Intratumoral injection of pEGFC1-IGFBP7 inhibits malignant melanoma growth in C57BL/6J mice by inducing apoptosis and down-regulating VEGF expression

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Abstract. Malignant melanoma (MM) is a type of aggressive skin cancer, and the effective therapy for MM is highly desired. Recently, genome-wide RNA interference screening study revealed that loss of expression of insulin-like growth factor binding protein 7 (IGFBP-7) is a critical step in development of MM, and this secreted protein plays a central role in apoptosis of MM. Furthermore, a prostatic carcinoma cell line stably transfected with IGFBP-7 cDNA showed poor tumorigenicity. Thus, we supposed it to be an efficacious agent for inhibiting melanomas. In this study, we constructed pEGFC1-IGFBP7 to try to obtain high expression of IGFBP7 and then we demonstrated that this plasmid inhibited proliferation of B16-F10 melanoma cells efficiently in vitro. Moreover, intratumoral injection of pEGFC1-IGFBP7 inhibited MM growth in C57BL/6J mice. The inhibition of MM growth is due to apoptosis and reduced expression of VEGF induced by pEGFC1-IGFBP7. These results suggest a potential new clinical strategy for MM treatment.

Introduction

Malignant melanoma is responsible for 80% of skin cancer deaths, and its incidence is increasing. Although the treatments such as surgery, chemotherapy and radiotherapy treatment are ongoing, the 5-year survival rate of MM patients at late-stage is only 10-20% because of the resistance to radiotherapy and chemotherapy (1-5). Therefore, a new effective therapy for MM is highly desired. Gene therapy has already been revealed to have potential in development of molecular biology. In previous studies, we demonstrated that the synthesis of vascular endothelial growth factor (VEGF) and growth of MM in xenograf models (3), were significantly inhibited by using small-interfering RNA (siRNA), which makes us believe that the modulation of aberrant signaling pathways in MM cells will probably provide more effective and potential nontoxic therapy for MM. However, this approach still has its shortcomings, in that VEGF is one of the downstream target genes of insulin-like growth factor (IGF), which is important in promoting tumor angiogenesis (6-9). Although pU-VEGF-siRNA directly inhibited MM cell proliferation by reducing VEGF expression, it could not induce valid apoptosis.

Immunohistochemical analysis of human skin, nevi, and melanoma samples implicates loss of IGFBP-7 expression as a critical step in melanoma carcinogenicity (10). Thus, the relationship between IGF axis and carcinogenesis has become the focus of research attention. The IGF system is composed of IGFs, IGF receptors, and insulin-like growth factor-binding proteins (IGFBPs). IGFBP-7 belongs to the IGFBP superfamily, also known as IGFBP-related protein 1 (IGFBP-rP1) or mac25, is a member of soluble protein family that binds IGFs with low affinity, and is expressed in a wide range of tissues (11,12).

In vitro studies demonstrated that IGFBP-7 induced apoptosis of many cancer cells (13,14), e.g., breast and prostate cancer cells, and plays a potential tumor suppressor role against colorectal carcinogenesis. Wajapeyee et al (10) showed that recombinant IGFBP-7 (rIGFBP-7) can efficiently induce apoptosis in human melanoma cell lines. These exciting data suggest that IGFBP-7 may be an efficacious anticancer agent for MM, since experiments have provided evidence that IGFBPs have both IGF-dependent and -independent antitumoral actions (14,15).

Previous data also demonstrated that a prostate carcinoma cell line stably transfected with IGFBP-7 cDNA showed poor
tumorigenicity both in vitro and in vivo (12). Meanwhile, in our previous study, we found that IGFBP-7 expression was low in B16-F10 cells (data not shown). However, it is still unclear whether IGFBP-7 cDNA inhibits proliferation of B16-F10 cells in vitro or B16-F10 MM growth in vivo. Therefore, in this study, we constructed the pEGFC1-IGFBP7 plasmid as an antitumor vaccine to investigate whether it is effective in treating mice bearing B16-F10 melanoma tumors.

