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ANTI-TUMOR ACTIVITY AND IMMUNE RESPONSES INDUCED BY HUMAN CANCER-ASSOCIATED MUCIN CORE PEPTIDE[1]

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Objective: To investigate the immune responses induced by apomucin which is a mixture of mucin core peptide, in mice for elucidating the role of mucin core peptide in the modulation of cancers. Methods: Apomucin was isolated from human pancreatic cancer cell line SW1990. The mice were immunized with this apomucin (10μg/time×6) plus DETOX. Results: When immunized, all mice developed delayed-type hypersensitivity (DTH) after challenged with apomucin or synthetic peptide MUC-2 or MUC-3, while the mice immunized with apomucin alone did not develop DTH. No antibodies were detected by ELISA after immunization. When the spleen cells of vaccinated mice were cocultured with this apomucin (10—50μg/ml) and rhIL-2(50U/ml) *in vitro*, the proliferated lymphocytes showed cytotoxicity against human cancer cells, including colon cancer, gastric cancer, pancreatic cancer and leukemia as measured by Cr-51 release assay. Antibodies against MUC-2 and MUC-3 could block the cytotoxicity. Conclusion: It was identified that a vaccine combined of apomucin and immune adjuvant DETOX can induce cellular immune response and anti-tumor cytotoxicity in mice.

Key words: Neoplasm, Immunology Mucin, Mucin core peptide

Mucins are expressed on the surface of most human adenocarcinoma cells, the molecular structure of cancer cells mucin is different from normal cells. The alteration of molecular structure of mucin carbohydrates has been significantly explored by monoclonal antibody techniques, but that of mucin core peptides is progressively elucidated in recent years with the proceedings of molecular biological techniques. Seven cDNAs of mucin core peptides were cloned, which have been separately named MUC1 to MUC7. Among them, only MUC1 was proved to be capable of inducing human humoral and cellular immune response.[1-3] A vaccine derived from MUC1 is now going on phase I and II clinical trials.[4] This study was to investigate the anti-tumor immune response induced by apomucin (which is a mixture of mucin core peptides, at least MUC2 and MUC3).

MATERIALS AND METHODS

Cell Lines and Antigens

Human pancreatic cancer cell line SW1990, gastric cancer cell line Kato-3, colon cancer cell line Ls174t, leukemia cell line K562 were cultured in DMEM medium with 5% FBS. synthetic peptides MUC2 and MUC3 were complimentary from Prof. Y S Kim in GI Res. Lab. of UCSF, USA.

Immunogen

Apomucin was isolated and purified from SW1990 in this Lab., which was a mixture of MUC2 and MUC3 immunogens at least and contained 88% amino acids. The molecular weight of it was from 28,000 to 90,000 deltons.

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Vaccination

Forty five female BALB/c mice about six to eight weeks old were divided equally into three groups, fifteen mice per group. The vaccine was given subcutaneous (s.c.) 6 times at a 14 days interval. Group one was immunized with 10μg apomucin alone. 0.9% NaCl as control.

Quantitation of Antibody

The humoral response to apomucin was determined by ELISA. Serum samples were collected at weekly interval and analyzed for the presence of antibody reacted with apomucin. Apomucin 10μg per well was coated overnight at 4℃, 2% bovine serum albumin was used to prevent nonspecific binding, followed by 2h incubation with 100 dilutions of test serum, then incubated with ABC kits which was purchased from Zymed Lab.

DTH Test

DTH assays were performed in mice by the measurement of footpad swelling. Seven days after the last immunization, 10μg apomucin were injected intradermal (i.d.) into the left hind paw, 10μg human serum albumin (HSA) was injected i.d. into the right hind paw. The thickness of footpads was measured at 0, 2, 6, 12, 24, 48, 72 and 96 h with a micrometer.

