RESEARCH ARTICLE

Cancer Preventive Effects of Whole Cell Type Immunization against Mice Ehrlich Tumors

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Abstract

Background: Effects of whole cell type immunization on mice Ehrlich tumours were evaluated. Materials and Methods: After preliminary study, mice were divided two major groups; 1x1000 and 100x1000 live Ehrlich cell transferred major groups, each divided into four subgroups (n: 10). Study groups were immunized with Ehrlich cell lysates in 0, 3, 7, 14th days and after 30 days of last immunization, live Ehrlich cells were transferred. Mice were observed for six months and evaluated for total and cancer free days. Results: Out of 100x1000 cell transferred solid type study group, all study group mean and tumour free periods were statistically longer than control groups. All 1x1000 Ehrlich cell transferred study groups survived significantly longer than 100x1000 Ehrlich cell transferred groups. Conclusions: Ehrlich mice tumours were prevented and survival prolonged with whole cell type immunization. Effects are related to the number of transferred tumor cells.

Keywords: Cancer - prevention - whole cell immunization - Ehrlich cell tumours

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Introduction

Of the National Cancer Institute's $4.8 billion total budget, only about 11% was allocated for cancer prevention and control in 2007 (Frieden et al., 2008). As to overall cancer mortality rates, no gains would have occurred since 1990 had it not been for reductions in smoking (Bailar et al., 1997; Jema et al., 2006). Two major approaches to cancer prevention are primary prevention through reduction in risk factors and changes to environmental factors that reduce human exposure to widely-consumed cancer-promoting agents. Immunization (vaccination) is an effective approach to specific virus-associated cancers, such as using human papillomavirus vaccine to prevent cervical cancer and hepatitis B virus vaccine to prevent hepatocellular cancer. Secondary prevention reduces cancer mortality through screening and early treatment (Bailar et al., 1997; Jema et al., 2006).

Antitumor immunizations are basically divided into two types: alive vaccines and dendritic cell based vaccines. Alive vaccines include live cancer cells cultured either directly or after being weakened by various methods (Pardoll., 2000; Reang et al., 2006). Dendritic cells develop in the bone marrow from hematopoietic stem cells. Preparation of dendritic cell based vaccine is difficult, expensive, and has limited efficacy (Banchereau, 1998; Sauter et al., 2000).

In this research, we aim to evaluate the effectiveness of immunization on cancer prevention and elucidate the relationship between the number of transferred malign cells. According to this aim, before different number of malign cells were transferred, mice were immunized with whole cell type vaccines. Cancer prevention and survey prolonged effects were evaluated.

Materials and Methods

This study was performed in the Istanbul University Cerrahpasa Faculty of Medicine Experimental Animals Research Laboratory and in the Yeditepe University Genetics and Bioengineering Department Laboratory. Research protocol was approved by the Istanbul University Local Animal Ethics Committee. All steps in this research were in accordance with the regulations governing the care and use of laboratory animals set forth in the declaration of Helsinki.

This research used 106 female Balb/c mice (mean age 4 months, mean weight 36±11g, out-bred produced). Twenty-six were used in the preliminary and 80 in the liminary research steps. All were kept in standard metabolic cages specifically designed for mice. A 12-hour light/12-hour dark cycle was used for illumination of the room where the mice were placed.

Preliminary study

We studied on 16 mice to reach the median and minimum cell numbers for tumor formation. From 100 to 500,000 cells were transferred in the intraperitoneal and

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subcutaneous neck areas. A minimum of 1000cell/1ml were needed to form a tumor either in the intraperitoneal or in the subcutaneous neck areas. For that reason, 1000cell/ml is defined as the "tumor forming minimal cell number," and 100 thousand/1ml is defined as the "tumor forming median cell number" (Table 1).

To evaluate self-effects and/or side-effects of immunization, 10 mice were immunized according to the study design (described below). In three months of observation, no self- or side-effects were seen in this group of subjects.

