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ENHANCEMENT OF TUMOUR REGRESSION IN VP3-BASED GENE THERAPY IN COMBINATION WITH IMMUNOMODULATORS

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JOHN SHIA KWONG SIEW

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine Universiti Putra Malaysia

February 2009

Dedicated with love and gratitude
to:

My wife, Dr. Winnie Lau

&

My beloved parents, brother, and sister

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement of the degree of Doctor of Philosophy

ENHANCEMENT OF TUMOUR REGRESSION IN VP3-BASED GENE THERAPY IN COMBINATION WITH IMMUNOMODULATORS

By

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February 2009

Chairman : Professor Dr. Mohd Azmi Mohd Lila

Faculty : Veterinary Medicine

The VP3 gene of Chicken Anemia Virus has the potential to be an effective anti-cancer therapy. *In vitro* studies showed that several types of transformed cells transfected with recombinant VP3 genes become apoptotic within 72 hours post-transfection. The apoptotic activities were confirmed by TUNEL assay, and the apoptotic activities were mainly found in the nuclei of transfected cells. Expression of VP3 gene also triggered apoptosis in tumour mass in immune-competent mice. Following an injection of 100µg recombinant plasmid containing the VP3 gene into a tumour mass, the tumour tissue started to regress from 47.9 ± 5.2 mm^3 on day-1 to 0.8 ± 0.4 mm^3 on day-11 post-injection and completely resolved by day-13 post-injection. Meanwhile, in control group, the tumour mass measured 54.1 ± 5.2 mm^3 (day-1), increased to 589.0 ± 0.4 mm^3 on day-11 post-injection and 808.8 ± 0.4 mm^3 by day-13 post-injection. In a different experiment, selected cellular (pVIVO-IL12/GM-CSF) or humoral (pBoost/IL4/IL13) immune modulator was injected together with the recombinant plasmid containing VP3 gene. In the presence of cellular immune modulator, the tumour sizes were significantly decreased from 48.6 ± 7.7 mm^3 on day-1 post-injection to 0.6 ± 0.4 mm^3 on day-9 post injection. The

tumour mass was totally resolved by day-11 post-injection. The most rapid and complete regression of tumour mass as determined on day-11 post-injection, suggested that an enhanced effect of tumour regression is attributable to the effect of IL12 and GM-CSF. A delay in tumour growth was also noticed in the treatment group receiving a single dose of pVIVO-IL12/GM-CSF plasmid only (100 µg/mouse). It is suggested that the anti-cancer mechanism is being induced following expression of IL12 gene in the tumor cells. This in turn will activate Th1 and NK cells. Meanwhile, an expression of GM-CSF will cause mobilization of granulocytes and macrophages. Combination of these cellular activities may produce more antigen presenting cells for the recognition of tumour-associated antigens that facilitate elimination of tumour cells. The treatment group receiving humoral immune modulating factors (IL4/IL13) in addition to VP3 gene therapy showed reduction in the size of tumour from 48.5 ± 5.3 mm^3 on day-1 post-injection to 4.1 ± 1.6 mm^3 on day-9 post-injection. The tumour was totally resolved by day-11 post-injection. However, its anti-cancer effect was still mediocre compared to VP3 gene therapy incorporated with IL12/GM-CSF. The flow cytometry analysis on the clusters of differentiation (CD) for the 3 treatment groups (Group 1: recombinant VP3 gene only; Group 2: recombinant VP3 gene + cellular immune modulator; and Group 3: recombinant VP3 + humoral immune modulator) further supported the rationale of choosing the particular cellular and humoral immune modulators for effective anti-cancer therapy. The percentage of CD4 cells detected in the thymus of tumour – bearing mice showed that cellular and humoral immune modulators enhanced the proliferation of CD4 lymphocyte population in the thymus. Meanwhile, the highest percentage of CD8 count was most distinguished in group 2 (39.02%) on day-10 post-injection. An increase in CD8 cell count was probably due to the enhanced

immune-stimulation by IL12 and GMCSF expressed by pVIVO-IL12/GM-CSF recombinant plasmids against tumour antigens. The distribution of CD4 and CD8 lymphocyte populations in the spleen of treated mice has a similar trend as in primary lymphoid organs (thymus). Again, the percentage of CD8 count was most distinguished in group 2 (38.00%) on day-10 post-injection. It is suggested that, the increase in CD8 cell counts could be due to the increase in mature cytotoxic T-cells produced by the thymus against tumour antigens, as a result of immune-stimulation of GM-CSF. These CD4 and CD8 cells may then be detected in the secondary lymphoid organs. The percentage of CD19 lymphocyte populations was only prominent in Group 3 (26.71%) on day-10 post-injection. This suggests that the increase of CD19 lymphocytes was due to the immune-stimulation by IL4/IL13. In conclusion, recombinant VP3 gene is best combined with cellular immune modulators (IL12/GM-CSF) for a more effective anti-cancer gene therapy.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan Ijazah Doktor Falsafah

