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Enhanced efficacy in anti-tumour activity by combined therapy of recombinant FGFR-1 related angiogenesis and low-dose cytotoxic agent

S.P. Zheng[a,b], S.J. Zheng[a,b,*], R.L. Wu[b], F.Y. Huang[a], L.M. Cao[b], C.L. Jiao[a]

[a]Pathology Department of Affiliated Hospital and Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571101, People's Republic of China

[b]Department of Pathology and Ultrasonography of Union Hospital, Tongji Medical College, Huazhong University Science and Technology, Wuhan 430030, People's Republic of China

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ABSTRACT

Fibroblast growth factor receptor-1 (FGFR-1) has been used as a target for anti-angiogeneic therapy of cancer. The strategies of combining anti-angiogenic biotherapy with chemotherapeutic drugs show potential and promise for cancer therapy. In this study, we evaluated the anti-tumour efficacy of chicken FGFR-1 (cFR-1) vaccine combined with low-dose gemcitabine in two mice tumour models. We found that both the cFR-1 vaccine and low-dose gemcitabine can suppress tumour growth to some extent. Remarkably, the combination strategy produces an apparent decrease in tumour volume, microvessel density and tumour cell proliferation, and an increase of apoptosis without obvious side-effects compared with either therapy alone. Moreover, the combination strategy also demonstrated synergistic indices against tumour growth and angiogenesis. Furthermore, auto-antibodies against mouse FGFR-1 were identified. These findings support the idea that the combination strategy synergistically strengthens anti-tumour activity via suppression of tumour angiogenesis without overt toxicity in tumour-bearing mice.

1. Introduction

It is well known that angiogenesis plays an essential role in tumour growth and metastasis, and anti-angiogenic therapy as a strategy for cancer therapy has been highly warranted.[1–3] More and more studies have confirmed that fibroblast growth factor receptor-1(FGFR-1) plays a critical role in tumour angiogenesis and tumour growth, which is markedly expressed both in active endothelial cells and in many different forms of tumour.[4–6] Accumulating evidence indicates that FGFR-1 mediated anti-angiogenesis targeted for tumour immunotherapy could suppress angiogenesis and further inhibit tumour growth.[7–10] Our recent data also found that immunotherapy of cancer with a vaccine based on chicken FGFR-1 (cFR-1) resulted in effective anti-tumour effect through the induction of autoimmunity against FGFR-1 in mice.[11,12]

Although anti-angiogenic therapy has proven effective at suppressing tumour growth in many preclinical studies,[12–14] it remains uncertain whether it is tumouricidal or not. Many studies have concluded that this therapeutic limitation may be overcome using a combination of angiogenic inhibitors with different cytotoxic agents.[15–17] Other findings have also demonstrated that anti-angiogenic therapy combined with chemotherapy could more effectively inhibit tumour growth without overt toxicity than either therapy alone.[17–19]

* Corresponding author. Tel.: +86 898 66893335; fax: +86 898 66893335.
E-mail address: zhenghoho@yahoo.com.cn (S.J. Zheng).
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Gemcitabine is a new deoxycytidine analogue that inhibits DNA synthesis and has shown cytotoxicity against cancer therapy.[20–22] Thus, the present study was to determine whether it would improve the anti-tumour efficacy by combining the cFR-1 protein vaccine with low-dose gemcitabine. Our findings showed that the combination strategy can synergistically enhance the anti-tumour activity without overt toxicity in two mice tumour models.

2. Material and methods

2.1. Vaccine preparation

The lyophilised recombinant proteins of cFR-1 were dissolved in normal saline (NS) and mixed with an equal volume of aluminum hydroxide adjuvant at 4 mg/mL for 60 min before use for vaccination.[11]