Materials and methods

Plasmid construction. The pEGFC1-IGFBP7 expression plasmid was constructed. IGFBP-7 gene (GenBank ID: 29817 No. AK156315.1) was amplified by RT-PCR from mRNA of splenocytes derived from C57BL/6J mice (IGFBP7 forward: 5'GAAGATCTATGGAGCGGCCGTCGCT-3', IGFBP7 reverse: 5'-CGGAATTCTTTATAGCTCGGCACCTTCA CCT-3'). IGFBP-7 cDNA was purified by Shanghai Biological Engineering Company. The eukaryotic vector expressing eGFP and IGFBP-7 was termed pE-IGFBP7, and pE-CONTROL only expressed eGFP. The inserted sequences were verified by DNA sequencing (ABI PRISM 310; CA, USA), and digested by restriction endonuclease (EcoRI, and BgIII enzyme). Escherichia coli DH5α were routinely cultured in LB (Luria-Bertani) medium with antibiotics ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

Cell cultures and in vitro transfected with pEGFC1-IGFBP7. B16-F10 cells were purchased from the Institute of Cell Biology, Shanghai Institute for Biological Sciences. Cells were seeded in six-well plates (2x10⁵ cells per well), cultured overnight in DMEM supplemented with 10% FBS at 37˚C and 5% CO2, then grown to 60% confluent prior to transfection. Subsequently, pE-IGFBP7, and pE-CONTROL were transfected using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Cells transfected with pE-CONTROL and parental cells served as controls. The experimental and two control groups were termed pE-IGFBP7, pE-CONTROL and B16-F10 cells, respectively.

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA from 1x10⁶ cultured cells was extracted using the TRizol reagent (Invitrogen, San Diego, USA). Then RNA was reversely transcribed and synthesized to cDNA using reverse transcriptase (Rever TraAce-AC II, Toyobo, Japan). Primer sequences for IGFBP-7 (forward: 5'-GGGAAGAGGGAGGACGCTGAGGT-3', reverse: 5'-CTGCTTGTCATAAAAGTGT TAGTGG-3') and β-actin (forward: 5'-CCGGTGAAAAATGT ACCCGATCC-3', reverse: 5'-TAGCACCAGCTGGTACCGG-3'). PCR and gelelectrophoresis conditions were as described previously (3). The expected fragment of IGFBP-7 and β-actin was 255 bp, 136 bp, respectively. PCR products were analysed by an image analysis system (Q550i W; Leica, Heidelberg, Germany).

Cell viability analysis. Cell viability was determined by the cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) and measured by microplate reader scanning at 450 nm as previously described (16).
ascertaining expression of IGFBP-7 in the cytoplasm. Negative controls used PBS as primary antibody, and positive controls used mouse thymus tissue sections which had confirmed positive expression of IGFBP-7.

The expression of caspase-3 and VEGF visualization is based on enzymatic conversion of a chromogenic substrate 3-amino-9-ethylcarbazole (AEC) into a colored red precipitate by horseradish peroxidase (HRP) at the sites of antigen localization, and nuclei were counterstained with haematoxylin. No significant difference in intensity of immunohistochemical staining was designated as negative, 0; positive, 1; strong positive, 2; and the percentage of positive cells was scored as <5%, 0; 5-25%, 1; 26-50%, 2; 51-75%, 3; or >75%, 4; of cells stained (21). Values in the parentheses were multiplied together to the scores for IGFBP-7, caspase-3, VEGF expression.

Detection of tumor apoptosis. Tumor apoptosis was detected using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL, Catalog # 11684809910, Roche, Germany) according to the supplier’s instructions, and apoptosis index (AI) was used to evaluate the results.

Statistical analysis. The statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA).
Statistical comparisons of mean values were performed using Student's t-test and Kruskal-Wallis test, the correlations was analyzed by Spearman's rho correlation analysis. All P-values were determined from two-sided tests. A significance criterion of P<0.05 was used in these studies.

