BMC Cancer

Research article

Dynamic distribution and expression in vivo of the human interferon gamma gene delivered by adenoviral vector

Jiangxue Wu1, Xia Xiao1, Hongyun Jia1,3, Jiemin Chen1, Yinghui Zhu1, Peng Zhao1, Huanxin Lin1 and Wenlin Huang*1,2

Address: 1State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-sen University, Guangzhou 510060, PR China, 2Institute of Microbiology, Chinese Academy of Science, Beijing 100080, PR China and 3College of fundamental Medical Science, Guangzhou University of Chinese Medicine, Guangzhou 510006, PR China

Email: Jiangxue Wu - gladysw@163.com; Xia Xiao - xiaoyue7604@yahoo.com.cn; Hongyun Jia - hongyun_jia@163.com; Jiemin Chen - cjmhl@163.com; Yinghui Zhu - hey_pigpig@hotmail.com; Peng Zhao - Peng_zhao007@126.com; Huanxin Lin - linhuanxin_888@126.com; Wenlin Huang* - hwenl@mail.sysu.edu.cn

* Corresponding author

Abstract

Background: We previously found that r-hu-IFNγ exerts a potent anti-tumor effect on human nasopharyngeal carcinoma xenografts in vivo. Considering the fact that the clinical use of recombinant IFNγ is limited by its short half-life and systemic side effects, we developed a recombinant adenovirus, Ad-IFNγ.

Methods: Dynamic distribution of the adenovirus vector and expression of IFNγ were evaluated by Q-PCR and ELISA after intratumoral administration of Ad-IFNγ into CNE-2 xenografts.

Results: Ad-IFNγ DNA was mainly enriched in tumors where the Ad-IFNγ DNA was injected (P < 0.05, compared to blood or parenchymal organs), as well as in livers (P < 0.05). Concentrations of Ad-IFNγ DNA in other organs and blood were very low. Intratumoral Ad-IFNγ DNA decreased sharply at high concentrations (9 × 10^5 copies/μg tissue DNA), and slowly at lower concentrations (1.7–2.9 × 10^5 copies/μg tissue DNA). IFNγ was detected in the tumors and parenchymal organs. The concentration of IFNγ was highest in the tumor (P < 0.05), followed by the liver and kidney (P < 0.05). High-level intratumoral expression of IFNγ was maintained for at least 7 days, rapidly peaking on day 3 after injection of Ad-IFNγ DNA.

Conclusion: An IFNγ gene delivered by an adenoviral vector achieved high and consistent intratumoral expression. Disseminated Ad-IFNγ DNA and the transgene product were mainly enriched in the liver.

Background

IFNγ, a homodimeric cytokine produced by T lymphocytes and natural killer cells, belongs to a family of glycoproteins that have antiviral, immunomodulatory, and anti-proliferative effects. The anti-tumor activities of IFNγ have been shown in various kinds of tumors, such as lymphomas, melanomas, and metastatic renal cell carcinomas [1-4]. Nasopharyngeal carcinoma (NPC) is a malignant disease of the head/neck region that is endemic in Southeast Asia [5]. We have reported that r-hu-IFNγ...
treatment exerts anti-proliferative effects in vitro on NPC cells, and leads to a profound anti-tumor effect in vitro [6]. However, although IFNγ has shown some promise in preclinical and clinical anticancer studies, it possesses properties that limit its clinical use.

High serum concentrations of IFNγ yield significant side effects and toxicities. These side effects include fever, fatigue, nausea, vomiting, neurotoxicity, and leukopenia. In addition, IFNγ has a short half-life of elimination, whether given intravenously, subcutaneously, or intramuscularly, and thus requires relatively frequent readministration. These limitations have prompted the investigation of alternate modes of delivering this cytokine to achieve better therapeutic outcomes while minimizing toxicity [1]. Studies have revealed that the most efficient and least toxic delivery system for IFNγ is a localized, sustained release dosage form [1,3,4]. Therefore, gene therapy with intratumorally injected recombinant adenoviral vectors is a promising approach because it offers the potential to achieve consistent therapeutic gene expression in the tumor [3,4].

