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Efficacy of *ex vivo* activated and expanded natural killer cells and T lymphocytes for colorectal cancer patients

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Abstract. Immune cell-based therapies using natural killer (NK) cells and cytotoxic T cells are under constant scrutiny, with the aim to design an effective and reduced-toxicity therapy, which will benefit patients via improved quality of life and improved prognosis. Four patients with stage IV colon cancer were administered 1, 3, 5 and 6 effector cell intravenous infusions, respectively. Peripheral blood was collected from the patients and the *ex vivo* activation and expansion of NK and T cells was performed in Good Manufacturing Practice-certified clean rooms for ~12-15 days. Immunophenotypic analysis of the peripheral blood mononuclear cells (PBMCs) and expanded NK and T cells was conducted using flow cytometry and the patients were followed up. On average, $4.8x10^7$ initial PBMCs and $2.7x10^9$ total expanded cells were obtained. The intravenous infusions of the expanded cells were not accompanied by adverse reactions. Improved prognosis, reflected by a considerable decrease in the cancer markers, accompanied by an improved quality of life in the patients were observed. In conclusion, potential strategies are currently under development for the large-scale production of effectors cells; therefore, autologous immune enhancement therapy (AIET) may be considered as a viable approach to cancer treatment.

Introduction

The Malaysian National Cancer Registry Report (2007) reported colorectal cancer to be the second leading type of cancer among men as well as among women, with a total of 2,246 diagnosed cases, including 1,235 affected men and 1,011 women in 2007 (1). With the progression of colorectal cancer, synchronous occurrence of liver metastases is identified in ≤50% of the patients, which requires a multimodality treatment approach (2).

Chemotherapy is currently considered to be the standard treatment method for stage III colon and stage II rectal cancer. In patients with terminal colorectal cancer, palliative care is considered to improve the quality of life (3,4).

The high rate of recurrence and metastasis of colorectal cancer underlines the need for novel treatment modalities. The combination of biotherapy with other standard treatment modalities is increasingly recognised as an effective method, particularly in cases with advanced-stage cancer (5-8).

Ex vivo natural killer (NK)- and T-cell expansion has been accepted worldwide as beneficial for the treatment of metastatic or minimal residual cancer and an improved prognosis has been reported with this application (9-11). A number of approaches have been investigated for the growth and expansion of NK (12-14) and T cells (15,16), in order to obtain a maximum-fold expansion. Takada *et al* (17) and Dewan *et al* (18) successfully expanded NK and T cells by severalfold from a low number of peripheral blood mononuclear cells (PBMCs) in order to develop a multipronged approach to cancer management.

Autologous immune enhancement therapy (AIET) involves the isolation and expansion of NK cells and T lymphocytes from the patients' own peripheral blood, followed by re-infusion of the activated cells to the patients intravenously. AIET may be combined with currently available treatment methods, in order to equip the immune system to efficiently recognize and eliminate tumor cells and may be used in passive or active immunotherapy for eradicating cancer stem cells (19). In this study, we report our experience with the application of AIET in 4 patients with stage IV colorectal cancer.

Patients and methods

Patients characteristics. One male and three female patients who were diagnosed with stage IV colon carcinoma were enrolled for AIET. Three patients received a hemicolorectomy, whereas the fourth patient was inoperable and exhibited poor cardiac function. Chemotherapy was prescribed to the patients with FOLFOX as first-line chemotherapy, followed by bevacizumab, cetuximab and capecitabine as second- and third-line treatment. The median age of the patients was 56 years.

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Abbreviations: NK, natural killer; PBMCs, peripheral blood mononuclear cells; AIET, autologous immune enhancement therapy

Key words: colorectal cancer, FOLFOX, autologous immune enhancement therapy

Table I. Details of the patients who underwent autologous immune enhancement therapy.

Cases	Age (years) /gender	Diagnosis	Surgical procedure	Cancer stage	Metastatic site	Standard therapy
1	50/F	Advanced colon CA	Hemicolectomy	IV	Liver to lung	FOLFOX[a], FOLFIRI[b] with bevacizumab
2	50/F	Advanced colon CA	Hemicolectomy, attempted liver resection	IV	Colon to liver	FOLFOX[a], FOLFIRI[b] with cetuximab
3	63/F	Advanced colon CA	Hemicolectomy	IV	Colon to liver	Cetuximab, FOLFOX[a], capecitabine
4	58/M	Advanced colon CA	Inoperable with poor cardiac function	IV	-	Refused

[a]Leucovorin, 5-fluorouracil and oxaliplatin. [b]Leucovorin, 5-fluorouracil and irinotecan. F, female; M, male; CA, colon adenocarcinoma.

Table II. Average number of cell population pre- and post-expansion.

