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MATRIX METALLOPROTEINASES-9 AS A PROMISING TARGET FOR ANTI-CANCER VACCINE: INHIBITION OF MELANOMA TUMOR GROWTH IN MICE IMMUNIZED WITH SYNGENEIC MMP-9 PEPTIDES

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Abstract – *Objective:* *The prevention and treatment of cancer remain a challenge. Current treatments are largely unsuccessful due to high toxicity. The most effective way to reduce global mortality from cancer is to block the initial stages of the disease, common to all types of cancer – invasion and metastasis. The elevated levels of ma-trix metalloproteinases, such as MMP-9 play a key role in tumorigenesis, angiogenesis, apoptosis, cancer invasion and metastasis. Among various therapeutic modalities, vaccines are the most effective and af-fordable approaches against diseases in general. In the global fight against cancer, a vaccine capable to impede MMP-2 and MMP-9 activity could open the door for effective prevention -- and even cure. We previously reported that mice immunized with synthetic oligopeptides containing specific amino acid se-quences from human MMP-2 and MMP-9 showed a significant reduction in melanoma tumors and tu-mor burden.*

Materials and Methods: *Here we tested a syngeneic approach to cancer vaccines by investigating whether immunization of mice with rodent derived MMP-9 oligopeptides would generate sufficient immune response and anticancer ef-ficacy. Accordingly, C57Bl/6 mice were immunized with three oligopeptides containing specific sequenc-es from rat MMP-9 and two oligopeptides from mouse MMP-9. All these peptides showed to be highly immunogenic in mice.*

Results: *Subsequently, the immunized mice challenged with B16FO melanoma cells developed significantly smaller tumors and had reduced tumor burden. The weight gain in vaccinated and control mice was com-parable. In addition, no significant differences were observed in serum clinical chemistry, hematological parameters and the histopathology of major organs in relation to test peptides in the immunized mice.*

Conclusions: *Our findings confirm that MMP peptide-based vaccines can be a viable strategy for cancer therapy.*

KEYWORDS: *Cancer vaccine, MMP-9, Immunogenic oligopeptides, Melanoma cancer, Tumor growth.*

INTRODUCTION

Effective and safe therapies for cancer remain a major challenge to global health. Most current cancer therapies are not fully effective, are highly toxic, non-specific and generate many health problems that impair quality of life of cancer survivors long after the cessation of treatments.

The most effective way to reduce global mortality from the cancer epidemic is to block the early stages of the disease, which is common to all types of cancer – invasion and metastasis. These stages are characterized by the presence of abnormally elevated levels of matrix metalloproteinases (MMPs) enzymes that digest the connective tissue. These enzymes enable tumor cells to invade the surrounding extracellular matrix (ECM), migrate in the tissue and penetrate into the vascular membrane to be carried in the bloodstream and metastasize to other organs[1,2].

The MMPs are zinc dependent endopeptidases that also play an important role during normal,

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physiological processes such as embryonic development, reproduction, tissue remodeling and others. In healthy conditions this process is under strict metabolic regulation[3]. In contrast, high and uncontrolled activity of metalloproteinases, particularly type MMP-2 and MMP-9, have been correlated with cancer progression and increased metastasis. Several studies, including our own, indicate that the spread of cancer, and also other diseases that are facilitated by the disintegration of ECM, can be controlled by curtailing the activity of MMPs[4-7]. Thus, we chose the blockage of MMP-2 and MMP-9 enzymes as our therapeutic target.

Regarding the path of delivery of such a new therapy, we selected vaccines as the most promising form. If selected and developed properly, vaccines are the most effective and affordable approaches against diseases in general. In the global fight against cancer, a vaccine capable to impede MMP-2 and MMP-9 activity could open the door for effective prevention - and possible cure. Our earlier studies in this field documented that synthetic peptides containing specific amino acid sequences from human MMP-2 and MMP-9 could be considered in the development of effective anti-cancer vaccine. Mice immunized with these oligopeptides showed a significant reduction in melanoma tumor growth with an average decrease in tumor volume by 76%. Notably in some vaccinated animals the tumor growth was completely prevented[8].

The anticipated target antigen in the development of future human anti-cancer vaccine is an oligopeptide(s) derived from human MMP sequences, which is a syngeneic approach. Therefore, in this study we evaluated whether immunization of mice with xenologous MMP-9 oligopeptide sequences derived from rodents will generate similar anti-cancer effects as observed earlier with oligopeptides selected from the human MMP-9. The C57Bl/6 mice were vaccinated with three oligopeptides containing rat MMP-9 sequences and two oligopeptides from mouse MMP-9. Subsequently, we tested the effects of this vaccination on growth of melanoma B16FO tumors. Moreover, in a separate mouse study, we assessed the metabolic impact of the immune response generated by these peptides in the body and organs of the test animals. Towards this end, we conducted a comprehensive evaluation of critical blood and safety parameters as well as histopathological changes in 23 organs in each immunized and control animals.