In the present study, we developed a recombinant adenovirus, Ad-IFNγ, encoding human interferon gamma. To determine whether intratumoral administration of Ad-IFNγ could achieve localized sustained expression of IFNγ, we sought to characterize the biodistribution of the adenoviral vector and transgene expression in the tumor, blood, liver, kidney, heart, spleen, and lung at days 1, 2, 3, 5, 7, 14, and 21 after injection. Results indicated that the IFNγ gene delivered by adenoviral vector achieved high intratumoral expression, lasting for at least 7 days after injection of Ad-IFNγ. Disseminated Ad-IFNγ DNA and the transgene product were mainly enriched in the liver.

**Methods**

**Adenoviral vectors**

E1-deleted, replication-deficient adenoviral vectors based on human adenovirus serotype Ad5 were used to produce the non-replicating adenovirus, Ad-IFNγ, encoding human interferon gamma [7]. The cDNA for IFNγ was inserted into the E1 region of the adenoviral genome with transgene expression driven by the cytomegalovirus (CMV) promoter. Adenoviruses were propagated in 293 cells (American Type Culture Collection, Manassas, VA, USA), harvested at 48 h after infection, and purified by cesium chloride gradient centrifugation according to a standard protocol [8], resulting in a particle:pfu ratio of 100:3.32. The viral vectors were stored at -80°C in virus preservation solution (10% glycerol, 0.585% NaCl, 0.02% MgCl₂·H₂O, 0.01 M Tris-HCl, pH 8.4) at a concentration of 1 × 10¹² VP/ml.

**Animals**

Female BALB/c nude mice (4–6 weeks old) were obtained from the Experimental Animal Center (Sun Yat-sen University, Guangzhou, China) and maintained in a specific pathogen-free (SPF) environment. After 1 week of adaptation, mice were inoculated subcutaneously in the scapular region with 2 × 10⁶ CNE-2 cells to generate tumors for subsequent experiments. When tumors reached 5–8 mm in diameter, mice were randomly assigned to groups (3 mice for each time point). Ad-IFNγ (diluted with 0.9% NaCl to 100 μl) or vehicle was delivered into the center of the tumor via a single injection with a 30-gauge needle. The dose used per injection was 1 × 10¹⁰ VP/tumor. Samples of blood, tumor, heart, liver, spleen, lung, kidney, and brain were harvested at the indicated time points after virus injection, and stored in aliquots at -80°C for analysis. All the animal experiments were conducted in accordance with “Guidelines for the Welfare of Animals in Experimental Neoplasia”.

**Quantitative analysis of adenovirus distribution**

Tissue samples (about 50 mg for tissue) were immersed in liquid nitrogen and ground into fine tissue powder in a mortar. Viral DNA was isolated from the tissue powder and whole blood (about 100 μl for blood) using a Total DNA Extraction Kit (TIANGEN, Beijing, China).

**Quantitative PCR**

Adenovirus copies in the samples were measured by hexon DNA PCR [9]. Primer and probe sequences were: hexon forward primer: 5’-CTTCGATGTGCCGACTGTCG-3’; hexon reverse primer: 5’-GGGCTCAGGTACTCCGAGG-3’; hexon probe: 5’-FAM-TTACATGCACATCTCGGGCCAG-GAC-TAMRA-3’. Amplification was performed in a 50 μl reaction volume under the following conditions: 10 ng of sample DNA, 1× Taqman Universal PCR Master Mix, 600 nM forward primer, 900 nM reverse primer, and 100 nM hexon probe. Thermal cycling conditions were: 2 min of incubation at 50°C, 10 min at 95°C, followed by 35 cycles of successive incubations at 95°C for 15 sec and 60°C for 1 min [9]. Quantification of adenovirus copy number was performed using a standard curve consisting of dilutions of adenovirus DNA from 1,500,000 to 15 copies in 10 ng of cellular genomic DNA.