2.2. Design of animal experiments

In the first part, the H22 hepatoma (H22) and the Meth A fibrosarcoma (Meth A) model were respectively established in syngeneic BALB/c mice to evaluate whether the combination of cFR-1 vaccine and low-dose gemcitabine would improve the anti-tumour efficacy. Six to 8-week-old female mice were transplanted with 1×10^6 live tumour cells. After the tumour had grown for 7 days, the mice were randomly divided into the following four groups of ten mice each. Group 1, combination of cFR-1 vaccine and low-dose gemcitabine(C + G), mice received cFR-1 vaccine plus low-dose gemcitabine as follows: after day 0 (7 days after tumour cell injection), cFR-1 vaccine was injected subcutanenously (s.c.) once weekly for 4 weeks with a dose of 10 μg per mouse. At day 7 (14 days after tumour cell injection), 10 mg/kg of gemcitabine was injected intraperitoneally (i.p.) twice weekly for 2 weeks. Group 2, cFR-1 vaccine alone(cFR), mice received cFR-1 vaccine in a scheme the same as that in group 1, but no gemcitabine. Group 3, low-dose gemcitabine alone(G), mice received the same dose of gemcitabine as group 1 but no cFR-1 vaccine. Group 4, NS control group(NS), mice received sterile NS s.c. as the scheme of vaccination and i.p. as in group 1, respectively. Tumour growth was evaluated every 3 days, and tumour volume was estimated using the formula for an ellipsoid ($0.5 \times \text{length} \times \text{width} \times \text{height}$).

In the second part, mice were not transplanted with tumour cells, and we investigated whether the combination therapy reduced the systemic toxicity. Forty mice were also randomly divided into four groups of ten mice each. Groups and treatments were similar to those in the first part. The exception was that after the fourth vaccination, the vaccination schedule was changed to once per month, and the other treatments in each group were continued for 6 months.

Mice treated with these schemes were investigated, in particular, for toxicity for a period of 6 months. Body weight and life span were recorded every 3 days, and gross measures such as ruffling of fur, behaviour and feeding were investigated. At the end of the experiment, the tumour tissues, major organs and blood samples of the mice were collected for subsequent histologic and immunologic investigations. All studies involving mice were approved by the Institute's Animal Care and Use Committee.

2.3. Western blot analysis

Western blot analysis was performed as described previously.[11] Briefly, the recombinant proteins were separated by 12% SDS-PAGE. Gels were transblotted with Mini Polyacrylamide Gel System (Bio-Rad, USA) onto a polyvinylidene difluoride membrane. Membrane blots were blocked at 4 °C in 5% nonfat dry milk, washed and probed with mouse sera at a 1:500 dilution. Blots were then washed and incubated with goat anti-mouse IgG HRP-labelled secondary antibody and then stained with the Vectastain ABC kit (Vector, Burlingame, USA).

2.4. ELISPOT assay

The enzyme-linked immunospot (ELISPOT) assay for the enumeration of anti-FGFR-1 antibody-producing B cells (APBCs) has been described.[11,12] Briefly, PVDF-bottomed, 96-well filtration plates (Millipore, Bedford, USA) were coated with 30 μg/ml of recombinant mFR-1 protein. Mononuclear cells prepared from spleen were incubated on the plates at 37 °C for 4 h. IgG bound to the membrane was revealed as spots with goat anti-mouse IgG bio-labelled secondary antibodies.

2.5. Immunohistochemistry

For MVD and cell proliferation analyses, frozen sections were fixed in acetone, incubated and stained with antibodies reactive to either CD31 or proliferating cell nuclear antigen (PCNA) (BD Pharmingen, USA), respectively. The MVD was determined by counting the number of microvessels and the proliferation index was calculated as the ratio of the proliferation cell number to the total cell number per high-power field in tumour sections as described.[23,24]

To identify the endothelial deposition of autoantibody by immunofluorescent staining, frozen sections were fixed in acetone, washed with PBS, and incubated with FITC-conjugated antibody against mouse IgG, IgA, or IgM (Sigma, St. Louis, USA). Moreover, sections of tissue were fixed with 1% paraformaldehyde in PBS and stained for apoptosis analysis using the TdT-mediated dUTP nick end labelling (TUNEL) assay according to the manufacture's instructions (In Situ Cell Death Detection Kit; Roche, UK). These slides were respectively imaged using a fluorescence microscope and the apoptosis index was calculated as the ratio of the apoptotic cell number to the total cell number in each high-power field.