Cancer cell preperation: A Ehrlich mice tumor cell line was obtained from the experimetal animal research laboratory of Istanbul University's Faculty of Science, Department of General Biology. Alive cells were injected into two Balb/C mice. In one mouse, cells were injected into the peritoneal cavity in order to produce] ascites type tumor cells. In the other mice, cells were injected subcutaneously into the neck area in order to produce solid type tumor cells. In the terminal period of time the mice were sacrificed and their tumors excised. After mechanical and enzymatic fragmentation with colleganese, tumor lysates were generated.

Immune Lysate Preparation: Cells were counted after suspension created. Degradation performed with frozen cell suspension in liquid nitrogen and defrosted at 37°C five times. Degradation were confirmed by microscopic evaluation.

Main study

Mice were divided into two major groups: the 1x1000/1ml alive Ehrlich cell transferred major group and the 100x1000/1ml alive Ehrlich cell transferred major group. These two groups were then divided into four subgroups each. According to power analysis with 0.05 accuracy and 0.95 power, the number of mice were determined to be 10 in each of the subgroups. Ascites Type Study Groups were immunized with Ehrlich cell lysates on the 0, 3, 7, and 14th days. After 30 days of last imunization, alive Ehrlich cells were intraperitoneally transferred to the mice. In the Ascites Type Control Groups, Ehrlich cells were only transferred into the peritoneal cavity. The same immunization procedure was used in the Solid Type Study Groups, but alive Ehrlich cells were transferred into the subcutanous nape area.

All subjects were observed for six months. When the mice were exitus related to tumor, autopsies were performed and tumors were resected for histopathologic evaluation. Alive mice were sacrificed after six month observation with 250mg/kg intraperitoneal thiopental sodium (Pentothal IV Ampul®, Abbott, Turkey).

Autopsies were performed and Ehrlich cells transferred areas were resected for histopathologic evaluation. All specimens were placed in formol, fixed in 70% alcohol, dehydrated, and embedded in paraffin wax. Sections were cut at a thickness of 5 mm and stained with hematoxylin and eosin.

The primary evaluation parameter of this research was total and cancer free surveys (days). Total survey was evaluted as days from transfer of the cancer cells to exitus of the mice. Cancer free survey was evaluted as total body weight gain in ascites groups and tumor palpation in solid groups. Secondary evaluation parameters were histopathologic changes of the tumors.

Statistical analysis

Statistical analyses were performed using IBM SPSS Ver. 19.0. In addition to descriptive statistical methods (mean, standard deviation, and median), we used the Fisher Exact test for pure frequency and the Log Rank test for survey comparisons. For in-group comparisons, Kaplan Meier and Chi Square tests were used.

Results

Mean total surveys and mean tumor free surveys for the 1x1000 and 100x1000 Ehrlich cell transferred groups are demonstrated in Tables 1 and 2 along with statistical analyses. Out of the 100x1000 cell transferred solid type

Table 1. Transferred Cell Number Based Tumor Positivity

Transferred Cell Number (thousand/1 ml)	Transfer Area	Results
500	Intraperitoneal	Tumor (+)
500	Subcutan Neck	Tumor (+)
300	Intraperitoneal	Tumor (+)
300	Subcutan Neck	Tumor (+)
100	Intraperitoneal	Tumor (+)
100	Subcutan Neck	Tumor (+)
10	Intraperitoneal	Tumor (+)
10	Subcutan Neck	Tumor (+)
5	Intraperitoneal	Tumor (+)
5	Subcutan Neck	Tumor (+)
1	Intraperitoneal	Tumor (+)
1	Subcutan Neck	Tumor (+)
0.5	Intraperitoneal	Tumor (+)
0.5	Subcutan Neck	Tumor (-)
0.1	Intraperitoneal	Tumor (-)
0.1	Subcutan Neck	Tumor (-)