MATERIALS AND METHODS

Materials and methods were followed as mentioned in one of our previous studies[8].

REAGENTS

Complete Freund's Adjuvant (Cat. No. F5881), Incomplete Freund's Adjuvant (Cat. No. F5506), Avidin (Cat. No. A9275, Anti-mouse IgG-HRP conjugated antibody (Cat. No. A4416) and TMB substrate (Cat. No. T0440) were obtained from Sigma-Aldrich (St. Louis, MO, USA).
Murine melanoma B16F0 cells obtained from Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mcg/ml streptomycin. The media and serum were obtained from ATCC (Manassas, VA, USA), while the antibiotics were from Gibco (Rockville, MD, USA).

ANIMALS

Male C57BL/6J mice of 5-6 weeks old were obtained from Simonsen Laboratory (Gilroy, CA, USA). The animals were acclimatized for a period of two weeks prior to the study. They were maintained on a standard rodent diet *ad libitum* with free access to water under controlled conditions of temperature and humidity on a 12 h light-dark cycle.

TEST PEPTIDES

The test MMP-9 oligopeptides were synthesized at GenScript (Piscataway, NJ, USA). Each peptide of 90% purity was synthesized in two forms, one covalently conjugated to keyhole limpet hemocyanin (KLH) protein for immunization, the other conjugated to biotin for IgG ELISA. The peptides (6 mg) were dissolved in 6 mL of 0.1 M $NaHCO_3$ and aliquots stored at -20°C until experimentation.

Rat Sequence	R#7	: D-T-D-R-K-Y-G-F
Rat Sequence	R#12	: H-F-P-F-T-F-E-G-R-S-Y-L-S-C
Rat Sequence	R#18	: D-K-A-D-G-F-C-P-T-R-A-D-V-T-V
Mouse Sequence	M#11	: D-K-D-G-K-F-G-F
Mouse Sequence	M#19	: D-Q-D-K-L-Y-G-F-C-P-T-R-V-D-A

PREPARATION OF PEPTIDE FOR IMMUNIZATION AND IMMUNIZATION SCHEDULE

On the day of immunization (Day 0), 0.8 mL of peptide solution was gradually added to 0.8 mL of Complete Freund's Adjuvant (CFA) and simultaneously vortexed thoroughly to get a uniform emulsion. The animals in treatment groups (5 animals per group) were injected intraperitoneally

TABLE 1. Experimental design.

Week -1 to 0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
	Week 1-7: Immunization and immune response							*Week 8-11 Efficacy evaluation*			
Quarantine/ Adaptation	Immunization of mice on day 0, 7, 14 and 28							Blood sampling Immune response measurements (Day 45)	B16FO melanoma cells implant	Clinical signs (daily) Body weight Tumor weight (after termination)	

with 100 ul solutions of the individual KLH conjugated peptides emulsified with CFA. On Days 7, 14 and 28, the animals were administered 100 µL of KLH conjugated peptides emulsified with Incomplete Freund's Adjuvant (IFA). The control group was administered with the adjuvant alone. The experimental timeline, including immunization, test and evaluation steps are presented in Table 1.

BLOOD SAMPLING

On day 45, animals were anesthetized using Isoflurane, and blood samples were withdrawn into 2 mL microcentrifuge tubes and centrifuged at 5,000 rpm for 10 min at 4°C for separation of serum. Samples were stored at -80°C until analysis.

IMMUNE RESPONSE ASSAY

The immune response was measured in serum samples on day 45 by using an antibody ELISA titer assay as described here. On day one, 96-well microtiter plates (Maxisorp®, Nunc™) were coated with 100 µL of avidin solution (10 µg/mL prepared in 0.05 M carbonate buffer, pH 9.5) and incubated for 24 h at room temperature. The next day, plates were washed 3 times with wash buffer (200 µL/well) followed by the addition of 100 µL biotinylated peptide solution per well (50 µg/mL concentration prepared in Binding buffer) and incubated for 1 h at 37°C. Post incubation, the well contents were discarded and the plate was washed 3 times with wash buffer. The serum samples to be analyzed were serially diluted with binding buffer to get a concentration of 1:100, 1:1,000 and 1:10,000. Then 100 µL of the diluted serum samples were added to the respective wells and incubated for 1 h at 37°C. After a serial wash with wash buffer, 100 µL of Anti-mouse IgG-HRP conjugated antibody diluted 1:2000 in binding buffer was added to each well and incubated for 30 min at 37°C. The plates were again washed 3 times with wash buffer, 100 µL of TMB substrate was added and incubated for 15 min at room temperature. The reaction was stopped by the addition of 100 µL of 0.3N HCl and the OD was measured at 450 nm in a microplate reader (Synergy™ HT, Biotek®). The IgG antibody titer was determined by quantification of color reaction and measured as OD units.

