vitro and re-inoculated into the mice for a second round of selection. The cell line consisting of cells prone to lung metastasis was obtained by repeating the procedure four times. NR-S1 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS at 37˚C in an incubator with 5% CO₂.

Preparation of NK4 plasmid DNA. The 1.4-kbp human NK4 cDNA was cloned in the mammalian expression vector pcDNA3 (14). NK4 plasmid DNA was prepared from bacterial cultures with endotoxin-free Qiagen columns (Qiagen, Tokyo, Japan).

Drug. Bristol Pharmaceuticals K.K (Tokyo, Japan) provided cisplatin (CDDP), commercially known as BRIPLATIN.

Preparation of cationised gelatin microspheres containing NK4 plasmid DNA. For in vivo transfection of plasmid DNA, cationised gelatin microspheres were used, prepared by chemically crosslinking gelatin in the water-in-oil emulsion state (15). To impregnate plasmid DNA into cationised gelatin microspheres, 20 μl of PBS containing 100 μg of plasmid DNA were mixed onto 1 mg of freeze-dried cationised gelatin microspheres and this mixture was kept at 4˚C for 16 h.

Tumour model and treatment. The animal studies conformed to the Kanagawa Dental College guidelines for research animal care. Back tumours were generated by subcutaneously injecting 1x10⁶ NR-S1 cells in 100 μl of PBS into the back of C3H/He mice (Japan SLC, Shizuoka, Japan) aged six weeks. Tumour volume was estimated using the formula: tumour volume (mm³) = length (mm) x width (mm)² x 1/2. Two weeks later, when the tumours had grown to 200-300 mm³, the mice were randomly divided into four treatment groups: PBS; cisplatin alone (Cis); NK4 plasmid DNA with cationised gelatin alone (NK4); and NK4 plasmid DNA with cationised gelatin plus cisplatin (NK4+Cis). For NK4 gene therapy, biodegradable cationised gelatin microspheres were used for the controlled release of NK4 plasmid DNA. On the second, third and fourth week after injecting the tumour cells, cationised gelatin microspheres containing NK4 plasmid DNA were administered by intratumoural injection. Cisplatin (1 mg/kg) was dissolved in 0.9% NaCl and administered via intraperitoneal injection one, two and three days after each round of NK4 gene therapy. The control group received intratumoural injections of PBS. Each treatment group contained ten mice.

Determination of spontaneous lung metastases. The mice were killed 35 days after tumour inoculation, the lung metastases were excised and the lung metastatic colonies were counted.

Immunohistochemical analysis. The mice were sacrificed and the implanted tumours were surgically removed. Implanted-tumour specimens were embedded in OCT compound, quickly frozen in dry ice and stored at –80˚C for immunohistochemical staining. Five-μm sections were treated in OCT by fixing them in 4% paraformaldehyde. The immunohistochemical analysis was as follows. Briefly, incubating slides in 3% H₂O₂ in methanol for 15 min inactivated endogenous peroxidase activity. Non-specific antibody binding was blocked with 10% normal goat serum in PBS for 30 min at room temperature before incubation with the appropriate dilution of anti-CD31 mAb (Pharmingen, San Diego, CA, USA) or anti-single-strand DNA (ssDNA) polyclonal Ab (DAKO Co., Carpinteria, CA, USA) at room temperature for 90min. After PBS rinses, bound primary Ab were detected using Histofine Simple Stain Mouse MAX-PO (Nichirei, Tokyo, Japan) according to the manufacturer’s instructions. Mayer’s haematoxylin was used as a counterstain.

HGF ELISA. Secretion of HGF within tumours in the NR-S1 xenografts was determined by a human HGF ELISA kit (Institute of Immunology Co., Ltd., Tokyo, Japan) according to the manufacturer’s instructions. Briefly, tumour samples were homogenised in a lysis buffer (Institute of Immunology Co., Ltd., Tokyo, Japan) at a 4:1 ratio of buffer volume (μl) to sample weight (mg) in order to normalise the influence of weight variance on the assay (15). The human HGF ELISA kit detects both human HGF and human NK4, but does not detect mouse HGF.

Statistical analysis. All data were expressed as mean ± standard error of the mean. The data were analysed with the unpaired Student’s t-test, and the results were considered to be statistically significant if p<0.05.

Results

In vivo expression of NK4/using cationised gelatin microspheres containing NK4 plasmid DNA in tumours. There was detectable NK4 protein expression in the tumour tissues after injecting cationised gelatin microspheres containing NK4 plasmid DNA, but there was no detectable NK4 protein expression in PBS- and cisplatin-injected tumour tissues (Figure 1).
Improved inhibition of tumour growth with combined treatment. At five weeks after NR-S1 tumour cell inoculation, the mean tumour volume of mice treated with antiangiogenic gene therapy with NK4 was less than that for mice in either the PBS group or the Cis group. However, no significant differences were found between the mean tumour volumes of the PBS, Cis and NK4 gene therapy alone groups. The combination treatment of NK4 gene therapy with cisplatin significantly inhibited tumour growth compared to both the NK4 gene therapy alone group and the Cis group (p<0.01) (Figure 2). The animals in all treatment groups appeared healthy. None of the animals lost weight or showed signs of systemic toxicity during treatment (data not shown).