Table 2. Tumor Free and Total Surveys

Survey (days)	Ascites		Log Rank	Solid		Log Rank
	Study Group	Control Group		Study Group	Control Group	
1x1000 Ehrlich cell transferred groups (Kaplan Meier, Chi square)						
Mean Total	127.6±21.46	25.00±0.81	χ^2=21.6, p<0.001	171.6±12.66	80.6±8.38	χ^2=21.4, p<0.001
Mean Tumor Free	122.3±23.51	9.00±1.06	χ^2=20.7, p<0.001	154.4±20.81	20.5±0.79	χ^2=21.6, p<0.001
100x1000 Ehrlich cell transferred groups (Kaplan Meier, Chi square)						
Mean Total	29.2±2.98	19.9±1.04	χ^2=6.72, p=0.01	103.6±12.66	80.6±0.47	χ^2=4.73, p=0.029
Mean Tumor Free	20.2±2.87	7.0±0.83	χ^2=19.9, p<0.001	50.1±21.01	16.9±0.18	χ^2=1.16, p=0.28

Table 3. Comparison of 100x1000 vs 1x1000 Ehrlich Cell Transferred Groups (Kaplan Meier, Chi square)

Survey (days)	Ascites		Log Rank	Solid		Log Rank
	Study Group 1000/1 ml	Control Group 100,000/1 ml		Study Group 1000/1 ml	Control Group 100,000/1 ml	
Mean Total	127.6±21.46	29.2±2.98	χ^2=19.7, p<0.001	171.6±12.66	103.6±12.66	χ^2=6.79, p=0.009
Mean Tumor Free	122.3±23.51	20.2±2.87	χ^2=13.9, p<0.001	154.4±20.81	50.1±21.01	χ^2=6.89, p=0.009

Table 4. Comparison of Tumor Presence Pure Frequency (Fisher Exact test)

n/N	Ascites		p	Solid		p
	Study Group	Control Group		Study Group	Control Group	
1x1000 cell	4-10	10-10	0.011	2-10	10-10	0.001
100x1000 cell	10-10	10-10	1.00	8-10	10-10	0.474
p	0.011	1.00	--	0.023	1.00	--

study group, all study groups' surveys were statistically longer than those of the control groups. Surveys of all 1x1000 Ehrlich cell transferred study groups were statistically longer than those of the 100x1000 Ehrlich cell transferred groups (Table 3).

Tumors occurred in the mice of all control groups, both in the1x1000 and in the 100x1000 Ehrlich cell transferred groups. In contrast, out of the 100x1000 Ehrlich cell transferred ascites group, all study groups included tumor free mice. In the 100x1000 Ehrlich cell transferred solid group 2 (p>0.05), in the 1x1000 Ehrlich cell transferred ascites group 6 (p<0.05), and in the 1x1000 Ehrlich cell transferred solid group 8 mice (p=0.001) were tumor free (Table 4).

Discussion

Tumor immunization (vaccination) is a new and important field in cancer prevention and treatment. Cancer immunization studies used different type of vaccines and different modelities. Success is highest when the specific tumor antigen or genome sequence is known (Bodey et al., 2000; Zhu et al., 2000; Liu et al., 2004; Niu et al., 2004). VhCDR3 is overexpressed in Murine B cell lymphoma. Tumor free survival is 60% in Murine B cell lymphoma when immunization is used with VhCDR3 epitope-based DNA (Rinaldi et al., 2008). For patients immunized with CDR3-based fusion vaccine, tumor free survival is 50% (Iurescia et al., 2010). The Wilms' tumor gene WT1 is overexpressed in leukemias and various types of solid tumors, and the WT1 protein was demonstrated to be an attractive target antigen for immunotherapy against these malignancies (Oka et al., 2004). Zeng et al investigated an immunotherapeutic strategy for rearrangement during transfection proto-oncogene (ret)-associated carcinomas in a transgenic MT/ret 304/B6 mice model in which spontaneous tumors develop due to overexpression of the ret gene. The systemic administration of the potent inhibitor of indoleamine 2,3-dioxygenase 1-methyl tryptophan (1MT) along with ret vaccine produced a significant increase in tumor-specific cytotoxic activity (Zeng et al., 2009). Specific tumor antigen based immunizations are successful, but the number of these tumors are few, expensive to produce, and difficult to use in clinical practice (Banchereau et al., 2001).