IMPLANTATION OF B16F0 MELANOMA CELLS IN THE IMMUNIZED MICE

Murine melanoma B16F0 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Sub-confluent monolayers were harvested, pelleted and re-suspended in cold PBS. Viable cells were counted using Trypan blue and 0.2 mL of the cell suspension in PBS containing $0.4x10^6$ cells were implanted subcutaneously in mice.

TUMOR EXCISION AND GROSS PATHOLOGY

After 3 weeks of cell implantation, the animals were sacrificed by CO_2 asphyxiation. A necropsy was performed to examine gross pathology and metastasis. Tumors were excised, their weight recorded, and pictures of the representative tumors were captured. Tumor volume was measured in terms of length (l) and width (w) by using a digital vernier caliper. The tumor volume was calculated by using the following formula: Tumor volume = length x width. Clinical signs were monitored daily and body weight was recorded twice weekly.

TOXICOLOGY EVALUATION

Toxicology due to the peptide vaccination was evaluated in a separate fully blinded experiment on 45 male B57Bl/6 mice separated into 9 dis-

tinct groups of 5 mice each. Test groups included: Group #1- injection with saline, Group #2 – injection with Freund's adjuvant, Group #3- injection with Freund's adjuvant + carrier protein Keyhole Limpet Hemocyanin (KLH), Group #4 – injection with mimicking peptide (endothelin-1); Group #5 – injection with R #7, Group #6 – injection with R #12, Group #7 – injection with R#18, Group #8 – injection with M #11, Group #9 – injection with M #19. As a part of the blinding procedure, details of the experimental design were not provided to the examiners and the mice were identified by the group number. No individual identification was provided. The evaluations included changes in body weight, hematology, comprehensive serum chemistry panel and histopathology examination of 23 organs, including injection-related evaluation of the abdominal wall (injection site) and lymph nodes proximal to the injection site.

STATISTICAL ANALYSIS

Statistical analysis of the data was performed by one-way ANOVA followed by Dunnett's test using GraphPad Prism 5.03 (San Diego, CA, USA).

RESULTS

Antibody generation by test MMP-9 peptides

Immune response in mice was measured by IgG ELISA on day 45 post immunization. As presented in Table 2, the immunization with five test peptides generated positive immune response indicated by a significant elevation in serum IgG levels when compared to control sera.

The results showed that the immune response was the highest at 1:100 serum dilutions and slightly decreased at 1:10,000. In up to 1,000 serum dilutions, the immune response was similar for all five test MMP-9 oligopeptides (M#11, M#19 and R#7, R#12 and R#18) and it stayed at similar level up to 1:10,000 dilutions except for peptide R#18, which induced significantly lower titer at this serum dilution.

TABLE 3. IgG Immune titer of MMP-9 Mouse Peptides #11 & #19 in B16FO Tumor Bearing Mice.

	Control Sera	*MMP-9 Peptide M#11*	*MMP-9 Peptide M#19*
1-100	0.126±0.004	2.408±0.10	1.938±0.10
1-1,000	0.123±0.004	2.295±0.23	2.06±0.11
1-10,000	0.119±0.002	2.505±0.04	1.97±0.09

Immune titer in mice immunized with test MMP-9 peptides after challenge with melanoma

The results in Table 3 and Table 4 present changes in IgG titer in mice immunized against mouse oligopeptides and rat oligopeptides, respectively, after inoculation with B16FO melanoma cells. The results indicate that the antibody titers did not markedly change in animals challenged with melanoma cells when compared to the pre-inoculation immune response (Table 2).

Inhibition of tumor development in mice vaccinated with MMP-9 oligopeptides

Mice immunized with the five peptides containing specific sequences of MMP-9 from rat and mice, and subsequently challenged with B16FO melanoma cells, developed distinctly smaller tumors compared to control mice at 3 weeks post melanoma cell implantation. As presented in Table 5 and Figure 1 (A, B) mice immunized with rat MMP-9 peptide sequence R #7 and mouse MMP-9 sequence M#19 had 55% reduction in the tumor weight ($p<0.001$ and $p<0.05$) compared to control mice. Mice immunized with MMP-9 mouse oligo-

TABLE 2. IgG Immune titer of MMP-9 Mouse Peptides #11 & #19 and MMP-9 Rat Peptides #7, #12 and #18 after immunization of C57BL/6 Male Mice.