Augmenting antiangiogenesis with combination treatment. To identify the mechanism that inhibits tumour growth, the angiogenesis of tumour tissues was examined by immunohistochemical staining. Angiogenesis within the tumour tissues was determined by directly counting the number of CD31+ cells on microvessels. The most highly vascularised areas of each tumour were identified and CD31+ vessels were counted in at least five fields (magnification: ×40). Angiogenesis was inhibited in the treatment with NK4 gene therapy alone, and there was a significant difference between the number of vessels counted for the PBS group. There was a pronounced inhibition of angiogenesis in tumours treated with the combination treatment of NK4 gene therapy with cisplatin compared with NK4-alone (Figure 3).

Increased apoptosis and decreased proliferation with combination treatment. Tumour growth is regulated by the balance between death and proliferation of tumour cells. In order to estimate the number of apoptotic and proliferating tumour cells, sections of tumour tissues from the different treatments were immunohistochemically stained for single strand DNA (ssDNA) and PCNA, respectively. NK4 gene therapy alone affected the rate of apoptotic tumour cells, whereas the rate of apoptotic tumour cells apparently increased with combined treatment (Figure 4A, 4B). PCNA staining was used to estimate the number of proliferated tumour cells. The greatest tumour cell proliferation was observed in the PBS, the Cis and NK4 gene therapy groups. Tumour proliferation in the combined-treatment collective group was significantly lower than in either the Cis group or the NK4 gene therapy group (Figures 4A, 4C).

Inhibition of the lung metastases formation with combination treatment. The effect of NK4 gene therapy plus cisplatin on lung metastases of NR-S1 tumours was investigated. NR-S1 tumour lung metastasis, PBS-, Cis-, and NK4-treated mice developed more than 50 metastatic nodules on lung surfaces,
whereas the number of metastatic nodules was significantly lower in lung tissues taken from mice treated with NK4 gene therapy plus cisplatin (Figure 5).

**Discussion**

Tumour growth and metastatic dissemination depend on the formation of new tumour microvessels. The process of tumour angiogenesis is a balance between proangiogenic and antiangiogenic factors. The use of antiangiogenic agents to restore this balance represents a useful approach to cancer treatment. Inhibition of tumour angiogenesis by antiangiogenic agents for anticancer therapy is widely accepted. NK4 is an antagonist of HGF and acts as an angiogenesis inhibitor (14). The efficacy of the NK4 gene therapy has already been demonstrated in animal models for many different types of solid tumours (16-18).

Antiangiogenic cancer therapy with antiangiogenic agents requires the peptide to be administered regularly or daily over a prolonged period, and this means that large quantities...
Figure 4. Immunohistochemical analysis of tumour apoptosis and proliferation. The excised xenograft tumours on day 28 were subjected to ssDNA for apoptosis and to PCNA for proliferation immunohistochemical staining. A: Representative images of treatment groups: PBS, cisplatin (Cis), NK4 gene therapy alone (NK4) and NK4 gene therapy with cisplatin (NK4+Cis). Magnification, ×100. Scale bars, 50 μm. B: The percentage of ssDNA-positive cells was considered to be the apoptotic index. C: The percentage of cells that showed positive nuclear staining for PCNA antigen was considered to be the proliferation index. In (B) and (C) data are represented as the mean±standard error of the mean of three mice in each group. *p<0.05, compared to PBS, Cis and NK4 groups.

Figure 5. Inhibition of spontaneous lung metastasis by combination treatment of NK4 gene therapies with cisplatin. A: Representative images of treatment groups: PBS, cisplatin (Cis), NK4 and NK4 gene therapy with cisplatin (NK4+Cis). B: Numbers of metastatic nodules of NR-S1 cells on the surface of lungs were counted at 5 weeks. Data are represented as the mean±standard error of the mean of three mice in each group. *p<0.05, compared to PBS, Cis and NK4 groups.
of the therapeutic agent are needed. These functional antiangiogenic proteins are expensive to produce, and frequently there are technical problems related to their physical properties and purity. Moreover, some therapeutic agents that would otherwise provide effective antiangiogenic cancer therapy have a biological half-life that is too short to sustain tumour regression. All of the above factors are obstacles that make it difficult to design effective therapies using exogenously injected antiangiogenic agents to inhibit tumour angiogenesis.

