P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wcjd@public.bta.net.cn www.wjgnet.com

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• LIVER CANCER •

Preparation of monoclonal antibody against apoptosis-associated antigens of hepatoma cells by subtractive immunization

Lian -Jun Yang, Wen-Liang Wang

Lian-Jun Yang, Wen-Liang Wang, Department of Pathology, Institute of Cancer Research, The Fourth Military Medical University (FMMU), Xian 710032,Shaanxi Province, China
Supported by Army Science Foundation, No.98M111
Correspondence to: Wen-liang Wang, Department of Pathology, Institute of Cancer Research, FMMU, Xi' an 710032, Shaanxi province, China. wlwang@fmmu.edu.cn
Telephone: +86-29-3284284 **Fax:** +86-29-3284284
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Abstract

AIM: To elucidate the expression of the apoptosis-associated molecules in human primary hepatocellular carcinoma (HCC) cells, and prepare the monoclonal antibodies (mAb) against the apoptosis-associated antigens of HCC cells.

METHODS: Human HCC cell line HCC-9204 cells were induced apoptosis with 60 mL·L^{-1} ethanol for 6 h and their morphological changes were observed by transmission electron microscope. The cell DNA fragmentations were detected by Terminal Deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, and the cell DNA contents by flow cytometry. Ten mice were immunized with ethanol-induced apoptotic HCC-9204 cells with the method of subtractive immunization, while the other 10 mice used as the control were immunized by the routine procedures. The tail blood of all the mice were prepared after the last immunization, and the produced antibodies were determined by the immunocytochemical ABC staining. The splenic cells of the mice whose tail blood sera-HCC-9204 cells serum reactions were most different between the apoptotic and the non-apoptotic were prepared and fused with the mouse myeloma cell line SP2/0 cells. The positive antibodies were selected by ELISA assay. The fusion rates of hybridoma cells and the producing rates of antibodies were calculated. The fused cells that secreted candidate objective antibody were cloned continually with the of limited dilution method, and then selected and analyzed further by the immunocytochemical ABC staining. The chromosomes of the cloned hybridoma cells that secreted objective mAb and the mAb immunoglobulin (Ig) subtype of the prepared mAb were also determined. The molecular mass of the mAb associated antigen was analyzed by Western blot assay.

RESULTS: HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h, manifested obvious apoptotic morphological changes, the majority of the cells were TUNEL-positive, and the sub-G1 apoptotic peak was evident. There were 2 mice in the experimental group whose tail blood serum reacted strongly with the apoptotic HCC-9204 cells, but weakly with their non-apoptotic counterparts.In the fusion rates of hybridoma cells as well as the producing rates of the antibody deseribed above, there did not show significant difference between the experimental and the control group, but weakly with non-apoptotic HCC-9204. However, the total producing rate of antibodies in the experimental group was significantly lower compared with the control ($P<0.01$), and so was the producing rate of the antibodies which reacted strongly with both apoptotic and non-apoptotic HCC-9204 cells($P<0.01$). After cloned continually for several times the cell that produce mAb which reacted strongly with the nuclei of ethanol-induced apoptotic HCC-9204 cells, but very weakly with that of non-apoptotic cells was selected out. Chromosome analysis revealed that the selected cell was with the universal characteristics of the monoclonal hybridoma cells which secreted mAb, and the Ig subtype of the prepared mAb was IgG1. The molecular mass of this mAb associated antigen of was about 75 ku.

CONCLUSION: Subtractive immunization is a useful method to prepare the mAb against the apoptosis-associated antigens of cells. The expression of some molecules increases to some extent in HCC-9204 cells in the process of apoptosis induced by low-concentration ethanol. The mAb that may be against ethanol-induced apoptosis-associated antigens of HCC cells was successfully prepared and primarily identified.