PENINGKATAN REGRESI TUMBUHAN BARAH DENGAN TERAPI GEN BERASASKAN VP3 DAN GABUNGAN MODULATOR IMUN

Oleh

JOHN SHIA KWONG SIEW

Februari 2009

Pengerusi : Profesor Dr. Mohd Azmi Mohd Lila

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Gen VP3 yang berasal daripada virus anemia ayam menpunyai potensi sebagai terapi anti-barah yang berkesan. Kajian *in vitro* menunjukkan bahawa pelbagai jenis sel terubah yang ditransfeksi dengan gen VP3 menjadi apoptotik dalam masa 72 jam pasca-transfeksi. Aktiviti apoptosis ini telah disahkan dengan ujian TUNEL, di mana kebanyakkan aktiviti apoptosis telah dikesan di dalam nukleus sel barah. Ekspresi gen VP3 juga memicu apoptosis dalam tumbuhan barah pada mencit yang berimun kompeten. Berikutan dengan suntikan 100 µg plasmid rekombinan yang mengandungi gen VP3 pada tumbuhan barah, tisu barah mula mengecil daripada 47.9 ± 5.2 mm^3 pada hari pertama pasca-suntikan, sehinggalah 0.8 ± 0.4 mm^3 pada hari ke-11 pasca-suntikan. Tumbuhan barah lesap pada hari ke-13 pasca-suntikan. Manakala untuk kumpulan kawalan, tumbuhan barah berukur 54.1 ± 5.2 mm^3 pada hari pertama pasca-suntikan, 589.0 ± 0.4 mm^3 pada hari ke-11 pasca-suntikan, sehinggalah 808.8 ± 0.4 mm^3 pada hari ke-13 pasca-suntikan. Dalam satu lagi kajian, modulator imun sellular (pVIVO-IL12/GM-CSF) atau modulator humoral (pBoost-IL4/IL13) yang terpilih telah disuntik bersama-sama dengan plasmid rekombinan yang mengandungi gen VP3. Dengan kewujudan modulator imun sellular, saiz

tumbuhan barah mengecil dari 48.6 ± 7.7 mm^3 pada hari pertama pasca-suntikan, sehinggalah 0.6 ± 0.4 mm^3 pada hari ke-9 pasca-suntikan. Tumbuhan barah lesap pada hari ke-11 pasca-suntikan. Kadar pengecilan tumbuhan yang paling cepat dan pelesapan tumbuhan barah pada hari ke-11 pasca-suntikan mencadangkan bahawa peningkatan kadar regresi tumbuhan barah adalah akibat daripada IL12 dan GM-CSF. Penangguhan tumbesaran tumbuhan barah juga diperhatikan dalam kumpulan rawatan yang menerima dos tunggal pVIVO-IL12/GM-CSF plasmid (100 µg/mencit). Ini mencadangkan bahawa, mekanisme anti-barah telah diaktifan berikutan ekpresi gen IL12 dalam sel barah yang mengaktifkan sel Th1 dan NK manakala ekpresi GM-CSF akan menyebabkan mobilisasi sel granulosit dan makrofaj untuk menghasilkan lebih banyak sel persembahan antigen untuk pengenalpastian antigen barah, dan seterusnya membantu pembasmian sel barah. Kumpulan rawatan yang menerima suntikan modulator humoral (IL4/IL13) menunjukkan pengecilan size tumbuhan barah dari 48.5 ± 5.3 mm^3 pada hari pertama sehinggalah 4.1 ± 1.6 mm^3 pada hari ke-9 pasca-suntikan. Tumbuhan barah lesap pada hari ke-11 pasca-suntikan. Akan tetapi, keberkesanan anti-barah adalah tidak sebaik jika dibandingkan dengan terapi gen VP3 dengan gabungan IL12/GM-CSF. Analisa aliran sitometri terhadap kelompok pembezaan (CD) terhadap ketiga-tiga kumpulan rawatan (kumpulan 1= gen VP3 sahaja; Kumpulan 2: gen VP3 + pVIVO-IL12/GM-CSF, dan Kumpulan 3: gen VP3 + pBoost-IL4/IL13) selanjutnya menyokong rasional pemilihan modulator-modulator imun tersebut, untuk terapi anti-barah yang berkesan. Peratus sel CD4 yang dikesan dalam timus pada mencit yang berbarah menunjukkan bahawa modulator sellular dan humoral telah meningkatkan penyebaran populasi limfosit CD4. Manakala, peningkatan peratus sel CD8 adalah paling ketara dalam kumpulan 2 (39.02%) pada hari ke-10 selepas suntikan. Peningkatan dalam jumlah sel CD8 ini

dipercayai atas stimulasi imun oleh IL12 dan GM-CSF, hasil daripada ekspresi plasmid pVIVO-IL12/GM-CSF terhadap antigen barah. Corak taburan populasi limfosit CD4 dan CD8 dalam limpa adalah serupa dalam timus. Peningkatan populasi sel CD8 adalah paling ketara dalam kumpulan 2 (38.00%) pada hari ke-10 pasca-suntikan. Ini mencadangkan bahawa peningkatan jumlah sel CD8 mungkin disebabkan oleh peningkatan populasi CD8 yang matang yang dihasilkan oleh timus terhadap antigen barah, hasil daripada stimulasi imun oleh GM-CSF. Sel-sel CD4 dan CD8 ini boeh dikesan dalam organ limfoid sekunder. Peningkatan peratus limfosit CD19 hanya ketara dalam kumpulan 3 (26.71%). Ini mencadangkan bahawa penigkatan CD19 adalah disebabkan oleh rangsangan imun IL4 dan IL13, hasil daripada suntikan pBoost-IL4/IL13. Kesimpulannya, terapi gen menggunakan gen VP3 dan pVIVO-IL12/GM-CSF merupakan kombinasi terbaik dan paling berkesan dalam terapi gen anti-barah.

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I certify that a Thesis Examination Committee has met on 23 February 2009 to conduct the final examination of JOHN SHIA KWONG SIEW on his thesis entitled "Enhancement of Tumour Regression in VP3-based Gene Therapy in Combination with Immunomodulators" in accordance with the Universities and University Colleges Act 1971 and the Constitution of Universiti Putra Malaysia [P.U.(A) 106] 15 march 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

JOHN SHIA KWONG SIEW

Date: 15 May 2009

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