**Quantitative analysis of IFN distribution**

After being immersed in liquid nitrogen and ground into fine tissue powder in a mortar, tissue samples (about 50 mg) were ground with PBS containing Protease Inhibitor Cocktail II (Upstate, Lake Placid, NY, USA). After centrifugation, the supernatants were extracted and stored at -80°C. IFNγ concentrations in the tumor, organ extracts, and plasma samples (about 100 μl) were detected with a human IFNγ ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s recommendations.
Ad-IFN\(\gamma\) concentrations in the tumor, heart, liver, spleen, lung, kidney, brain, and blood at different time points after intratumoral injection of Ad-IFN\(\gamma\). The dose of injection was 1 \(\times\) 10\(^{10}\) VP/tumor. Data represent mean ± SD of three mice. Representative results from two independent experiments are shown.
The results were then converted to the concentrations of IFNg in the different tissues based on the sample volume or weight. Sensitivity of the kit was up to 16 pg/ml.

**Statistical Analysis**
Results were evaluated using a t-test by SPSS 11.0 software (SSPS Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

**Results**

**Systemic distribution of recombinant human IFN adenovirus**

1 × 10^{10} VP of Ad-IFNg was injected into CNE-2 tumors. At the indicated time points post-injection, samples of tumor, parenchymal organs, and blood were harvested. To demonstrate adenovirus distribution, fluorescent real-time quantitative PCR was used to quantify viral copies in the samples.

Samples from tumor, heart, liver, spleen, lung, kidney, brain, and blood on days 1, 2, 3, 5, 7, 14, and 21 were tested for the amount of targeted DNA. The copy number of the targeted DNA in samples was deduced according to the standard curves. As shown in Fig. 1, Ad-IFNg DNA was detected in tumor, blood, and parenchymal organs. Ad-IFNg DNA was mainly enriched in tumors where the Ad-IFNg DNA was injected (P < 0.05, compared to blood or parenchymal organs), then in livers (P < 0.05, compared to blood or other parenchymal organs). Concentrations of Ad-IFNg DNA were very low in other organs and blood (Fig. 1). Ad-IFNg DNA persisted for at least 7 days in the tumors. Intratumoral Ad-IFNg DNA decreased sharply at high concentrations (9 × 10^5 copies/μg tissue DNA), and slowly at lower concentrations (1.7–2.9 × 10^5 copies/μg tissue DNA) (Table 1). Ad-IFNg DNA in livers also decreased in a time-dependent manner and became undetectable at day 14 after intratumoral administration (Table 1).

|                  | tumor (copies/μg tissue DNA) | liver (copies/μg tissue DNA) |
|------------------|-----------------------------|-----------------------------|
| day 1            | 897927.2 ± 188201.2         | 206274.7 ± 67269.5          |
| day 2            | 645995.1 ± 76137.4          | 168531.6 ± 46526.4          |
| day 3            | 289441.2 ± 75017.3          | 109217.2 ± 5478.3           |
| day 5            | 197677.1 ± 220320.1         | 90712.8 ± 68176.5           |
| day 7            | 169882.6 ± 69067.4          | 11465.8 ± 2383.5            |
| day 14           | 0                           | 0                           |
| day 21           | 0                           | 0                           |

The dose of injection was 1 × 10^{10} VP/tumor. Data represent mean ± SD of three mice. Representative results from two independent experiments are shown.

Discussion

Nasopharyngeal carcinoma is a major malignant disease of the head and neck region that is endemic to Southeast Asia and the Mediterranean basin. NPC affects a predominantly young population and the current treatment regimen of radiation therapy, even combined with cisplatin chemotherapy, yields a 5-year survival rate of approximately 70%. Therefore, evaluation and development of novel therapeutic approaches are critical [9-14].

Our study revealed that r-hu-IFNg treatment is a promising novel approach for the treatment of NPC. However, the clinical application of recombinant IFNg is limited by the short half-life and the systemic side effects experienced by patients. Therefore, continuous localized release of IFNg is a desirable goal. To overcome these difficulties, we developed a recombinant adenovirus, Ad-IFNg. Our results suggested that high and sustained expression of IFNg could be achieved in CNE-2 xenografts, after intratumoral injec-
Figure 2
Human IFNγ concentrations in the tumor, heart, liver, spleen, lung, kidney, and brain at different time points after intratumoral injection of Ad-IFNγ. The dose of injection was $1 \times 10^{10}$ VP/tumor. Data represent mean ± SD of three mice. Representative results from two independent experiments are shown.
tion of Ad-IFNγ (Fig. 2). Consistent with the anti-tumor effects of r-hu-IFNγ, Ad-IFNγ produced a significant dose-dependent inhibition of tumor growth in CNE-2 models. Intratumoral administration of Ad-IFNγ at doses of 1 × 109 and 5 × 108 pfu/week for three weeks led to inhibition rates of 67.9% and 58.64%, respectively [see Additional file 1, Zhu YH, et al., unpublished data].