Results

Identification of pE-IGFBP7 plasmid. To determine whether increasing IGFBP-7 mRNA expression induces apoptosis of B16-F10 cells, we constructed pE-IGFBP7. The sequence analysis of constructed pE-IGFBP7 by a DNA sequencer showed the same sequence of eukaryotic IGFBP7 mRNA as designed. Meanwhile, recombinant pE-IGFBP7 plasmid was confirmed by restriction enzyme analysis. These results indicated that the pE-IGFBP7 vector was constructed successfully. Then pE-IGFBP7 and pE-CONTROL were transfected into cells by Effectene Transfection Reagent, termed pE-IGFBP7 cells and pE-CONTROL cells, respectively, with transfection rate being ~60%.

Effect of pE-IGFBP7 plasmid on IGFBP-7 mRNA expression. As seen in Fig. 1A and B, IGFBP7 mRNA level with in vitro plasmid transfection was increased 1 to 6 times the original level in a dose-dependent manner. Plasmid (2 μg) induced the highest IGFBP7 expression, but it also caused considerable cell necrosis, which was not conducive for observation of time-course experiment. To avoid this, we selected 1 μg as the optimal amount of plasmid for transfection. According to the time-course experiments, it was found that the IGFBP-7 mRNA levels in pE-IGFBP7-transfected B16 cells were increased by ~4-, 8-, 7-, 6-fold on days 1, 3, 6 and 12, respectively, compared with the control group (Fig. 1C). However, no change of IGFBP7 expression in pE-CONTROL groups (Fig. 1C) (P>0.05) was found, suggesting that pE-IGFBP7 vector specifically promotes expression of IGFBP7, without affecting β-actin mRNA.

pE-IGFBP7 suppresses B16-F10 cells growth in vitro. Fig. 1D shows that the proliferation of pE-IGFBP7-transfected cells was significantly suppressed compared with control cells (P<0.01). The highest suppression effect of pE-IGFBP7 was found at 48 h post-transfection, and no significant difference in proliferation between pE-CONTROL and untransfected cells was observed (P>0.05), indicating that transfection of pE-IGFBP7 blocks the proliferation of B16-F10 cells by increasing IGFBP-7 synthesis and secretion.

Quantification of B16-F10 cell apoptosis by flow cytometry. To evaluate apoptosis-induced effect of pE-IGFBP7 in melanoma cells, B16-F10 cells at 48 h post-transfection was monitored by FCM. Fig. 1E shows that the apoptosis rate in pE-IGFBP7 group (26%) was significantly higher than that in control groups (P<0.01). However, no marked apoptosis was observed in pE-CONTROL (6.2%) and B16-F10 (5.3%). Our finding mentioned above indicated that the long-term IGFBP7 expression possibly establishes a desirable basis for the therapeutic effect in vitro.

Effect of pE-IGFBP7 on IGFBP-7 expression and growth of MM homeograft in vivo. To evaluate the therapeutic potential of pE-IGFBP7 on B16-F10 MM homeograft in vivo, we carried out plasmid in vivo intratumoral injection. Tumor growth curves showed that pE-IGFBP7 inhibited tumor growth. As shown in Fig. 2A and B at the time of sacrifice, the volumes of MM in B16-F10 cell group and pE-CONTROL group were 587 ± 35 mm³ and 566 ± 34 mm³, respectively, being ~8 times the starting volume. Whereas the volumes of
B16-F10 tumors injected with pE-IGFBP7 were 256±25 mm³, with the volume increase being only 2.8-fold. The delay in tumor growth was statistically significant (P<0.001) from day 5 after the beginning of therapy until the day the mice were sacrificed. To evaluate the expression of IGFBP-7 in tumor homeograft, the proteins were determined by Western blotting. As shown in Fig. 2C, IGFBP-7 expression in the pE-IGFBP7 group was significantly higher than in pE-CONTROL and B16-F10 cell groups (P<0.01), whereas there was no significant difference in IGFBP-7 expression between pE-CONTROL and B16-F10 cells groups (P>0.05). These results are in agreement with those determined by immuno-histochemistry (Fig. 3A-C).