As early as the 1970s, Hanna et al. (1979) pioneered the whole cell type immunization technique with irradiated tumor cells in various animal models (Hana et al., 1979; de Gruijl et al., 2008). Whole cell type immunization is cheap, simple to use in clinical practice, and effective for cancer prevention. Malignant tumor cells have different kinds of carcinogenic antigenic structures. Whole cell type immunizants contain all of the intra- and extracellular proteins of the tumor cells. Effects of whole cell type immunization is associated with the content of these rich antigenic structures (de Gruijl et al., 2008). In the literature, whole cell type immunization has been used for colorectal cancer (Hanna et al., 2001), malignant melanoma (Baars et al., 2000; Berd et al., 2004), renal cell cancer (Jocham et al., 2004), and prostate cancer (Michael et al., 2005).

Ehrlich tumor is a specific and aggressive malignant tumor isolated first from mice breast tissue (Ehrlich, 1905). Ehrlich tumor has ascites and solid subtypes. The ascites subtype is rapidly prolifering because H2 histocompatibility antigens are not featured (Chen 1970; Pessina et al., 1980). Ehrlich tumors are used largely in experimental cancer treatment, prevention, and modeling studies because tumor ocurrence rates after transplantation is very high and tumor growth is extremely rapid. On the other hand, a number of studies related to prevention of Ehrlich tumor (either ascites or solid subtypes) are few (Mashanova et al., 2010; Jukanti et al., 2011; Niang et al., 2011; Salem et al., 2011).

In this research, we hypothesised that whole cell type immunization may prevent tumor occurrence and/or prolong survey. In order to evaluate the accuracy of the hypothesis, we preferred to use with Ehrlich tumors because of the need for an aggressive tumor model. Many clinical and experimental research studies in the literature used whole cell type immunization, but most of them are related to treatment, not to prevention (Baars et al., 2000; Jaffee et al., 2001; Michael et al., 2005; Small et al., 2007; De Gruijl et al., 2008).

Immunization research on cancer follows one of two routes: treatment or prevention. Cancer treatment via immunization is not as effective as prevention. Nagorsen et al. (2006) reviewed 108 vaccination studies for colorectal carcinomas. Different immunization types were used in these studies: dendritic cell and/or peptid (12 studies), genomic (7 studies), antigenic (4 studies), whole cell type (5 studies) and other type (4 studies). In all studies the humoral immune response was 59% and the cellular immune response was 44%. The clinically respected objective immune response rate was 0.9% (Nagorsen et al., 2006).

Of the few studies on cancer prevention, the most important is by Suckow et al. In their research, rats were immunized subcutaneously with complete Freund's

adjuvant (CFA) plus glutaraldehyde-fixed (GFT) whole cell or potassium thiocyanate extract (PTE) preparations derived from in vivo tumors. Rats were immunized each month for 3 to 12 months. Compared with the media-immunized controls, groups of 30 GFT cell-immunized rats and PTE-immunized rats showed a 90% and 50% reduction, respectively, in the occurrence of de novo prostate tumors. The researchers concluded that prostate cancer may be prevented by whole tumor derived immunization (Suckow et al., 2005).

When we transferred 1x1000 Ehrlich tumor cells, cancer occurred 10/10 in the control groups, 2/10 in the immunized solid-type groups, and 4/10 in the immunized ascites-type groups. When we transferred 100x1000 Ehrlich tumor cells, cancer occurred 10/10 in the control and immunized ascites-type groups, but 8/10 in the immunized solid-type group. Tumor free surveys and total surveys were statistically longer in the immunized groups than in the control groups.

In this study, we demonstrated that Ehrlich mice tumor, an aggressive tumor model, is prevented and survey is prolonged with whole cell type immunization. Effects are related to the number of transferred tumor cells. Cancer biology is chaotic and has numerous unknown steps, but the general opinion is that cancer is generated by malign transformation of a few or even only a single cell (Fernandez et al., 1980; Kennedy et al., 1980). Accordingly, whole cell type immunization may be more effective to prevent human cancers. In this research, as in many cancer prevention studies we studied with the external cancer cell transferring model, most cancers occur not to transferred malign cells out of the body, but by a malign transformation of self-cells. In clinical practice, effects of whole cell immunization for prevention of human cancers is not clear. New studies are needed to evaluate whole cell type immunization on cancer prevention.

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