Intratumoral injection is an advantageous method for local viral gene delivery [15,16]. Researchers often assume that viral vectors and transgene expression are confined to solid tumors after the infusion, thereby causing minimal toxicity in normal tissues. However, the dissemination of the viral vector and its encoded gene product could be a serious impediment for vectors that encode cytokine genes, such as IFNγ, that are not only expressed in situ, but also secreted into the system and have considerable normal tissue toxicity [17-20]. Therefore, we investigated transgene expression in the blood and major organs in this study.

We found that there was potential dissemination of viral DNA and transgene product into parenchymal organs. A significant amount of IFNγ was detected in the liver and kidney, which indicated that the clearance of IFNγ in vivo is through hepatic metabolism and glomerular filtration. Our results also suggested that most of the disseminated viral DNA was enriched in the liver, which is consistent with reports of efficient uptake of adenovirus by the liver. There was a minimal amount of viral DNA in the blood. One possible explanation for this is that the number of infectious viruses present in the blood was significantly reduced through rapid uptake of the virus by Kupffer cells in the liver [21,22].

Studies have revealed that dissemination of transgene products in normal organs, such as in the liver, may exceed normal tissue tolerance if the products are highly toxic [19]. Toxicological studies conducted in this lab have indicated that intratumoral injection of Ad-IFNγ doesn't induce significant toxic effects in mice [unpublished data]. Therefore, adenovirus-mediated intratumoral delivery of the IFNγ gene is a promising approach because it achieves high and sustained local IFNγ expression in the tumor.

Conclusion
Adenovirus-mediated intratumoral delivery of the IFNγ gene is a promising approach because it achieves high and sustained local IFNγ expression in the tumor. Disseminated Ad-IFNγ DNA and the transgene product were mainly enriched in the liver.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JXW, XX, HYJ, JMC, YHZ, PZ and HXL participated in data acquisition. WLH supervised and participated with JXW in the design and coordination of the study. JXW drafted and wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Antitumor activity of Ad-IFN on CNE-2 xenografts. The data provided represent the antitumor activity of Ad-IFN on CNE-2 xenografts. Female athymic nude mice were inoculated s.c. in the scapular region with 2 × 106 CNE-2 cells in 100 l sterile PBS. When tumors reached a volume of 30 to 40 mm3, animals were randomly assigned into 6 experimental groups of 6-7 animals: Ad-IFN (1 × 109, 5 × 108 or 1 × 107 pfu/week), 1 × 107 pfu/week of Ad-LacZ, 1 × 106 IU/kg/d of r-hu-IFN or PBS alone was intratumorally injected. Mice were killed after 3 weeks of treatment and tumors were resected and weighted. Columns, average weight of tumor from 6–7 mice; bars, SD. *, p < 0.05, compared with the PBS-treated and the Ad-LacZ group.
Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2407-9-55-S1.tif]

Acknowledgements
This work was supported by grants from the State Key Development Program of Basic Research of China (973 Program, No. 2004CB518801), the National High Technology Research and Development Program of China (863 Program, No. 2007AA021202 and 2007AA021203), the National Natural Science Foundation of China (30801360), and Science Funds for Young Scholar of Cancer center of Sun Yat-sen University.