Dilution	*Control Sera*	*MMP-9 Mouse Peptides*		*MMP-9 Rat Peptides*		
		Peptide M#11	*Peptide M#19*	*Peptide R#7*	*Peptide R#12*	*Peptide R#18*
1-100	0.142	2.280±0.3	2.246±0.3	2.559	2.232	2.120±0.1
1-1,000	0.126	2.41±0.44	2.403±0.09	2.608	2.109	1.798±0.17
1-10,000	0.123	1.961±0.2	1.920±0.16	1.696	1.768	0.865 ±0.07

TABLE 4. Immune response to MMP-9 Rat Peptides #7, #12, and #18 in B16FO Tumor Bearing Mice.

	Control Sera	*MMP-9 Peptide R#7*	*MMP-9 Peptide R#12*	*MMP9 peptide R#18*
1-100	0.156	2.54±0.21	2.71±0.21	2.26±0.12
1-1,000	0.140	2.87±0.96	2.9±0.12	2.49±0.12
1-10,000	0.138	2.56±0.1	2.38±0.16	2.30±0.09

TABLE 5. Tumor weight (g) at the end of the study.

Groups	*Tumor weight*	*Tumor weight reduction (%)*	*Rank in tumor weight reduction*
Control	3.2 +/-0.52	–	
Peptide R # 7	1.4+/-0.46	55	#4
Peptide R # 12	0.78+/-0.24	77	#1
Peptide R # 18	1.0+/-0.37	70	#2
Peptide M # 11	1.24+/- 0.40	62	#3
Peptide M # 19	1.5 +/-0.41	55	#4

peptide M#11, and rat MMP-9 oligopeptides R#12 and R#18, showed even more pronounced average reduction in tumor weight, by 62%, 77% and 70%, respectively. Peptide R#12 ranked number one in tumor weight reduction in the vaccinated animals.

Evaluation of tumor volume (length x width) in immunized mice is presented in Table 6 and Figure 2 (A, B). The results show that tumors developed in mice immunized with R#12 and R#18 MMP-9 oligopeptides had the lowest average volume (63% reduction) compared to mice immunized with two mice derived MMP-9 oligopeptides and the rat oligopeptide R#19. As such, tumor volume in mice immunized with rat peptide R#7, mouse peptides M#11 and M#19 was reduced by 44%, 43% and 36% respectively, compared to controls. Changes in tumor volume show that immunization with the oligopeptides R#12 and R#18 ranked number 1 in tumor volume reduction.

Photographs of the excised tumors underscore the marked anti-tumor efficacy of the tested oligopeptide vaccines (Figure 3).

The results on Figure 4A and Figure 4B show that animals immunized with the test MMP-9 oligopeptides from mice and rats, respectively, had a normal rate of weight gain, similar to the unvaccinated animals. The macroscopic inspection of essential organs did not show any pathology.

Toxicology evaluation

The effect of test peptide vaccination on the body weight, hematology profile, blood chemistry and weight and histopathology of major organs in the test animals was evaluated in a separate blinded experiment as described in Materials and Meth-

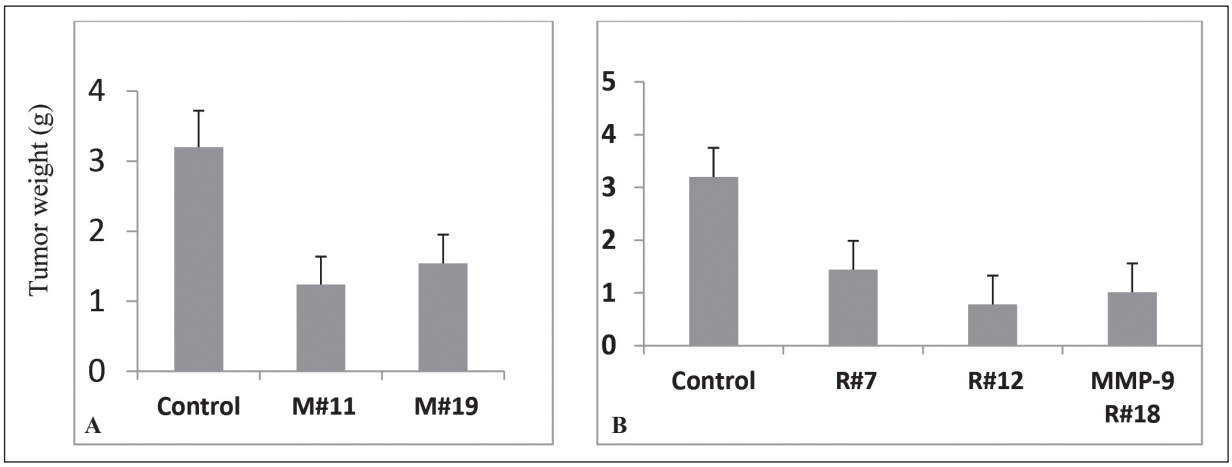

Fig. 1. Effects of vaccination on melanoma tumor weight. Tumor weight inhibition in mice immunized with mice MMP-9 oligopeptides M#11 and M#19 ***(A)*** and rat MMP-9 oligopeptides R#7, R#12 and R#18 ***(B)***.