Cases	No. of AIET infusions	PBMC average count/collection (mean ± SD) ($x10^6$)	Average no. of cells/infusion (mean ± SD) ($x10^6$)	Average initial cell count for NK-cell culture ($x10^6$)	Average final cell count for NK-cell culture ($x10^6$)	Average initial cell count for T-cell culture ($x10^6$)	Average final cell count for T-cell culture ($x10^6$)
1	6	52.2±15.5	4,731.5±712.6	32.7	1,444.1	19.5	3,287.3
2	3	38.5±13.0	3,017.7±808.9	25.7	1,170.8	12.8	1,846.8
3	1	33.0	859	22	848	11	1,100
4	4	77.1±41.4	2,024.1±737.2	45.9	1,095.1	31.2	928.9

AIET, autologous immune enhancement therapy; PBMCs, peripheral blood mononuclear cells; NK, natural killer; SD, standard deviation.

One patient refused to undergo chemotherapy and received 6 infusions of AIET. The medical history of the patients who underwent AIET infusions is summarized in Table I.

In vitro isolation of PBMCs, activation and expansion. The peripheral blood collected from the patients was processed in a Good Manufacturing Practice-certified clean room. The isolated PBMCs were cultured for 12-15 days using gas-permeable culture bags as previously described (17,18). Interleukin-2 and the patient's own plasma were used as growth supplements throughout the culture process to enhance the expansion. The cells were harvested after 12-15 days for intravenous administration. Pre- and post-expansion immunophenotyping was performed to determine the initial and final percentages of NK and T cells from the PBMCs and the expanded cell population. Multiple intravenous infusions were administered to the patients, with a maximum of 6 infusions.

Results

Total expanded cell count. The cell counts were determined with the trypan blue dye exclusion test for the isolated PBMCs and expanded NK cell and T lymphocyte populations. Cell growth was increased from day 7 or 9 and logarithmic expansion was observed until the cells were harvested. Table II shows the average population of cells seeded to respective anti-CD3 and anti-CD16 coated flasks and the retrieved cell count on the day of harvest in accordance with the deviation noticed during processing of each sample. On the day of the harvest, the maximum-fold increase compared to the initially isolated cell count was found to be 150.

The pre- and post-expansion immunophenotyping analysis of various lymphocyte markers revealed a steady increase in the number of effector cells following expansion. The list of the analyzed markers, the average percentage of the population and the corresponding number of infusions administered to each of the patients, is presented in Table III, whereas the absolute cell numbers and fold expansions are presented in Table IV.

Intravenous infusion and follow-up. The harvested cells were suspended in 100 ml sterile saline solution and administered intravenously. The patients were followed up after the AIET infusions. The tumor marker evaluation revealed a significant decrease, which was accompanied by an improvement in the patients' quality of life and a considerable increase in survival rates. The prognosis of the patients following administration of AIET is summarized in Table V.

Discussion

Previous studies on the positive outcome of immunotherapy using *in vitro* expanded lymphocytes (14,19-21) reported

Table III. Results of immunophenotyping analysis (average), pre- and post-expansion.

Cases	No. of infusions	PBMCs (%)			NK-cell culture (%)			T-cell culture (%)		
		$CD3^+$	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD3^-CD56^+$	$CD3^-CD56^+$	$CD3^-CD16^+$	$CD3^+$	$CD3^+CD4^+$	$CD3^+CD8^+$
1	6	77.2	40.5	36.5	7.08	39.7	21.2	80.3	33.6	71.4
2	3	52.9	34.9	19.4	22.5	67.8	35.6	69.6	44.5	53.9
3	1	78.2	48.4	26.2	10.1	57.6	57.2	63.9	40.2	24.5
4	4	86.1	63.9	21.5	6.9	78	71.9	73.8	24.1	54.8

PBMCs, peripheral blood mononuclear cells; NK, natural killer; CD, cluster of differentiation.

Table IV. Absolute cell number (average) pre- and post-expansion.

Cases	No. of infusions	PBMCs			NK-cell culture			T-cell culture		
		T cells ($x10^6$)	$CD4^+$ T cels ($x10^6$)	$CD8^+$ T cells ($x10^6$)	NK cells before ($x10^6$)	NK cells after ($x10^6$)	NK-cell fold expansion	T cells ($x10^6$)	$CD8^+$ T cells ($x10^6$)	$CD3^+CD8^+$ T-cell fold expansion
1	6	40.3	21.1	19.1	3.7	573.3	155	2,639.7	1,104.5	58
2	3	20.4	13.4	7.5	8.7	793.8	91	1,285.4	821.8	110
3	1	25.8	16.0	8.6	3.3	488.4	148	702.9	442.2	51
4	4	66.4	49.3	16.6	5.3	854.2	161	685.5	223.9	13

PBMCs, peripheral blood mononuclear cells; NK, natural killer; CD, cluster of differentiation.