References
1. Younes HM, Amsden BG: Interferon-gamma therapy: evaluation of routes of administration and delivery systems. J Pharm Sci 2002, 91(1):2-17.
2. Pang KR, Wu JI, Huang DB, Tyring SK, Baron S: Biological and clinical basis for molecular studies of interferons. Methods Mol Med 2005, 116:1-23.
3. Liu M, Acres B, Bailoul JM, Bizouarne N, Paul S, Slos P, et al.: Gene-based vaccines and immunotherapeutics. Proc Natl Acad Sci U S A 2004, 101 Suppl 2:14567-14571.
4. Chada S, Ramesh R, Mhashilkar AM: Cytokine- and chemokine-based gene therapy for cancer. Curr Opin Mol Ther 2003, 5(5):463-474.
5. Chan AT, Teo PM, Huang DP: Pathogenesis and treatment of nasopharyngeal carcinoma. Semin Oncol 2004, 31(6):794-801.
6. Wu JX, Xiao X, Zhao P, Xue G, Zhu YH, Zhu XF, et al.: Minicircle-IFNα: Induces Anti-proliferative and Anti-tumoral Effects in Human Nasopharyngeal Carcinoma. Clinical Cancer Res 2006, 12:4702-13.
7. Zhao P, Zhu Y, Wu JI, Liu R, Zhu X, Xiao X, et al.: Adenovirus-mediated delivery of human IFN-gamma gene inhibits prostate cancer growth. Life Sci 2007, 81(9):695-701.
8. Graham FL, Prevec L: Methods for construction of adenovirus vectors. Mol Biotechnol 1995, 3(3):207-220.
9. Spano JP, Busson P, Atlan D, Bourhis J, Pignon JP, Escobar C: Nasopharyngeal carcinomas: an update. Eur J Cancer 2003, 39(15):2121-2135.
10. Young LS, Murray PG: Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene 2003, 22(33):5108-5121.
11. Wakisaka N, Pagano JS: Epstein-Barr virus induces invasion and metastasis factors. Anticancer Res 2003, 23(2133-2138).
12. Weinrib L, Li JH, Donovan J, Huang D, Liu FF: Cisplatin chemotherapy plus adenoviral p53 gene therapy in EBV-positive and -negative nasopharyngeal carcinoma. Cancer Gene Ther 2001, 8(3):352-360.

13. Li JH, Lax SA, Kim J, Klamut H, Liu FF: The effects of combining ionizing radiation and adenoviral p53 therapy in nasopharyngeal carcinoma. Int J Radiat Oncol Biol Phys 1999, 43(3):607-616.

14. Li JH, Shi W, Chia M, Sanchez-Sweatman O, Siatskas C, Huang D, et al.: Efficacy of targeted FasL in nasopharyngeal carcinoma. Mol Ther 2003, 8(6):964-973.

15. Lu Y, Carrarah J, Zhang Y, Armstrong J, Lerner J, Rogers WP, et al.: Delivery of adenoviral vectors to the prostate for gene therapy. Cancer Gene Ther 1999, 6:64-72.

16. Rein DT, Breidenbach M, Curie1 DT: Current developments in adenovirus-based cancer gene therapy. Future Oncol 2006, 2(1):137-143.

17. Lohr F, Huang Q, Hu K, Dewhirst MW, Li CY: Systemic vector leakage and transgene expression by intratumorally injected recombinant adenovirus vectors. Clin Cancer Res 2001, 7(11):3623-3628.

18. Paielli DL, Wing MS, Rogulski KR, Gilbert JD, Kolozsvary A, Kim JH, et al.: Evaluation of the biodistribution, persistence, toxicity, and potential of germ-line transmission of a replication-competent human adenovirus following intraprostatic administration in the mouse. Mol Ther 2000, 1(3):263-274.

19. Wang Y, Hu JK, Krol A, Li YP, Li CY, Yuan F: Systemic dissemination of viral vectors during intratumoral injection. Mol Cancer Ther 2003, 2(11):1233-1242.

20. Wang Y, Yang Z, Liu S, Kon T, Krol A, Li CY, et al.: Characterisation of systemic dissemination of nonreplicating adenoviral vectors from tumours in local gene delivery. Br J Cancer 2005, 92(6):1414-1420.

21. Xu Z, Tian J, Smith JS, Byrnes AP. Clearance of adenovirus by Kupffer cells is mediated by scavenger receptors, natural antibodies and complement. J Virol 2008, 82(23):11705-11713.

22. Smith JS, Xu Z, Tian J, Stevenson SC, Byrnes AP. Interaction of systematically delivered adenovirus vectors with Kupffer cells in mouse liver. Hum Gene Ther 2008, 19(5):547-54.

Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/9/55/prepub