TABLE 6. Changes in tumor volume.

Groups	*Tumor volume (Mean ± SE)*	*Tumor volume reduction (%)*	*Rank in tumor volume reduction*
Control	394±52	0	
Peptide R# 7	219±21	44	#2
Peptide R # 12	146.3±35	63	#1
Peptide R # 18	144±24	63	#1
Peptide M# 11	226±37	43	#3
Peptide M# 19	253±39	36	#4

Tumor volume calculation = length x width.

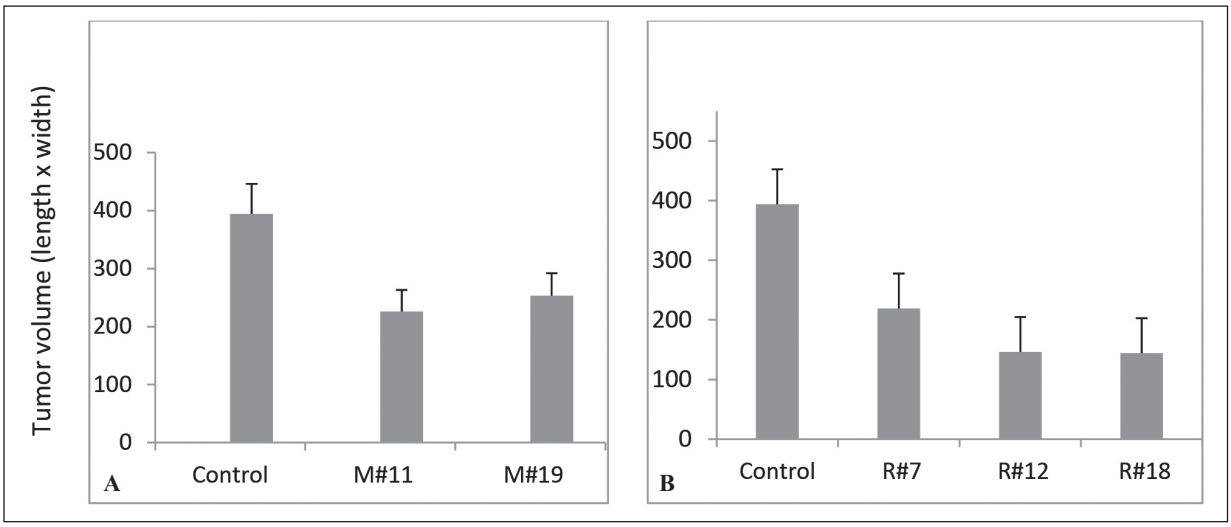

Fig. 2. Effects of vaccination on melanoma tumor volume. Tumor volume inhibition in mice immunized with mouse MMP-9 oligopeptides M#11 and M#19 ***(A)*** and rat oligopeptides R#7, R#12 and R#18 ***(B)***.

ods. Mice were assigned to five groups injected with test rat and mice peptides and four control groups with: injection with saline only, with Freud's adjuvant only, with Freud's adjuvant together with a carrier protein and with injection of mimicking peptide only.

Histopathology findings evaluating pathological changes in 23 organs in each mouse indicated that most animals had the presence of granulomatous inflammatory alterations around the abdominal organs particularly in the pelvis near the injection site (spleen, liver, pancreas, etc.).

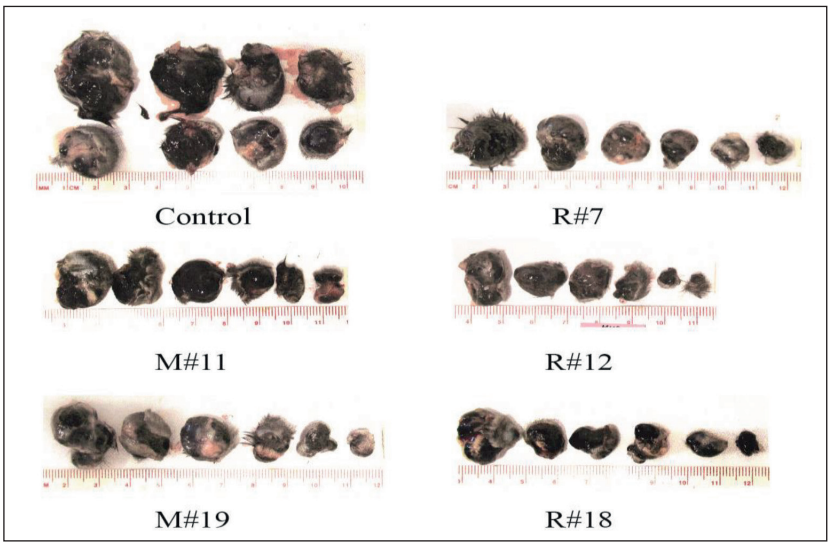

Fig. 3. Melanoma tumors in vaccinated and control mice. Photographs of melanoma tumors developed in mice immunized with MMP-9 oligopeptides M#11, M#19, R#7, R#12 and R#18 and in control mice