Table V. Prognosis of the colon cancer patients following AIET.

Cases	Age (years) /gender	Number of AIET infusions	Prognosis
1	50/F	6	18 months, (succumbed to ARDS following radiotherapy to the mediastinum)
2	50/F	3	32 months (succumbed to the disease)
3	63/F	1	20 months (succumbed to the disease)
4	58/M	4	12 months (alive, stable disease, continuing AIET)

AIET, autologous immune enhancement therapy; F, female; M, male; ARDS, acute respiratory distress syndrome.

that this type of treatment may even target cancer stem cells, which are considered to be a major target of eradication (22), to achieve a disease-free survival. Extensive investigation has been focused on expanding the desired cell population of immune cells to considerable numbers that may efficiently target the tumor cells (21).

It was suggested that adequate quantities of clinical grade immune effectors (13,23) and the safety of multiple infusions as showin in previous treatments (24-26) remain a major concern in immunotherapy. In this study, it was substantial to achieve a maximum 161-fold expansion of NK cells without using any feeder cells for 2 weeks (14).

There were no adverse reactions following the administration of the *ex vivo* expanded lymphocytes. The infused cells were able to boost the immune response under *in vivo* conditions, which was reflected by the improvement in the quality of life of the patients. The safety of the intravenous administration of immune cells to cancer patients was previously reported (14,24,27). The follow-up of the patients after administration revealed improved survival in all the patients who underwent immunotherapy. In fact, the patient who did not undergo any chemotherapy (case 4; Table I) remains alive with a good quality of life and is still under follow-up.

One of the most promising anticancer approaches is the employment of autologous immune cells to eradicate tumor cells. In that regard, NK cells (28) and cytotoxic T lymphocytes (9) are receiving increasing attention worldwide. Various concepts and strategies were introduced and discussed regarding the adoptive transfer of various kinds of immune cells, mainly focusing on cancers such as breast (29),

prostate (30) and ovarian cancer (31). In addition, adoptive immunotherapies were further investigated for melanoma (32), pediatric malignancies (33), thyroid carcinoma (34) and renal cell malignancies (35).

In conclusion, our results are encouraging, although they require further validation regarding the safety of the expanded population of NK cells and T lymphocytes for advanced colon cancer patients. With the combined efficiency of chemotherapy and immediate referrals it may be feasible to generate an optimal method for the management of the increased number of colon cancer patients, as this type of cancer is considered to be a major cause of mortality in Malaysia. The current technology has enabled maximization of the lower available cell population to a considerable number that may prove beneficial to cancer patients as an adjuvant/alternative therapeutic option.