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INTRODUCTION

Apoptosis is a process of active programmed cell death(PCD), which can be regulated by many kinds of biological factors encoded by a lot of mammalian genes[1-16]. There is not only the increase or decrease of the expression of some already-existed proteins in the process of apoptosis, but also the production and presentation of some new apoptosis-associated molecules that do not express in non-apoptotic cells[17-20]. Currently, the knowledge about apoptosis-associated molecules is still limited. The conventional process to discover new apoptosis-associated molecules is to clone and sequence the apoptosis-associated genes of cells by the methods such as differentiated PCR and phage display first, and then performe the experiments to study the function of the expressed product of the candidate apoptosis-associated genes[21-25]. Preparing the antibodies against the associated antigens of apoptotic cells is also a very hopeful way to investigate apoptosis-associated molecules. There are already some reports about the successful preparation of the polyclonal antibodies against apoptosis-associated molecules while their antigens are unclear and even monoclonal antibodies (mAb) at the condition that their associated antigens are specific[19, 26,27]. However, there has been no report until now about preparing the mAb against apoptosis-associated antigens at the condition that its associated antigens are still unspecific. In the present study, some mice were immunized by the method of subtractive immunization, and the splenic cells of the effectively immunized mice were fused

with mouse myeloma cells to prepare the mAb against the apoptosis-associated antigens of human primary hepatocellular carcinoma (HCC) cells, so as to elucidate the expression of the apoptosis-associated molecules of HCC cells and investigate the methodology and feasibility to prepare mAb against the apoptosis-associated molecules of cells at the condition that the associated antigens are not clear.

MATERIALS AND METHODS

Cells, animals and main reagents

Human HCC cell line HCC-9204 was established previously by our department. Balb/c mouse myeloma cell line SP2/0 was kindly provided by Mrs. Su-Zhen Zhang, Department of Genetic and Developmental biology of our university. Ten-wk old female Balb/c mice were provided by Expereimental Animals Center of our university. Cyclophosphamide (CP), PEG4000, HT (The compound of hypoxanthine and thymidine), HAT (The compound of hypoxanthine, aminopterin and thymidine), 3,3' -diaminobenzine tetrahydrochloride (DAB), 3-aminopropyltriethoxysilane (APES), propidium iodium (PI), colchicine and Giemsa were the products of Sigma Chemical Co., USA. RNase A was the product of Promega Co., USA. HRP-labeled sheep anti-mouse IgG mAb was the product of Dako Co., USA. Terminal Deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit was the product of Boehringer Mannheim Co., Germany. Immunohistochemical ABC kit was the product of Vector Laboratories Inc., USA. Mouse hybridoma immunoglobulin (Ig) subtyping kit was the product of Roche Molecular Biochemicals, USA.

Apoptosis induction and detection

Apoptosis induction HCC-9204 cells at log phage were cultured with RPMI1640 medium containing 60 mL· L^{-1} ethanol for 6 h to induce cellular apoptosis, and non-treated HCC-9204 cells was used as control.

Electron microscope observation The cells were fixed in glutaraldehyde, fixed further in osmium tetroxide, dehydrated and embedded in epikote. The sections were ultra thin, stained doubly with uranyl acetate and lead citrate, and bobserved with transmission electron microscope (JEC Co., Japan).

TUNEL assay[28] The cells cultured on cover slips were fixed in formitrol, and then put into penetrating solution (1 g· L^{-1} sodium citrate containing 1 g· L^{-1} Triton X-100) and reacted at 4 ℃ for 2 min. The cells were stained with TUNEL reaction solution contained FITC-labeled nucleotide and deoxynucleotide terminal transferase at 37 ℃ for 60 min, and then observed with fluorescence microscope.

DNA contents analysis The cells were fixed in 700 mL· L^{-1} cold ethanol. RNase A (1 g· L^{-1}) was added into the suspending solution of cells and reacted at 37 ℃ for 30 min. Then PI (0.1 g· L^{-1}) was added with the four times volume of that of the above solution and reacted at 4 ℃ for 30 min. The DNA contents of different cell cycles were analyzed with flow cytometer (Coulter Co., USA).

Methods of antibody detection

ELISA assay The cells adhered to 96-well culture plate with polylysine were fixed in 0.2 mL· L^{-1} glutaraldehyde. The supernatant of the culture medium of hybridoma cells were used as the primary antibody, and the PBS as its negative control. The HRP-labeled sheep anti-mouse IgG mAb was used as the second antibody. The indirect ELISA assay was performed with the standard procedures. Optical absorbance (*A*) was measured at 490 nm with ELISA detector (Bio-Rad Co., USA).

