THE INFLUENCE OF GENETIC VARIABILITY OF TUMOR CELL POPULATION OF MOUSE HEPATOMA MH–22a ON INTERINDUCTION OF APOPTOSIS BETWEEN TUMOR HEPATOCYTES AND SPLENOCYTES

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ВЛИЯНИЕ ГЕНЕТИЧЕСКОЙ ВАРИАБЕЛЬНОСТИ ПОПУЛЯЦИИ ОПУХОЛЕВЫХ КЛЕТОК ГЕПАТОМЫ МЫШИ МГ–22a НА ИНДУКЦИЮ АПОПТОЗА В КЛЕТКАХ ГЕПАТОМЫ И СПЛЕНОЦИТАХ

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Heterogeneity of tumors by different features increases during tumor progression and determines the level of its malignancy. To reveal possible correlations between different characteristics, the method of clonal analysis of the tumor cell population should be used. The goal of this study was to reveal the influence of DNA-polymorphism level of mouse hepatoma cells MH-22a on their abilities to differentiate upon the growth in the eye anterior chamber (EAC), to induce apoptosis of splenocytes at cocultivation, and to resist to splenocyte-induced apoptosis. The DNA-polymorphism was revealed using RAPD-PCR with three random primers. The ability of tumor hepatocytes for differentiation was determined by transplantation into the EAC of syngenic mice C3HA. The capability for apoptosis interinduction between tumor hepatocytes and syngenic splenocytes was determined upon cocultivation *in vitro* with the use of electrophoresis of low molecular DNA fractions and by the method of clonal survival. It was shown that the clonal lines MH-22a with a high level of genetic variability were unable to differentiate if they are grown in EAC, were stable to apoptosis induction by splenocytes, but could induce apoptosis in splenocytes. The basic population of tumor hepatocytes and clonal lines characterized by low level of genetic variability differentiated during the growth in EAC and had capability for interinduction of apoptosis with syngenic splenocytes.
Key Words: **genetic variability, RAPD-PCR, tumor, apoptosis, lymphocytes, differentiation.**

Гетерогенность опухолей по ряду признаков нарастает в процессе прогрессии и определяет злокачественность опухоли в целом. Для выявления возможных корреляций между отдельными признаками необходимо проведение клонального анализа исследуемой популяции опухолевых клеток. Целью настоящей работы явилось использование клоновых линий гепатомы мыши МГ-22а для изучения влияния разной степени ДНК-полиморфизма опухолевых гепатоцитов на их способность к дифференцировке при росте в передней камере глаза (ПКГ), на способность индуцировать апоптоз спленоцитов при совместном культивировании, а также на устойчивость к апоптозу, вызываемому спленоцитами. ДНК-полиморфизм выявляли с помощью RAPD-ПЦР с тремя случайными праймерами. Способность опухолевых гепатоцитов к дифференцировке определяли при их трансплантации в ПКГ сингенных мышей С3НА. Способность к взаимоиндукции апоптоза между опухолевыми гепатоцитами и сингенными спленоцитами выявляли при их совместном культивировании *in vitro*. Наличие апоптоза определяли методом электрофореза низкомолекулярных фракций ДНК, а в гепатоцитах, кроме того, и методом клоногенной выживаемости. Установлено, что клоновые линии МГ-22а с высоким уровнем генетической вариабельности не способны к дифференцировке в ПКГ, устойчивы к апоптозу, вызываемому спленоцитами, и обладают способностью индуцировать апоптоз спленоцитов. Основная популяция опухолевых гепатоцитов и клоновые линии с низким уровнем генетической вариабельности дифференцируются при росте в ПКГ и обладают способностью к взаимоиндукции апоптоза с сингенными спленоцитами.
***Ключевые слова*: генетическая вариабельность, RAPD-PCR, опухоль, апоптоз, лимфоциты, дифференцировка.**

Disturbances of tumor caryotype, such as chromosome and chromatid rearrangements have been studied very intensively. However, small genetic disturbancies occupying only some dozens or hundreds of nucleotides could be studied effectively only after the appearance of a method of polymerase chain reaction (PCR) in its different variations [17]. During the last decade the first attempts to apply PCR technique on the study of such disturbances in the tumor cell genome were made.

Numerous studies of apoptotic events in malignant cells have shown that apoptosis as the genetically programmed cell death is implicated also in embryogene–

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Abbreviations used: AF DNA — amplified fragments of DNA; CS — clonal surviving; EAC — eye anterior chamber; EF — electrophoresis of low molecular fractions of DNA; RAPD–PCR — random amplified polymorphic DNA; SCT — subcutaneous connective tissue.

sis and normal functioning of organs and tissues as well as in pathology of different kinds [2, 5, 9, 13]. Thus, the combined approach in studying apoptosis in malignant cells is required. Earlier, we have studied possible relation between genetic variability of tumor cells and interinduction of apoptosis by PCR with primers that were homologous to B1 repetitive elements of mouse [3]. It was shown that the clonal lines of hepatoma MH–22a differed in capability for apoptosis interinduction upon their cocultivation with splenocytes and that this capacity correlated with the higher level of genetic variability in clonal lines. The goal of this work was to study interrelations between quantity of genetic rearrangements in hepatoma MH–22a clonal lines revealed by RAPD–PCR and their capability for interinduction of apoptosis at their cocultivation with syngenic splenocytes. RAPD–PCR instead of B1–PCR was applied for obtaining of higher amounts of amplified fragments of DNA (AF DNA) in each spectrum and of DNA fragments with higher molecular masses. Also, the possible correlation between the quantity of revealed genetic rearrangements in clonal lines and their ability to differentiate *in vivo* upon the growth in the EAC was examined.