References

1. Ministry of Health Malaysia, National Cancer Registry: Malaysian cancer statistics - data and figure peninsular Malaysia 2006. http://www.makna.org.my/PDF/MalaysiaCancerStatistics.pdf. Accessed, May 01, 2013.
2. Cai GX and Cai SJ: Multi-modality treatment of colorectal liver metastases. World J Gastroenterol 18: 16-24, 2012.
3. Amersi F, Stamos MJ and Ko CY: Palliative care for colorectal cancer. Surg Oncol Clin N Am 13: 467-477, 2004.
4. Ronnekleiv-Kelly SM and Kennedy GD: Management of stage IV rectal cancer: palliative options. World J Gastroenterol 17: 835-847, 2011.
5. Cunningham D, Atkin W, Lenz HJ, *et al*: Colorectal cancer. Lancet 375: 1030-1047, 2010.
6. Kannagi R: Carbohydrate antigen sialyl Lewis a - its pathophysiological significance and induction mechanism in cancer progression. Chang Gung Med J 30: 189-209, 2007.
7. Sato H, Usuda N, Kuroda M, *et al*: Significance of serum concentrations of E-selectin and CA19-9 in the prognosis of colorectal cancer. Jpn J Clin Oncol 40: 1073-1080, 2010.
8. Wasserberg N and Kaufman HS: Palliation of colorectal cancer. Surg Oncol 16: 299-310, 2007.
9. Rosenberg SA, Restifo NP, Yamg JC, *et al*: Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 8: 299-308, 2008.
10. Hunder NN, Wallen H, Cao J, *et al*: Treatment of metastatic melanoma with autologous $CD4^+$ T cells against NY-ESO-1. N Engl J Med 358: 2698-2703, 2008.
11. Srivastava S, Lundqvist A and Childs RW: Natural killer cell immunotherapy for cancer: a new hope. Cytotherapy 10: 775-783, 2008.
12. Alves PC, Andrade LA, Petta CA, *et al*: Ex vivo expansion of $CD56^+$ NK and NKT-like lymphocytes from peripheral blood mononuclear cells of patients with ovarian neoplasia. Scand J Immunol 74: 244-252, 2011.
13. Somanchi SS, Senyukov VV, Denman CJ and Lee DA: Expansion, purification, and functional assessment of human peripheral blood NK cells. J Vis Exp: 2540, 2011.
14. Terunuma H, Deng X, Dewan Z, *et al*: Potential role of NK cells in the induction of immune responses: implications for NK cell-based immunotherapy for cancers and viral infections. Int Rev Immunol 27: 93-110, 2008.
15. Teschner D, Wenzel G, Distler E, *et al*: In vitro stimulation and expansion of human tumour-reactive $CD8^+$ cytotoxic T lymphocytes by anti-CD3/CD28/CD137 magnetic beads. Scand J Immunol 74: 155-164, 2011.
16. Hippen KL, Merkel SC, Schirm DK, *et al*: Massive ex vivo expansion of human natural regulatory T cells (T_{regs}) with minimal loss of in vivo functional activity. Sci Transl Med 3: 83ra41, 2011.
17. Takada M, Terunuma H, Deng X, *et al*: Refractory lung metastasis from breast cancer treated with multidisciplinary therapy including an immunological approach. Breast Cancer 18: 64-67, 2011.
18. Dewan MZ, Terunuma H, Toi M, Tanaka Y, *et al*: Potential role of natural killer cells in controlling growth and infiltration of AIDS-associated primary effusion lymphoma cells. Cancer Sci 97: 1381-1387, 2006.
19. Sun Z, Shi L, Zhang H, *et al*: Immuno modulation and safety profile of adoptive immunotherapy using expanded autologous activated lymphocytes against advanced cancer. Clin Immunol 138: 23-32, 2011.
20. Rao B, Han M, Wang L, *et al*: Clinical outcomes of active specific immunotherapy in advanced colorectal cancer and suspected minimal residual colorectal cancer: a meta-analysis and system review. J Transl Med 9: 17, 2011.
21. Jursik C, Prchal M, Grillari-Voglauer R, *et al*: Large-scale production and characterization of novel $CD4^+$ cytotoxic T cells with broad tumor specificity for immunotherapy. Mol Cancer Res 7: 339-353, 2009.
22. Iovino F, Meraviglia S, Spina M, *et al*: Immunotherapy targeting colon cancer stem cells. Immunotherapy 3: 97-106, 2011.
23. Sangiolo D: Cytokine induced killer cells as promising immunotherapy for solid tumors. J Cancer 2: 363-368, 2011.
24. Barkholt L, Alici E, Conrad R, *et al*: Safety analysis of ex vivo-expanded NK and NK-like T cells administered to cancer patients: a phase I clinical study. Immunotherapy 1: 753-764, 2009.
25. Cruz CR, Hanley PJ, Liu H, *et al*: Adverse events following infusion of T cells for adoptive immunotherapy: a 10-year experience. Cytotherapy 12: 743-749, 2010.
26. Rabinowich H, Sedlmayr P, Herberman RB and Whiteside TL: Increased proliferation, lytic activity, and purity of human natural killer cells cocultured with mitogen-activated feeder cells. Cell Immunol 135: 454-470, 1991.
27. Cho D, Kim SK and Carson WE III: NK cell-based immunotherapy for treating cancer: will it be promising? Korean J Hematol 46: 3-5, 2011.
28. Terme M, Ullrich E, Delahaye NF, *et al*: Natural killer cell-directed therapies: moving from unexpected results to successful strategies. Nat Immunol 9: 486-494, 2008.
29. Zhou J and Zhong Y: Breast cancer immunotherapy. Cell Mol Immunol 1: 247-255, 2004.
30. McNeel DG: Cellular immunotherapies for prostate cancer. Biomed Pharmacother 61: 315-322, 2007.
31. Chu CS, Kim SH, June CH and Coukos G: Immunotherapy opportunities in ovarian cancer. Expert Rev Anticancer Ther 8: 243-257, 2008.
32. Katipamula R and Markovic SN: Emerging therapies for melanoma. Expert Rev Anticancer Ther 8: 553-560, 2008.
33. Cooper LJ: Adoptive cellular immunotherapy for childhood malignancies. Bone Marrow Transplant 41: 183-192, 2008.
34. Stift A, Sachet M, Yagubian R, *et al*: Dendritic cell vaccination in medullary thyroid carcinoma. Clin Cancer Res 10: 2944-2953, 2004.
35. Wysocki PJ, Zolnierek J, Szczylik C, *et al*: Recent developments in renal cell cancer immunotherapy. Expert Opin Biol Ther 7: 727-737, 2007.