Immunocytochemical staining[29] The cells cultured on cover slips were fixed in 950 mL· L^{-1} ethanol. The supernatant of the culture medium of hybridoma cells was used as the primary antibody, and the PBS as its negative control. The immunocytochemical ABC staining was performed with routine procedures illuminated by the protocol of Vector Laboratories Inc.

Subtractive immunization for mice

Twenty Balb/c mice were divided randomly into 2 groups. The ten mice in the experimental group were immunized firstly with the non-apoptotic HCC-9204 cells at log phase through cavum abdominis. Then the mice were injected with CP (0.1 g· kg^{-1} body mass) into the cavum abdominis at 10 min, 24 h and 48 h respectively after the first immunization. At the 14th, 21st and 28th d after the first immunization, the mice were further immunized with the ethanol- induced apoptotic HCC-9204 cells through the same route. The ten mice in the control group were immunized with the ethanol-induced apoptotic HCC-9204 cells through cavum abdominis for 3 times at an interval of 2 wk. The tail blood sera of animals in both group were respectively prepared after the last immunization, and the immunocytochemical ABC staining was performed as described above to detect the production of antibodies.

Cell fusion and cloning

The mice whose tail blood sera-HCC-9204 cells reactions were most different between the apoptotic and the non-apoptotic were selected from the experimental group. The splenic cells of the selected mice were prepared at the 3rd day after the last immunization, and fused with SP2/0 cells at log phase promoted by PEG4000. Two 96-well culture plates were used for every mouse. The fused cells were cultured continually with RPMI1640 medium containing HAT and HT. Five d later, the culture wells where the monoclonal hybridoma cells were growing were marked and the producing rate of the clones, i. e. the fusion rate, of hybridoma cells was calculated. When the growing hybridoma cells had covered about 1/2 of the bottom area of a culture well, the antibodies that reacted strongly with ethanol-induced apoptotic HCC-9204 cells, but weakly with the non-apoptotic were selected by the ELISA assay as described earlier and the producing rate of antibodies was calculated. The data was statistically analyzed by χ^2 test. The interested cells were cloned continually by the method of limited dilution, selected and analyzed further by the immunocytochemical ABC staining.

Identification of the characteristic of mAb

Chromosomes of hybridoma cells The cells cultured on cover slips were treated with colchicine and stained with Giemsa by the routine procedures. One hundred hybridoma cells at metaphase were counted and observed.

Ig subtype of mAb The detection and analysis of the Ig subtype of mAb was performed with standard procedures illuminated by the protocol of Roche Molecular Biochemicals.

Molecular mass of mAb-associated antigen Some ethanol-induced apoptotic and non-apoptotic HCC-9204 cells were burst and shattered respectively by ultrasonic and the proteins of cells were prepared by the routine methods. Then SDS-PAGE was performed. The supernatant of the culture medium of hybridoma cells that secreted objective mAb was used as the primary antibody. HRP-labeled sheep anti-mouse IgG mAb was used as the second antibody. The proteins in the gel of SDS-PAGE were transferred to colloxylin filter film by Western blot system (Bio-Rad Co., USA) and stained following the protocol of Bio-Rad Co.. The color was developed with freshly dispensed DAB.

RESULTS

Detection of apoptotic cells

Electron microscope observation The majority of the cells treated with 60 mL· L^{-1} ethanol for 6 h showed cellular pyknosis and were densely stained. The processes and microvilli on the cell surface decreased significantly or even disappeared. Granular, crescent or cricoid nuclei (Figure 1), chromatin margination, as well as cytoplasm bubbling were observed. Endoplasmic reticulums expanded and its vacuolation occurred in some cells.