2.6. Calculation of synergistic indexes

Mean values of tumour volume, MVD, cell apoptosis and proliferation were used for calculation of the correspondent synergistic indexes using the methods as described previously.[23,24] Calculated synergistic indexes of tumour volume, MVD, cell apoptosis and proliferation are further detailed in Table 1. An index of greater than 1 indicates a synergistic effect, whereas an index of less than 1 indicates a less than additive effect.

Table 1 – Synergistic indexes of combination treatment relative fraction[a]

Day[b]	FGFR-1	Gemcitabine	Combination treatment		Index[d]
			Expected[c]	Observed	
H22 model					
Tumour volume	0.33	0.41	0.14	0.13	1.08
MVD	0.39	0.59	0.23	0.18	1.28
Apoptosis	2.23	2.52	5.62	4.78	1.18
Proliferation	0.59	0.66	0.39	0.34	1.15
Meth A model					
Tumour volume	0.27	0.44	0.12	0.12	1.00
MVD	0.37	0.60	0.22	0.17	1.29
Apoptosis	2.23	2.90	6.47	5.41	1.20
Proliferation	0.66	0.57	0.38	0.30	1.20

a Relative fraction (RF) = mean tumour volume (or MVD, apoptosis and proliferation) experimental/mean tumour volume (or MVD, apoptosis and proliferation) untreated on day 33 respectively.
b Day after tumour cell transplantation.
c RF of gemcitabine × RF of FGFR-1 vaccine.
d Obtained by dividing the expected RF by the observed RF. An index > 1 indicates a synergistic effect; an index < 1 indicates a less than additive effect.

2.7. Statistical evaluation

For comparison of individual time points, ANOVA and an unpaired Student's *t*-test were used. Survival curves were constructed according to the Kaplan–Meier method. Statistical significance was determined by the log-rank test. Changes were considered to be significant when $p < 0.05$.

3. Results

3.1. The combination strategy suppressed tumour growth synergistically and extended survival time

In the first part, low-dose gemcitabine or cFR-1 vaccine treatment resulted in the inhibition of tumour growth to a certain extent compared with the untreated control group. Remarkably, the combination therapy resulted in more significant anticancer activity (Fig. 1A, B). The relative ratio of tumour volume in the combination group showed a synergistic relationship about 33 days after tumour cell transplantation in both tumour models (Table 1). In addition, the survival of tumour-bearing mice was similar results to that of tumour growth (Fig. 1C, D).

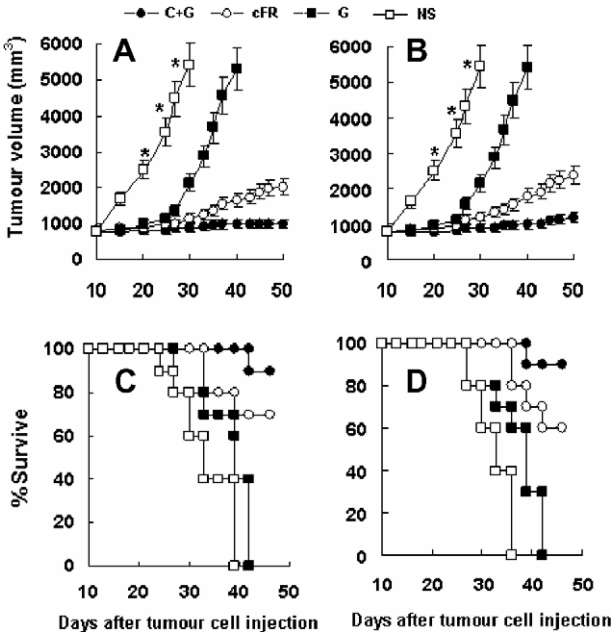

Fig. 1 – The combination treatment inhibits tumour growth synergistically and extended survival time. Mice (ten mice per group) were transplanted s.c. with 1×10^6 H22 (A and C) or Meth A (B and D) live cells. *Significant difference compared to untreated group ($p < 0.05$).