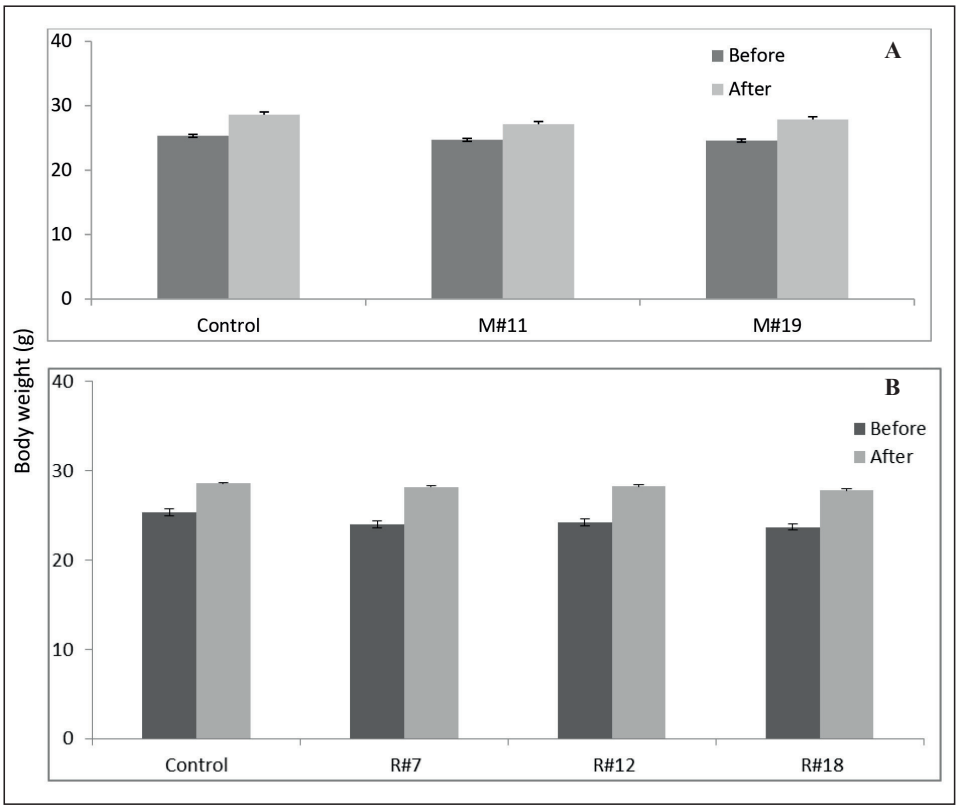

Fig. 4. Effect of immunization on mice body weight. Weight of male C57Bl/6 mice immunized with mouse MMP-9 oligopeptides M#11 and M#19 ***(A)*** and rat MMP-9 oligopeptides R#7, R#12 and R#18 ***(B)*** before and after challenging them with melanoma B16FO xenografts.

These changes are characteristic of the antigenic challenge with oil-containing adjuvants, such as Freund's adjuvant used in the vaccination and are most probably, indicative of non-specific reaction to intraperitoneal administration of test vaccines. The histopathological findings were consistent among all test animal groups and were omnipresent in the controls and immunized groups, except for group 1 (control-saline) displaying no signs of immunization related changes. The pathologist report did not indicate any peptides related to organ and metabolic toxicity.

The results in Figure 5 indicate that there were no significant changes in total body weight between test and control groups. No differences in weights of major organs were observed as well (results not shown).

The analysis of blood hematology and chemistry panels (Table 7) showed no statistically significant differences between the groups for the majority of parameters tested. Statistically significant differences were noted for total protein, which was associated with the globulin rather than albumin levels and not related to the immunization with test rat and mice peptides. There were variations in AST, ALT, and BUN between the groups; however all were within normal ranges for each parameter and not related to test oligopeptides (Table 7).

Professional expertise evaluating tissue and organ pathology and toxicity in this double-blind study concluded that changes induced by test-articles represent response to intraperitoneal administration of immunogenic substances, presumably antigen solutions emulsified with Freund's adjuvant. No specific toxic effects attributable to the test-oligopeptides themselves could be identified. Due to the large volume of data, only the summary findings, but not particular results, are presented.