**Effect of pEGFC1-IGFBP7 on IGFBP-7, caspase-3, VEGF and apoptosis expression in vivo.** We studied IGFBP-7, caspase-3, VEGF expression and apoptosis in tumor homeograft tissues. As shown in Fig. 3, IGFBP7, caspase-3 and VEGF were mainly expressed in the cytoplasm of tumor cells. IGFBP-7 was determined by fluorescent immuno-
hypothesis, as shown in Fig. 3A-F that IGFBP-7 and caspase-3 in the pE-IGFBP7 group are significantly higher than in the pE-CONTROL and B16-F10 cell groups (IGFBP-7, P<0.002; caspase-3, P<0.004), but VEGF expression in the pE-IGFBP7 group is significantly lower than both in the pE-CONTROL and B16-F10 cell groups (P<0.006) (Fig. 3G-I) respectively, and no significant difference in IGFBP-7, caspase-3 and VEGF expression were found between the pE-CONTROL and B16-F10 cell groups (P>0.05). According to these results determined by immunohistochemistry, there would be significantly more apoptotic cells in the pE-IGFBP7 group than in the pE-CONTROL and B16-F10 cell groups (P<0.031).

As shown in Fig. 3J-L, morphological characteristics of apoptotic cells include cell shrinkage, deformation and loss of contact with neighbouring cells. Fig. 3J shows more apoptotic cells in the pE-IGFBP7 group than in the pE-CONTROL (Fig. 3K), and B16-F10 cell groups (Fig. 3L), which contained almost the same numbers of apoptotic cells. The expression of IGFBP-7 is positively correlated with caspase-3 and cell apoptosis rate (r=0.704, r=0.806, respectively; P<0.01). However, there is negative correlation between IGFBP-7 and VEGF (r=-0.564, P<0.01). These results suggest that pE-IGFBP7 inhibited the proliferation of MM cells by up-regulating IGFBP7 and caspase-3 expression and down-regulating VEGF expression in vitro, resulting in slowing down of MM growth.

Discussion

It has been confirmed that transfection with anti-tumor plasmids is more specific, more efficient, and longer lasting for anti-tumor therapy, than recombinant protein. Transfection of anti-tumor plasmids has some advantages over the application of rIGFBP7, namely less risk of immunological rejection and low cost of synthesis and purification (3). In addition, MM cells transfected with eukaryotic expression plasmids have stable and effective expression of IGFBP-7 gene. Our research demonstrated that pE-IGFBP7 vector could promote expression of IGFBP-7 specifically and have a long-lasting effect. However, it is conflicting to our previous hypothesis that IGFBP7 expression should elevate gradually over time, the possible explanation for this phenomenon was attributed to the high-performance P<sub>SV</sub> promoter contained in pE-IGFBP7, which would exhaust and be toxic to tumor cells since it ad infinitum synthesized IGFBP-7. Augmentation of IGFBP-7 in cell supernatant would induce apoptosis of part of tumor cells and therefore, the synthesis of IGFBP7 also decreases with reduction of tumor cells.

To determine therapeutic potential of pE-IGFBP7 in vitro, we analyzed cells viability and apoptosis rates by the Cell Counting kit-8 and FCM. We found pE-IGFBP7 blocks the proliferation of B16-F10 cells, and induces apoptosis by increasing IGFBP-7 synthesis and secretion, and no significant apoptosis were observed in control groups. Our results are in agreement with the research of Sprenger et al (14), indicating that the growth of a tumorigenic SV40 prostate cell line, M12, was suppressed by transfecting the IGFBP-rP1 cDNA. Also, prostatic carcinoma cells were stably transfected with IGFBP-7 cDNA showed poor tumorigenicity in both in vitro and in vivo (22). Moreover, IGFBP7 acts to inhibit BRAF-MEK-ERK signaling and induce apoptosis through autocrine/paracrine pathways (10). However, our findings contrast with the results of Jiang et al, who indicated that IGFBP-7 was highly overexpressed in glioma tissues, and mediating glioma cell growth and migration (23). In addition, the expression pattern of IGFBP-7 varies with tumor types. Both up-regulated (24) and down-regulated expression (25,26) of IGFBP-7 is observed in different types of cancer. Together, these results suggest that IGFBP-7 plays different roles in different tumor or host environments. Therefore, we need to evaluate the therapeutic potential of pE-IGFBP7 on B16-F10 in vivo.