3.2. The combination strategy induces anti-tumour autoimmunity

The possibility that the cFR-1 vaccine alone or the combination treatment induces production of anti-tumour autoimmunity in two tumour models was examined by using Western blot analysis, ELISPOT assay and immunofluorescent staining respectively.

In Western blot analysis, sera from these cFR-1-immunited mice recognised a protein as indicated by the positive staining of a ~40 kDa band (Fig. 2A, C) that was not stained by sera from the untreated control or low-dose gemcitabine treatment groups (Fig. 2B, D).

The number of APBCs, which were detected by ELISPOT assay, was significantly elevated in the spleen of mice immunised with cFR-1 vaccine, both in the vaccine alone and in the combination treatment groups, as compared with those in non-immunised groups (Fig. 2E). At the same time, the number of APBCs was not different between the vaccine alone and combination treatment groups, which suggested that the low-dose gemcitabine scheme did not inhibit the immune response to cFR-1 immunisation.

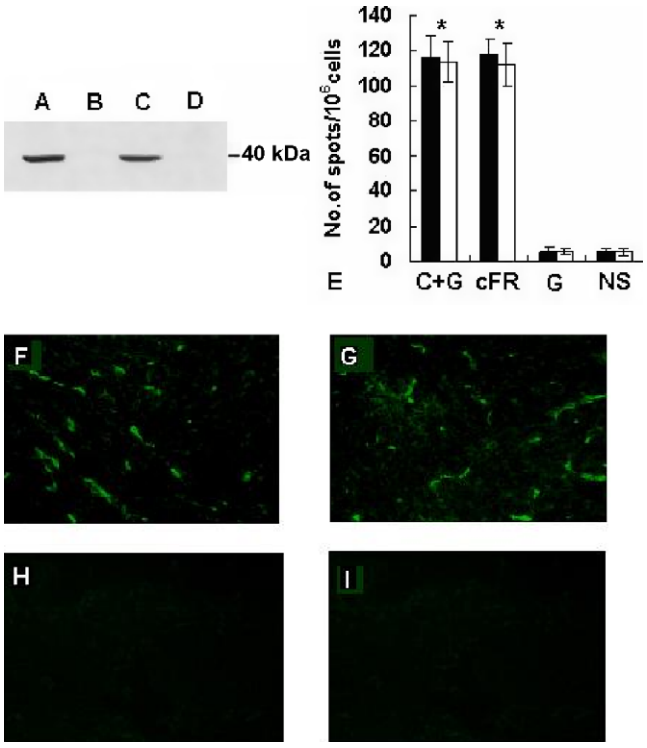

Fig. 2 – Induction of autoantibodies by cFR-1 vaccine alone or combination therapy. Recombinant FGFR-1 was recognised by sera from cFR-1-immunised mice in both the vaccinated groups of the H22 tumour model (A, C), but not in gemcitabine alone and untreated group (B, D) by Western blot analysis. Quantification of the number of APBCs for cFR-1 was analysed by ELISPOT (E). *There was no significant difference between the two vaccination treatment groups ($p < 0.01$). In addition, the deposition of autoantibodies on the endothelial cells from cFR-1-immunised mice was observed by immunofluorescent staining (F and G). No fluorescent signal was detected within the tumour tissues from control groups (H and I). Similar results were also found in Meth A model.

The endothelial deposition of auto-antibodies was found within tumour tissues from cFR-1-immunised mice, as detected by immunofluorescent staining (Fig. 2 F–I); however, this deposition was not detected in the non-immunised control groups. In addition, detectable deposition of auto-antibodies was not found within the major organs of immunised and non-immunised mice.