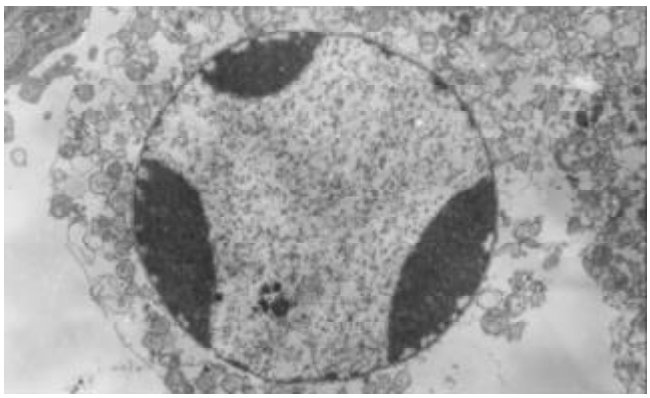

Figure 1 Morpholoical changes of HCC-9204 cells treated with 60 mL·L^{-1} etjanol for 6 h TEM ×6000

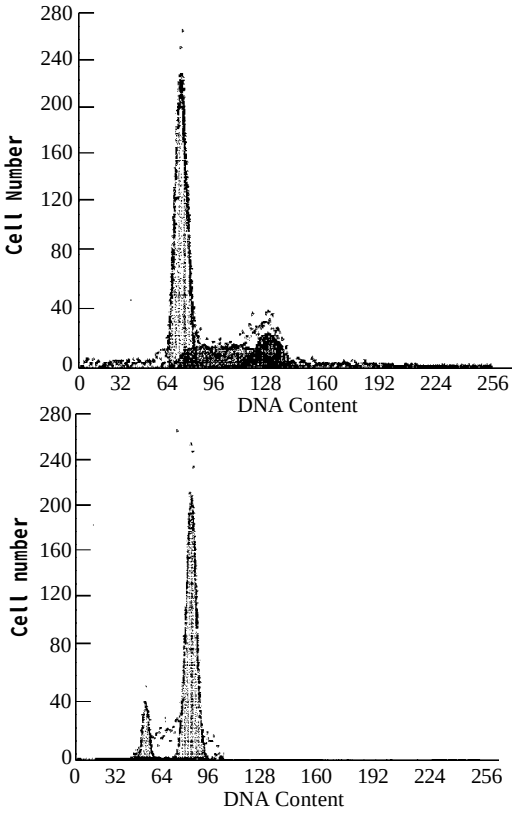

Figure 3 DNA contents of HCC-9204 cells treated with 60 mL·L^{-1} for 6 h. A: Non-treated HCC-9204 cells; B:HCC-9204 cells treated with 60 mL·L^{-1} etjanol for 6 h

TUNEL assay

The positive signal of TUNEL reaction was green or yellow in color within the round, oval, crescent or cricoid, nuclei of cells. The majority of the apoptotic cells in the experimental group were TUNEL-positive (Figure 2).

DNA contents analysis

Compared with that in the control of non-treated HCC-9204 cells, there was obvious sub-G1 apoptotic peak in the experimental group (Figure 3).

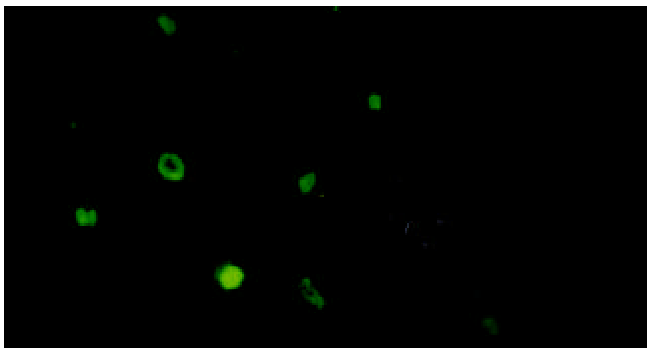

Figure 2 Detection of HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h by TUNEL assay ×200