Lymphocyte Proliferation Assay

Seven days after last vaccination, spleens were mechanically minced and dispersed through stainless-steel mesh, erythrocyte and dead cells were removed by centrifugation over a Ficoll-Hypaque gradient. Lymphocytes were washed and resuspended in culture medium, which consisted of RPMI1640, 5% human serum, apomucin 50μg/ml, and rhIL-2 50U/ml, for up to 2 days, cultures were pulsed with (^{3}H) thymidine 1850Kbq/ml, 10μg/well, for 4h. Cells were collected, and incorporated radioactivity was measured by liquid scintillation spectrometer (LKB 1219).

Cytotoxic Assay

Seven days after the last vaccination, spleen cells were removed and processed as described above. 1×10^6 cancer cells were radiolabelled with $Na_2{}^{51}CrO_4$ (Institute of Chinese High Energy Physics) for 120min at 37℃, followed by thoroughly washing to remove unincorporated isotopes. T cells and target cells (1×10^4 cells/well), both resuspended in culture medium, were then combined at a ratio of 10: 1 for effector to target cells, centrifuged 100g for 5 min to initiate cell contact and then incubated for 4h at 37℃ with 5% CO_2. After incubation, the supernatants were collected and radioactivity was quantitated in a gamma counter (FT-646). Spontaneous release of ^{51}Cr was determined by incubation of target cells in the absence of effectors. The maximum release of 51Cr was determined by lysis of target cells with distilled water. Percentages of specific release of ^{51}Cr was determined by the following equation:

Percent specific release (lysis%)=
(experimental release-spontaneous release)/
(maximum release-spontaneous release)
×100

Also, the cytotoxicity of supernatants of cultured spleen cells was quantitated as above. Blocking the cytotoxicity of spleen cells was examined by adding the antibodies of anti-MUC2 and MUC3 into target cells at 37℃ for 60 min before adding the effectors. Antibodies against apomucin in the supernatants of cultured spleen cells were also tested with ELISA.

RESULTS

Anti-apomucin Antibody

No antibody was examined in serum of vaccinated mice.

DTH

DTH was induced in all mice in group one immunized with apomucin and DETOX after the mice challenged with apomucin or synthetic peptide of MUC-2 and MUC-3, but it was not in other mice in group two and group three. No footpad swelling was found when mice challenged with HSA (Table 1).

Lymphocyte Proliferation

Lymphocyte proliferation in mice of group one

was higher than that in mice of group two, and lymphocyte proliferation in both group 1 and 2 were higher than that in group 3. The rate of spleen cell proliferation increased following the amount of apomucin added into culture medium (from 10μg/ml to 40μg/ml), but decreased while the amount of mucin core peptide was over 50μg/ml (Table 3).

Table 1. DTH response after vaccination (30h after antigen injected)

Group	Immunogen	Stimulated antigen	Animal No.	Footpad thickness (0.01mm)
1	Apo+Detox	apomucin	5	48.0+11.14
2	Apo+Detox	MUC-2	5	42.2+19.9
3	Apo+Detox	MUC-3	5	42.0+15.9
4	Apo+Detox	HSA	5	6.8+5.02
5	Apo	apomucin	5	15.2+4.15
6	Apo	HSA	5	10.4+6.23
7	NaCl	apomucin	5	4.4+1.67
8	NaCl	HSA	5	4.8+4.15

1 vs 4, 2 vs 4, 3 vs 4, and 1,2,3, vs 7,8 $P<0.001$

Table 2. Lymphoproliferative response of mice immunized with apomucin

Immunogen	Animal No.	(^{3}H)thymidine incorporation (cpm)	P value
apomucin+Detox	3	962.49+188.93	<0.001
apomucin	3	516.57+292.84	<0.02
NaCl	3	218.00+96.78	

Table 3. Lymphoproliferation with various quantity of apomucin

Apomucin(μg/ml)	(^{3}H)thymidine corporation (cpm)	Animal No.
50	1178.64+285.14	6
40	1521.64+774.21	6
30	1070.82+460.68	6
25	962.49+188.93	6
20	868.22+532.58	6
10	844.59+319.39	6
0	790.84+157.92	6