3.3. *The combination strategy synergistically enhances antitumour activity*

Although single agent alone can suppress tumour growth to some extent, the combination strategy produces an apparent decrease in tumour volume, microvessel density and tumour cell proliferation, and an increase of apoptosis when compared with either therapy alone. MVD was significantly lower in the combination group than in cFR-1, gemcitabine, and control group [(6.8 ± 1.3) % versus (15.3 ± 2.9) % versus (17.8 ± 2.2) % versus (27.4 ± 3.7) %, $P < 0.05$] in the H22 model

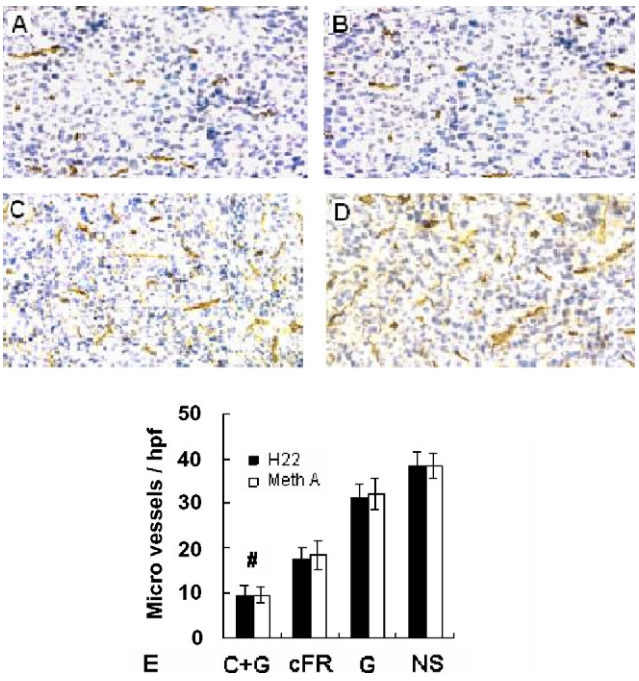

Fig. 3 – Synergistic inhibition of angiogenesis. Section of frozen Meth A model tumour tissue obtained from mice treated with the combination strategy (A), cFR-1 vaccine alone (B), gemcitabine alone (C) and untreated (D). Vessel density was determined in H22 and Meth A tumour sections stained with antibody reactive to CD31 (E), as described in Materials and methods. # Synergistic index > 1.

(Fig. 3 A–E). Tumour cell apoptosis(Fig. 4A)was significantly higher in the combination group [(31.3 ± 3.3) %] than that of cFR-1 group [(13.8 ± 2.0) %], gemcitabine group [(14.7 ± 2.3) %], and control group [(2.4 ± 0.8) %] ($P < 0.05$ for all). Proliferation index (Fig. 4B) was significantly higher in the combination group than in cFR-1, gemcitabine, and control group (6.8 ± 1.3 versus 15.3 ± 2.9, 17.8 ± 2.5, 27.4 ± 3.6, $P < 0.05$). About 33 days after tumour cell transplantation, the synergistic indexes of tumour volume, MVD, cell apoptosis and proliferation in the combination therapy group respectively were 1.08 versus 1.28 versus 1.18 and 1.15 in H22 model (Table 1). The similar findings were confirmed in the Meth A model.

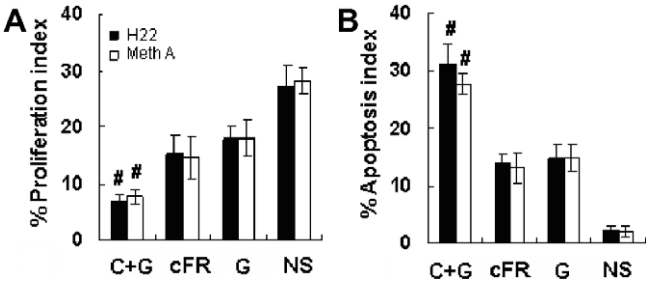

Fig. 4 – Synergistic induction of apoptosis and suppression of proliferation. Section of frozen Meth A model tumour tissues were stained for apoptosis (A) by TUNEL and for proliferation (B) by PCNA. #Synergistic index > 1.

3.4. *Without overt toxicity*

In the second part, potential toxicity in gross measures was not observed in either the combination or single agent groups. The gross measures include such things as ruffling of fur, behaviour, body weight and life span. In addition, no pathologic changes in liver, lung, kidney or heart tissue sections, which were stained with haematoxylin and eosin (HE), were observed by microscopic examination. Furthermore, no detectable toxicity of bone marrow, kidney or liver was found by complete blood count and enzyme analysis (data not shown).