Discussion

The results presented in this work corroborate our earlier findings which showed that oligopeptides containing human MMP-9 and MMP-2 sequences can generate immune response in mice and, as a result, significantly inhibit melanoma tumor growth in these animals[8]. These earlier studies prompted us to further pursue the development of a human anti-cancer vaccine by target-

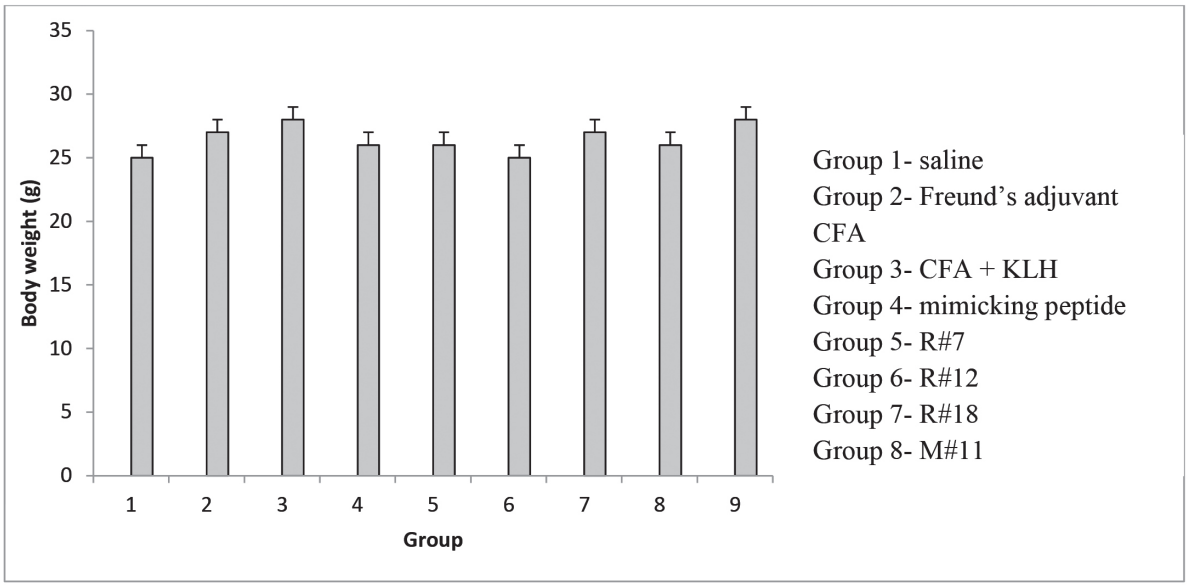

Fig. 5. Changes in body weight in mice challenged by different immunization articles. Control groups: 1- injection with saline only, 2- Freud's adjuvant only, 3-Freud's adjuvant together with a carrier protein (KLH), 4- mimicking peptide endothelin-1 only. Peptide challenged groups: rat peptides: 5- R# 7, 6–R# 12, 7-R#18, mouse peptides: 8-M#11, 9–M#19.

ing human MMPs as critical enzymes involved in cancer growth, angiogenesis and metastasis. As a next step, we tested whether immunization of mice with rodent MMP oligopeptides (a syngeneic approach) will stimulate immunogenicity and anti-cancer efficacy similarly to human MMPs peptides.

Here we have shown that two synthetic oligopeptides containing amino acid sequences from mice and three oligopeptides from rat MMP-9 were able to generate immune response in mice without significant tissue and metabolic toxicity. Subsequent challenge of the vaccinated mice with melanoma cancer cells resulted in a significant decrease in tumor weight and tumor volume. Interestingly, all five test peptides generated similar antibody titers in mice, and we could not detect a direct correlation between strength of the immune response and a decrease in tumor weight or volume. Among the MMP-9 peptides, the R#12 and R#18 were most effective in decreasing tumor volume (by 63%), and R#12 in reducing weight of melanoma tumors (by 77%). It is anticipated that the combinations of these peptides would increase their anti-tumor efficacy, which is under evaluation.

Conjointly, the *in vivo* results obtained from our earlier studies using oligopeptides derived from human MMPs and rodents (this study), show that targeting of MMP-9 and/or MMP-2 activity through vaccination has a distinct therapeutic potential towards the development of a universal vaccine effective against all types of cancer at the same time.

Our study confirms that effective control of cancer could be achieved through increasing ECM stability as already proposed by Rath and Pauling already in 1992[9]. Many studies already documented that anti-cancer effects of some individual natural compounds can be mediated through inhibition of activity and expression of MMP-9 and/or MMP-2[10, 11]. We have also shown that a synergistic combination of natural components has anti-cancer efficacy mediated by increased synthesis and integrity of ECM in addition to other effects. This has been documented in over 50 types of human cancer cells[12].

Also, in the past 20 years, various pharmacological approaches including the development of MMPs inhibitors to halt the spread of cancer, have been exercised. However, clinical trials using first generation MMP inhibitors proved to be disappointing[13,14].

Conclusions

This and our previous study confirm that developing a vaccine targeting the MMPs can be a viable option for effective control of cancer. If successful, this could lead to a significant reduction of the global human and economic burden of cancer – and long-term to the potential termination of this epidemic.