Although the apoptosis-inducing effect of pE-IGFBP7 in cultured cells was shown for in vitro applications, its therapeutic applications in vivo represent an altogether more daunting challenge. To elevate transfection efficiency, we employed Invivofectamine (a new in vivo plasmid delivery reagent) to carry pE-IGFBP7 transfected into tumors tissue. Our data clearly showed that intratumoral injection of the Invivofectamine pE-IGFBP7 complex was able to slow down the growth of B16-F10 MM homograft, and its transfection efficiency was ~70%. Most importantly, it had a lasting effect on tumor development, being effective for at least 20 days, because stable expression of IGFBP7 by using pE-IGFBP7. We focused on the therapeutic mechanisms of the Invivofectamine pE-IGFBP7 complex in B16-F10 MM homograft. The antitumor research of IGFBP has provided evidence that IGFBPs may have both IGF-dependent and independent actions. We hypothesized that IGFBP7 inhibits MM growth by an IGF-dependent manner, and reduces VEGF expression through preventing IGF-1 binding to its receptors. In addition, IGFBP7 induces MM apoptosis through a novel IGF-independent pathway (15).

To confirm the presumption, we studied the expression of IGFBP-7, caspase-3, VEGF and apoptosis in tumor homograft tissues. The results of the immunohistochemistry and TUNEL showed that IGFBP-7 and caspase-3 expression in pE-IGFBP7 group are significantly higher than in pE-CONTROL and B16-F10 cells groups, but VEGF expression in the pE-IGFBP7 group are significantly lower than both control groups, and no significant difference in IGFBP-7, caspase-3 and VEGF expression were found between the pE-CONTROL and B16-F10 cell groups. According to these results, determined by immunohistochemistry, there were significantly more apoptotic cells in the pE-IGFBP7 group than in control groups. This was considered possibly to relate to the effectiveness of IGFBP7 in promoting apoptosis. However, our findings contrast with the results of Adachi et al (27), who found that high expression of IGFBP7 in invasive tumor cells was associated with poor prognosis. This discrepancy may be due to the difference in the immunohistochemical scoring (21,28). We used the composite score to evaluate the expression of IGFBP7, which seems to be one of the most promising and accurate scoring systems currently defined.
In our study, we demonstrated that the expression of IGFBP-7 is positively correlated with caspase-3, and cell apoptosis rate. In addition, there is negative correlation between IGFBP-7 and VEGF. Those results suggested that pE-IGFBP7 can up-regulate IGFBP-7, caspase-3 expression, and down-regulate VEGF expression in vivo to inhibit the proliferation of MM cells, which resulted in slowing down of MM growth. Angiogenesis is essential for tumor development, and increasing evidence shows that IGF-I plays a crucial role in tumor growth by up-regulating the VEGF expression and neovascularization (29). A recent study indicated that IGFBP7 might exhibit angiogenesis-modulating properties, reducing VEGF expression by regulating IGF availability in body fluids and tumor tissues and modulating combination of IGF-I to its receptors (29,30). Moreover, the reduction of VEGF-induced tube formation by IGFBP-7 could be mainly mediated by inhibition of MAP kinase cascade through c-Raf, and BRAF-MEK-ERK signaling (31). Although our study imply that IGFBP-7 blocks VEGF-induced angiogenesis and VEGF expression by interfering with IGF-I, its role in tumor angiogenesis remains poorly understood. The mechanisms by which IGFBP-7 induces apoptosis and inhibit neovascularization should be further explored.

In conclusion, our data showed that increasing IGFBP7 expression by using the pE-IGFBP7 plasmid suppresses MM growth, which is mediated by apoptosis and reduction of VEGF in vitro and in vivo. Intratumoral injection of pE-IGFBP7 holds promise as a clinical gene therapy approach for MM, which provides a framework for further study of its broader applicability to a range of human tumors. However, it would be difficult to make uniform distribution of pEGFIC1-IGFBP7 in tumor tissue by intratumoral injection of Inivofectamin and a more specific and efficient lentiviral vector transfer system need be to be used in further studies. Secondly, there is no suitable MM cell line available that express high level of IGFBP7 to prove the specificity of anti-tumor effect of pEGFIC1-IGFBP7. Moreover, many biological roles of pEGFIC1-IGFBP7 remain to be elucidated.

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References