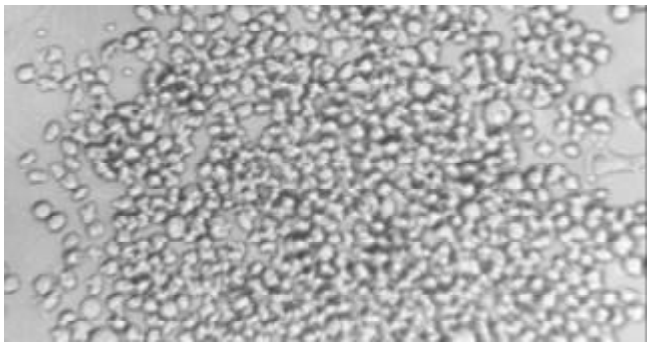

Figure 4 Monoclonal hybridoma cells growing in culture well. ×100

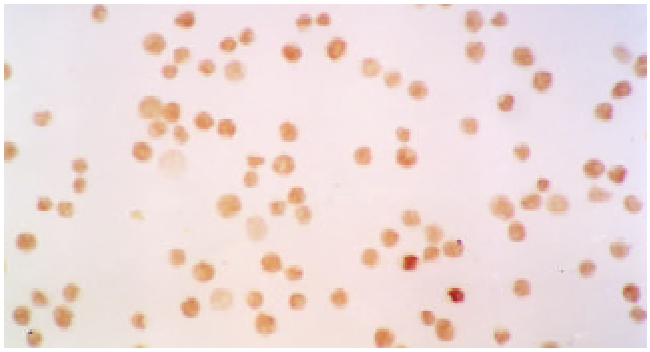

Figure 5 Immunocytochemical localization of the prepared mAb in HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h. ABC ×200

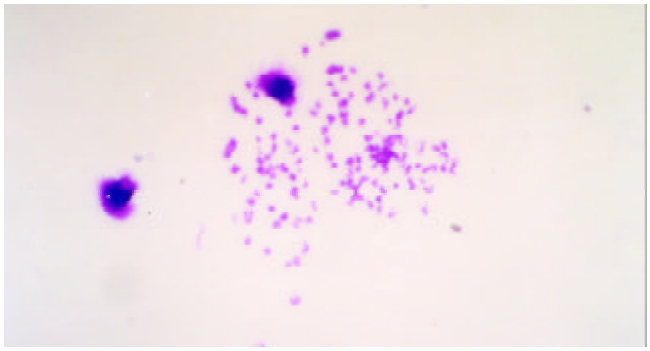

Figure 6 The chromosomes of the hybridoma cells which secrete objective mAb. ×400

Table 1 The fusion rates of hybridoma cells and the producing rates of antibodies

Group	Fusion wells / Incubated wells (Fusion rate)	I(I / Fusion wells)	II(II / Fusion wells)	III(III / Fusion wells)	(I+II+III) ((I+II +III) / Fusion wells)
Experiment	241 / 384(62.8%)	46(19.1%)[a]	5(2.0%)	0(0%)	51(21.2%)[a]
Control	262 / 384(68.2%)	101(38.5%)	4(1.5%)	1(0.4%)	106(40.5%)

[a]$P<0.01$ *vs* Control. I: The wells whose antibodies reacted strongly with both apoptotic and non-apoptotic cells; II: The wells whose antibodies reacted strongly with apoptotic cells, but weakly with non-apoptotic cells(Difference is>8 times); III: The wells whose antibodies reacted weakly with apoptotic cells, but strongly with non-apoptotic cells (Difference is >8 times).

Subtractive immunization for mice

The tail blood sera of 8 mice in the experimental group and 10 mice in the control reacted strongly with both apoptotic and non-apoptotic HCC-9204 cells. However, the tail blood sera of the 2 other mice in the experimental group reacted strongly with apoptotic, but weakly with non-apoptotic HCC-9204 cells.

Cell fusion and cloning

Monoclonal hybridoma cells were growing in many wells of the culture plates at the 5th d after cell fusion (Figure 4).

In the fusion rate of hybridoma cells, as well as the producing rate of the antibodies that reacted strongly with apoptotic, but weakly with non-apoptotic HCC-9204 cells, there did not show significant difference between the experimental and the control group. The total producing rate of antibodies in the experimental group was significant. However, lower compared with that in the control group ($P<0.01$), and so was the producing rate of the antibodies that reacted strongly with both apoptotic and non-apoptotic HCC- 9204 cells ($P<0.01$, Table 1).

An mAb that reacted strongly with the nuclei of ethanol-induced apoptotic, but very weakly with that of non-apoptotic HCC-9204 cells was obtained after the hybridoma cells which secreted candidate objective mAb had been cloned continually for several times (Figure 5).

Characteristic identification of mAb

Chromosomes of hybridoma cells The number of the chromosomes in the hybridoma cells which secreted objective mAb was 83 - 105, their modal number was 94 - 98, and the majority of the chromosomes were the type of terminal centromere, which is consonant with the universal characteristics of the hybridoma cells which secrete mAb (Figure 6).