TABLE 7. Serum chemistry in mice injected with control and test oligopeptides. Group #1- injection with saline, Group #2 – injection with Freund's adjuvant, Group #3- injection with Freund's adjuvant + carrier protein Keyhole Limpet Hemocyanin (KLH),Group #4 – injection with mimicking peptide (endothelin-1); Group #5 – injection with R #7, Group #6 – injection with R #12, Group #7 – injection with R#18, Group #8 – injection with M #11, Group #9 – injection with M #19.

TEST	Mice Groups									ANOVA
	1	2	3	4	5	6	7	8	9	
TP, g/l	4.91±0.33	4.92±0.13	5.23±0.13	5.26±0.21	5.46±0.11	5.11±0.16	5.16±0.15	5.39±0.32	5.04±0.16	F (8, 36)=4.41, p=0.0009
Alb, g/l	3.02±0.16	2.94±0.06	3.02±0.11	3.07±0.14	3.12±0.08	3.09±0.06	3.14±0.07	3.04±0.09	2.96±0.07	F (8, 36)=2.43, p=0.0329
Glob, g/l	1.89±0.21	1.98±0.09	2.21±0.04	2.19±0.15	2.35±0.06	2.02±0.13	2.01±0.09	2.35±0.27	2.09±0.12	F (8, 36)=6.17, p<0.0001
Gluc, mmol/l	7.46±1.02	6.45±2.19	7.57±0.58	6.90±2.54	6.63±2.35	8.08±1.33	7.18±2.11	7.62±1.95	7.73±2.41	F (8, 36)=0.39, p=0.9200
Chol, mmol/l	3.33±0.41	2.99±0.17	2.92±0.28	3.19±0.28	3.24±0.42	3.39±0.09	3.00±0.25	3.23±0.17	2.66±0.17	F (8, 36)=3.69, p=0.0031
TG, mmol/l	0.79±0.05	0.76±0.10	0.76±0.08	0.66±0.08	0.67±0.13	0.80±0.28	0.83±0.12	0.51±0.13	0.75±0.19	F (8, 36)=2.20, p=0.0512
AST, u/l	89.5±28.4	92.7±10.8	89.2±16.3	120.4±22.6	106.0±22.5	77.5±12.2	99.4±15.8	74.0±9.3	97.9±35.6	F (8, 36)=2.28, p=0.0438
ALT, u/l	35.5±9.1	37.7±4.4	33.4±2.1	51.9±18.3	39.1±11.7	31.0±7.0	35.4±5.6	29.4±3.9	35.5±7.7	F (8, 36)=2.57, p=0.0248
LDH, u/l	686±345	693±123	739±261	1001±492	631±196	545±158	681±296	646±198	760±352	F (8, 36)=0.94, p=0.4936
ALP, u/l	269±52	271±42	236±26	286±36	295±35	265±29	285±49	248±38	274±31	F (8, 36)=1.17, p=0.3426
Amy, u/l	444±30	426±17	451±54	409±52	453±20	464±48	424±59	477±55	408±25	F (8, 36)=1.58, p=0.1645
Crea, µmol/l	28.4±12.8	28.5±3.8	28.4±8.0	34.3±1.7	30.0±5.2	31.2±7.4	29.3±5.9	31.4±4.1	32.5±5.4	F (8, 36)=0.42, p=0.9024
BD, µmol/l	1.93±0.96	2.05±1.18	1.90±0.89	1.15±0.76	2.57±1.08	1.54±0.56	1.69±0.93	1.92±0.61	1.54±0.56	F (8, 31)=0.88, p=0.5453
BT, µmol/l	1.78±0.64	2.35±0.89	1.59±1.52	1.84±1.17	1.83±1.14	2.19±0.78	1.77±1.95	1.45±0.70	1.49±0.50	F (8, 29)=0.32, p=0.9508
BUN, mmol/l	5.88±0.59	5.71±1.54	6.48±1.00	5.99±2.02	6.17±0.53	5.36±0.95	6.06±1.01	4.29±0.44	5.16±1.23	F (8, 36)=1.66, p=0.1413
Ca, mmol/l	2.61±0.14	2.66±0.14	2.64±0.03	2.73±0.20	2.61±0.07	2.48±0.09	2.54±0.08	2.52±0.04	2.47±0.07	F (8, 36)=3.28, p=0.0065
Na, mmol/l	156±2	158±1	159±2	158±3	154±2	154±2	156±3	155±2	158±4	F (8, 36)=2.60, p=0.0237
K, mmol/l	5.15±0.70	5.86±0.98	5.53±0.27	6.61±0.68	6.19±0.93	5.55±0.47	5.21±0.60	5.32±0.49	5.59±0.42	F (8, 36)=2.69, p=0.0200

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

Authors declare no conflict of interest.

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