Gene transfer represents an attractive and promising alternative method to deliver antiangiogenic agents. Gene therapy has the potential to produce the antiangiogenic agent in high concentrations in a tumour area for a sustained period, thereby avoiding the problems encountered with long-term administration of recombinant proteins. In addition, there are various methods by which to transfect antiangiogenic genes into cells. One method is to use a viral vector to incorporate the gene into the cells (19, 20). While this method allows an effective supply of the antiangiogenic gene products to be established in the cell, there is an unacceptably large risk that the virus will cause harm to the host. Several non-viral vectors, including naked plasmid DNA, complexes formed with cationised liposomes, and complexed formed with cationised gelatin microspheres, have the advantages of being less harmful to the host and easier to prepare. The system of cationised gelatin microspheres allows controlled biodegradation of the local delivery agent and nuclease protection of plasmid DNA from rapid degradation (21, 22).

Although it has been reported that a single intratumoural injection of NK4 plasmid DNA in cationised gelatin microspheres allows the continuous release of plasmid DNA and prolonged expression of NK4 gene, time gradually erodes its viability, and in any case, when the cell divides, the transfected plasmid cDNA does not survive (15). In the present study, these limitations were addressed by using repeated injections of plasmid DNA in cationised gelatin microspheres. After the three injections of cationised gelatin microspheres containing NK4 plasmid DNA into the tumour mass, NK4 protein was detected in the tumour tissue. Apparently, the positively charged complex of plasmid DNA with cationised gelatin electrostatically interacted with the cell membrane so that the complex was transported into the cell interior. Clearly, antiangiogenic plasmid DNA was expressed at the injected site and secreted into the tumour tissues.

In the present study, however, NK4 gene therapy alone did not effectively inhibit tumour growth. Angiogenesis in tumour tissues was not significantly reduced, tumour cell proliferation was not significantly reduced, and apoptosis was not significantly increased compared to the PBS-treated mice. In the lung metastases formation assay, the mouse lungs were collected on day 35 after tumour inoculation and the nodules of NR-S1 cells were counted. For lung metastasis, mice treated with PBS, Cis, or NK4 gene therapy alone developed more than 50 metastatic nodules on their lung surface (Figure 6).

One of the strategies to enhance the inhibitory effect of antiangiogenic gene therapy on lung metastases is to use chemotherapy. For the treatment of gliomas, a strategy combining continuous low-dose chemotherapy and antiangiogenesis has been developed (23). The combination of low-dose chemotherapy and antiangiogenic treatment suppresses tumour growth more effectively than conventional chemotherapy alone. Cisplatin has been widely used for the treatment of oral squamous cell carcinoma with advanced tumour stages or distant metastases. Therefore, in the present study, cisplatin was used as a chemotherapeutic drug. The study was designed to determine whether low-dose cisplatin potentiates or enhances the antiangiogenic ability of NK4 gene therapy. The results of the present study showed that the combined treatment with cisplatin and NK4 gene therapy led to an enhanced inhibition of tumour growth, tumour angiogenesis and the increased induction of apoptosis compared with NK4 gene therapy, and cisplatin alone. Moreover, there were fewer lung metastases in the lungs from mice treated with NK4 gene therapy plus cisplatin.

A treatment combining low-dose chemotherapy with an antiangiogenic agent has been developed for experimental tumours such as glioma, breast cancer and neuroblastoma (23-25). It is well known that during the break periods between successive cycles of maximum tolerable dose chemotherapy, tumour regrowth and drug resistance may be accelerated by increased mobilisation of circulating endothelial progenitors (CEP) (26). In contrast, a strategy of continuous low-dose chemotherapeutic regimens may reduce the bone-marrow-derived CEP mobilisation and viability; this has been termed antiangiogenic or metronomic chemotherapy (27). Cisplatin has been reported to influence the process of tumour vascularisation and cause vascular cytotoxicity (28). Antiangiogenic gene therapy increases vascular permeability, which may lead to increased tumour exposure to chemotherapeutic agents and induce apoptosis of tumour cells. It was, therefore, hypothesised that the low-dose cisplatin amplifies the antitumour effect of NK4 gene therapy. Although the exact mechanism remains unclear, combining NK4 gene therapy with cisplatin reduced tumour volume, CD31+ tumour vessels and the rate of proliferation of tumour cells and it increased the induction of apoptosis. Moreover, mice given combined treatment did not experience any side-effects.

In summary, the data of the present study suggested that the combination of NK4 gene therapy using cationised gelatin microspheres and low-dose cisplatin is effective in the treatment of experimental squamous cell carcinoma, based on the increased inhibition of tumour angiogenesis and lung metastasis. Further studies are needed to establish optimal protocols for antiangiogenic gene therapy, including dosage of the chemotherapeutic agents and scheduling characteristics.
Acknowledgements

The Ministry of Education, Culture, Sports Science and Technology of Japan supported this work with a Grant-in-Aid for the High-Tech Research Center Project.

References


Received August 23, 2010
Revised October 12, 2010
Accepted October 13, 2010