Ig subtype of mAb The subtype Ig of the prepared mAb was IgG1.

Molecular mass of mAb-associated antigen The molecular mass of the prepared mAb associated antigen was about 75 ku (Figure 7).

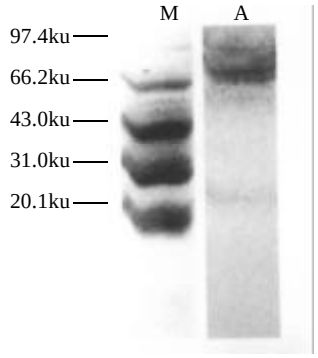

Figure 7 The molecular mass of the associated antigen of the prepared mAb. M: Standard molecular mass; A:HCC-9204 cells treated with 60 mL·L^{-1} ethanol for 6 h

DISCUSSION

To investigate the changes of the apoptosis-associated molecules on cell surface and the production of new antigens in the process of apoptosis is important in many fields of biomedical studies such as the mechanism of signal transduction and molecular regulation in cells and cellular immunology, *etc*., and many mammalian gene-encoded proteins can induce or suppress apoptosis in a variety of cell types[1-16]. It was discovered previously by the method of 2-dimentional gel electrophoresis that there are 17 new proteins, whose molecular mass are 12 - 80 ku, expressed in some cell types undergoing apoptosis induced by the transfection of wild-type *p53*[17]. A mitochondrial membrane protein 7A6 expresses only in some kinds of apoptotic but not in non-apoptotic cells, and its mAb has been used in the detection of apoptosis in some cell types[19, 26, 30-32]. The facts cifed above indicate that in the process of apoptosis, there exists not only the up or down-regulation of some proteins expressed already in non-apoptotic cells, but also the production of new proteins that did not exist in non-apoptotic cells.

Low-concentration ethanol can induce apoptosis in some cell types such as T lymphocytes, natural killer cells, macrophage, neutrophils, nerve cells, reproduction stem cells, mucosa epithelial cells, salivary gland cells, osteoblast, thyroid epithelial cells, leukemia cells, hepatocytes and HCC cells, *etc*.[33-40]. The results of present study showed that the majority of the HCC-9204 cells treated with 60 mL· L^{-1} ethanol for 6 h manifested marked morphological changes of apoptosis,the majority of the cells were TUNEL-positive, and the sub-G1 apoptotic peak detected by flow cytometry was obvious, suggested that low-concentration ethanol could induce apoptosis obviously in HCC-9204 cells and the model of ethanol-induced apoptosis in HCC cells was established successfully. The previous study with SDS-PAGE and folium scan in our laboratory have revealed that at least 7 kinds of new proteins whose molecular mass are more than 67 ku expressed in the HCC-9204 cells treated with 60 mL· L^{-1} ethanol for 6 h but not in non-apoptotic cells. It is well known that HCC is a common malignant tumor in China and some other places in the world, and its oncogenesis and development are related with apoptosis[41-60]. Therefore the ethanol-induced apoptotic HCC-9204 cells were used as antigen to prepare mAb against the apoptosis-associated antigens of HCC cells in this experiment.

Most of the proteins encoded by apoptosis-associated genes express only instantaneously in cells, and the quantity of the expressed products of apoptosis-associated genes is usually very little. The general procedures of the routine of immunization for the preparation of mAb are that the mice were further immunized twice respectively with the same antigen as the first immunization at an interval of 2 wk. In our previous studies, more than ten rabbits have been immunized with ethanol-induced apoptotic HCC-9204 cells by routine immunization procedures, there was no rabbit found whose

ear blood serum reacted strongly with apoptotic, but weakly with non-apoptotic cells. There is also no mouse whose tail blood serum reacted differently with apoptotic and non-apoptotic cells in all the 10 mice used as control in this experiment. The those results suggest that the antibodies against cell apoptosis-associated antigens are difficult to be prepared by the routine immunization procedures because of the interference of the large amounts of antigens not associated with apoptosis in the apoptotic cells.