Cytotoxicity

Lymphocytes from group one mice can mediate lysis of cells SW1990 and Kato-3 and Ls174t and weakly lysis of cells K562. The lysis percentage of spleen cells from group one mice was higher than that from group two mice, and both were higher than that of group three mice (Table 4). Cytotoxicity of spleen cells from group one mice were inhibited at various degrees when antibodies of anti-MUC2 or anti-MUC3 added into the medium. The inhibition rates were 26—52% against SW1990 and 33% against Kato-3 by LSB (anti-MUC2), 25—46% against SW1990 and 7—20% against Kato-3 by M3P (anti-MUC3). These results indicate that the epitopes of MUC2 and MUC3 on cancer cells surface can be recognized by spleen cells of vaccinated mice, if they were blocked by anti-MUC2 or anti-MUC3 antibodies, the cytotoxicity of mice spleen cells can be inhibited.

Table 4. Cytotoxic splenic cells mediate lysis against tumor cells by ^{51}Cr release assay E/T=10

Immunogen	Animal No.	SW1990	Target cells Kato-3	Ls174t	K562
apomucin+Detox	3	11.4+2.5	20.5+7.5	25.4+4.4	12.2+2.7
apomucin	3	4.1+1.4	7.0+1.5	9.8+1.2	7.5+1.8
NaCl	3	1.6+1.4	2.0+1.3	3.7+1.2	4.4+0.3

Table 5. Supernatant of splenic cells mediate lysis against tumor cells by ^{51}Cr release assay E/T=10

Immunogen	SW 1990	Target cells Kato-3	Ls174t	K562
apomucin+Detox	10.29	4.75	27.07	27.47
apomucin	3.47	9.59	18.23	16.35
NaCl	5.54	0	0	0

Cytotoxicity of Supernatant of Cultured Spleen Cells

The supernatant of cultured spleen cells from group 1 mice also mediated lysis of tumor cells (Table 5).

DISCUSSION

Some human tumor associated antigens can induce humoral and cellular immune response,[5,6] especially cellular immune response that plays an important role in leading to tumor growth suppression.

In this study, it was identified that a vaccine combined of apomucin and immune adjuvant DETOX can induce cellular immune response and anti-tumor cytotoxicity in mice. While without DETOX, the apomucin alone could induce weakly cytotoxicity but it could not induce DTH reaction. This means immune adjuvant DETOX is important and potential in cellular immune response induced by apomucin vaccine, and may play an important role in antigen presentation. At the same time, this vaccine did not induce humoral immune response, this may be due to: (1) the quantity of antigen was too small, (2) immune adjuvant DETOX may inhibit humoral immune response.

The apomucin used in this study, was a mixture of two mucin core peptides, MUC2 and MUC3 at least. Whether MUC2 and MUC3 could induce cellular immune response is not understood clearly. The results in this experiment show that MUC2 and MUC3 could induce cellular immune response also, because (1) both MUC2 and MUC3 injected subcutaneously can induce DTH following apomucin vaccination (2) antibodies against MUC2 and MUC3 could partly inhibit the cytotoxicity of spleen cells of vaccinated mice. Peptide MUC2 and MUC3 may be the epitopes recognized by T cells and play an important role in the immune response induced by this apomucin vaccine. Whether the purified MUC2 and MUC3 can induce the immune response and what amino acid motifs of MUC2 and MUC3 function mainly in the cellular immune response induced by this apomucin vaccine remain to be further studied.

Even though this vaccine induce strongly cytolysis against adenocarcinoma cells which expressed the epitopes of MUC2 and MUC3, it also induce weakly cytolysis against K562 cells that do not express the epitopes of MUC2 and MUC3. Whether any epitopes of K562 cells are included in this apomucin which recognized by T cells or NK cells that are activated by this vaccine need to be clarified. Whether the cytotoxicity induced by this vaccine is toxic to normal cells and tissues also remains to be further studied.

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