4. Discussion

Angiogenesis is critical to the growth and metastasis of tumour. Due to the genetic stability and accessibility to systemically delivered therapeutic agents, endothelial cells that line tumour blood vessels are attractive targets for anticancer therapy.[2,3] Since FGFR-1 is an important molecule of angiogenesis in solid tumour, as described previously, it is thus conceivable that the breaking of immune tolerance against FGFR-1-involved angiogenesis in solid tumours should be used as a useful and new approach for cancer therapy with active immunity. Sequence comparison analysis shows that chicken FGFR-1 is about 80% identical with mouse FGFR-1 at the amino acid level. Our previously studies have demonstrated that active immunotherapy with xenogeneic homologous molecules-involved angiogenesis can suppress tumour growth in animal models.

Although anti-angiogenic therapy, which targets various stages of angiogenesis, provides a particular opportunity to arrest tumour growth and prevent metastasis and has proven to be effective at inhibiting tumour growth in many preclinical studies, it remains uncertain whether it is tumouricidal. Many studies have concluded that this therapeutic limitation may be overcome by using a combination of angiogenic inhibitors with different cytotoxic agents such as cisplatin, gemcitabine and vinflunine, etc.[15–17] Thus, these strategies of combining chemotherapeutic drugs with anti-angiogenic biotherapy show potential and promise for anticancer therapy.

Both acquired drug resistance and considerable systemic toxicities are major reasons for the limited advances made in cancer chemotherapy and have resulted in the failure of treatments. Gemcitabine is a new deoxycytidine analogue that has been widely applied in preclinical and clinical anticancer therapy. Many studies have also confirmed that the combination of low-dose gemcitabine with anti-angiogenic biotherapy strategies can suppresses tumour growth more effectively than conventional chemotherapy or anti-angiogenic biotherapy alone, including reversal of acquired drug resistance and minimisation or elimination of systemic toxicity.[19–22] The purpose of our study was to evaluate the anticancer efficacy of cFR-1 protein vaccine combined with low-dose gemcitabine and the potential toxicity of the treatments in two animal models.

Our present studies show that the mechanism responsible for the interaction between cFR-1 vaccine and low-dose gemcitabine alone therapy may involve a synergistic anti-angiogenic effect and synergistic apoptosis of both tumour and endothelial cells. On one hand, the immunotherapy with cFR-1 vaccine could induce special anticancer immunity reaction through inducing the production of auto-antibodies against self-FGFR-1, which was confirmed by Western blot analysis and ELISPOT assay, as proved in our study previously. On the other hand, low-dose gemcitabine therapy, being cytotoxic agents, could interfere with DNA synthesis and induce DNA breakage, thus resulting in tumour cell apoptosis.[20–22,25] In addition, gemcitabine, by inhibiting the enzyme, can reduce the synthesis of deoxyctidine and thereby inhibit the synthesis of tumour DNA and inhibit the growth of tumour.[26,27] Moreover, our data clearly demonstrate that cFR-1 vaccine and low-dose gemcitabine resulted in more effective inhibition of tumor growth, not only by induction of more effective anti-angiogenesis, but also by promotion of apoptosis and up-regulation of the suppression of cell proliferation in tumor tissues as compared with either therapy alone or with untreated groups, without obvious side-effects. Furthermore, our data also indicate that the combination therapy strategy could potentiate each other's anticancer effects as was demonstrated in the synergistic indexes of tumour volume, MVD, apoptosis and proliferation. At the same time, the potential toxicity of the treatments in the second part of the animal study did not find any significant side effects in the different groups. These results may provide a paradigm by which the combination strategy attains the same treated effects as conventional chemotherapy without the systemic toxicity.

In conclusion, our findings demonstrate that the combination strategy effectively enhances anti-cancer effect via inhibition of tumour angiogenesis without systemic toxicity in mice.

Conflict of interest statement

None declared.

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