Subtractive immunization is a special method of differentiated immunization used for the preparation of antibodies when the antigen components are complicated, unclear or cannot be extracted and purified[61-65]. The mechanism of subtractive immunization is that when certain antigens induce the immunoreaction of a animal body, the lymphocytes will proliferate, and the cells in split phase are prone to be selectively killed by the drug containing alkyls such as CP that is in common use in the procedures of subtractive immunization. When the same antigen is injected into the animal body again after the first immunization, the immunoreaction to the antigen is suppressed. CP can effectively inhibit the production of the antibodies against non-objective components of the antigen, but the production of objective antibodies cannot be influenced obviously, and neither are the fusion rates of hybridoma cells. Therefore the selection workload in the process of preparing mAb could reduce remarkably. The standard procedures of subtractive immunization are as the followings. Firstly, 2 groups of mixed antigens A and B whose components were similar were prepared. There is objective antigen in group A, but no objective antigen in group B. Secondly, group B is firstly used to immunize some animals. At the meantime, the immunized animals are treated with CP, or CP is injected 10 min, 24 h and 48 h later. Thirdly, the animals are further immunized with group A for 3 times respectively at an interval of 1 wk. At this time, the immunoreaction of the animal body to the common components of group A and group B is suppressed. Therefore the interference of non-objective antigens to the preparation of objective mAb is wakened or even may be eliminated, the immunoreaction to the objective antigens in group A is enhanced relatively, and the probability to obtain specific mAb increases significantly[48].

There were 2 mice whose tail blood sera reacted strongly with ethanol-induced apoptotic HCC-9204 cells, but weakly with non-apoptotic HCC-9204 cells in 10 mice immunized by subtractive immunization in this experiment, indicating that there are certain antigens which express strongly in apoptotic cells, but weakly or even do not express in non-apoptotic cells in the process of HCC cells apoptosis. The subtractive immunization is therefore a useful method to prepare the antibodies against the apoptosis-associated antigens of cells. The preferable results obtained only in 20 % (2 / 10) of the mice immunized by subtractive immunization, suggesting that there is obvious individual difference in the effect of subtractive immunization with CP. In order to get ideal effect, more mice are needed to be immunized in an experiment. The fusion rate of hybridoma cells in the experimental group did not differ distinctly from that in the control group, but the total producing rate of antibodies was lower significantly than that in the control group, suggesting that CP can suppress remarkably the production of antibodies while the fusion rate of hybridoma cells is not greatly influenced. The producing rate of the antibodies which reacted strongly with both apoptotic and non-apoptotic cells in the experimental group is significantly lower than that in the control, whereas there is no remarkable difference on the producing rate of the antibodies which reacted strongly with apoptotic, but weakly with non-apoptotic cells between the two groups, indicating that CP had partly suppressed the production of the antibodies against non-objective antigens to some extent, but not influenced that of the antibodies against objective antigens. The subtractive immunization can significantly decrease the selection workload in the process of preparing mAb when the antigen of mAb is unknown.

The mAb whose associated antigen located within the nucleus of ethanol-induced apoptotic HCC-9204 cells and reacted weakly with non-apoptotic HCC-9204 cells was prepared successfully and identified primarily in this experiment, which is consistent with some previous reports that injecting apoptotic cells into the cavum abdominis of mice can lead to the production of anti-nucleus antibodies[66]. The results of Western blot analysis revealed that the molecular mass of the associated antigen of this mAb was about 75 ku, which is consistent with the result of our previous study that new proteins whose molecular mass are more than 67 ku are discovered in 60 mL· L^{-1} ethanol-induced apoptotic HCC-9204 cells. The associated antigen of the mAb prepared in this experiment is the certain molecule that expresses in the nucleus of HCC-9204 cells and may be related with the ethanol-induced apoptosis. No antibody that reacted strongly with apoptotic cells, but completely absent with non-apoptotic cells is obtained in this experiment, suggesting that the antibodies against new apoptosis-associated antigens that do not exist in non-apoptotic cells is difficult to be prepared by the method of cell fusion used in this experiment. Low-concentration ethanol can not only induce apoptosis in HCC-9204 cells, but may also suppresses their proliferation and startup the expression of some molecules related with some other effect of ethanol on cells. The relationship between the mAb prepared in this experiment and its associated antigen and apoptosis in HCC cells is still to be determined and should be studied further. This experiment gives us a hopeful prospect to discover some new apoptosis-associated molecules in HCC cells.

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