MATERIALS AND METHODS

The cell line of mouse hepatoma MH–22a was obtained from the Cell Cultures Museum of the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg, Russia). The cultivation of the hepatoma cell line was carried out under standard conditions. To obtain the clones, hepatoma was spread on 25 mm Petri dishes (250–300 cells/dish), and cultured under the same conditions as the total basic cell population.

The genetic heterogeneity in the cell population of MH–22a hepatocytes was revealed by RAPD–PCR. As primers for RAPD–PCR, three synthetic decanucleotides were used: 447 (5′–AACGGTCACG–3′), 452 (5′–CCGGCTACGG–3′) and 453 (5′–AGCTGCCGGG–3′) [6]. The total DNA of the hepatocytes was isolated by the standard procedure [4]. The cell DNA (0.05 μg) was amplified with each primer (50 pm), Taq–polymerase (1.25 unit), dNTP (100 μM), and $MgCl_2$ (2.5 μl) in reaction buffer (50 μl) during 40 cycles. The thermal cycling was performed as follows: denaturation at 94 °C for 30 s, annealing at 40 °C for 1 min, synthesis of DNA at 72 °C for 1 min. The time of denaturation in the first cycle and the time of elongation in the last one was 5 min. The products of RAPD–PCR were separated electrophoretically on 2% agarose gel in Tris–borate buffer, and the DNA fragments were stained with ethidium bromide. RAPD–PCR probes obtained with primer but without DNA and with DNA without primer were used as the controls.

The hepatoma MH–22a and its clonal lines were transplanted into subcutaneous connective tissue (SCT) by injection of 10^6 cells to syngenic C3HA mice. Usually, the tumors, 1–1.5 cm in diameter, were detected in 2 weeks. The transplantation of the tumor cells into the EAC was performed using intraperitoneal Nembutal narcosis (concentration — 8 mg/ml, 100 μl per mouse). The eye was washed with a penicillin solution and then treated with one drop of 1% atropine and one drop of 0.25% dicain after dilatation of pupil for several min. The suspension of hepatoma cells (25–50 μl) was injected into the EAC using 1 ml syringe. In 15–20 days after transplantation the growth of transplants was checked. The tumors were fixed in 10% neutral paraformaldehyde, passed through ethanol of different concentrations and embedded in paraffin.

For induction of apoptosis, splenocytes from the spleen of the 6–9–week old syngenic C3HA male mice were used. All mice were sacrificed after narcosis. The experiments for interinduction of apoptosis between hepatocytes and splenocytes were performed in the DME medium for 18 h. Splenocytes and hepatocytes were mixed in the ratio 50:1. Splenocytes and hepatocytes cultivated separately for 18 h were used as a control. The low molecular DNA was isolated by a modified Hirt's method [21]. For the detection of the oligonucleosomal DNA fragmentation the electrophoresis in 1% agarose gel was applied.

The level of apoptosis in hepatocytes was evaluated by the clonal survival test. Approximately 250 tumor hepatocytes were placed in three Petri dishes after their cocultivation with splenocytes. The number of clones was counted, when they reached the diameter 1–2 mm. Tumor hepatocytes incubated without splenocytes were used as the control.

RESULTS

In the basic cell population of hepatoma MH–22a cells and in its five clonal lines, DNA was studied for genetic variability with three random primers — 447, 452, and 453. In each case, the spectra of amplified DNA fragments (AF DNA) consisted of 14–17 AF DNA and differed in their molecular weight range: for primer 447 — from 300 to 3000 bp, for primer 452 — from 900 to 3000 bp, and for primer 453 — from 450 to 1300 bp (Fig. 1). The analysis of AF DNA spectra has allowed to reveale the differences between the fragments with respect to their variability: the appearance and disappearance of new fragments, the shift of the fragments, and an increase or decrease of fluorescence level of AF DNA. According to the data obtained with use of all three primers both *in vitro* and *in vivo*, the most variable DNA fragments were 500, 750, 900, and 1400 bp.

Although similar results were obtained using all three primers, the most pronounced changes were observed in the AF DNA spectra obtained with the use of primer 447. To analyze genetic variability, the AF DNA spectra of clonal lines were compared *in vitro* with the AF DNA spectrum of the basic cell population MH–22a. It was shown that the clonal lines differed in the amount of genetic rearrangements from the basic population and between themselves (Table). The genetic variability in the basic population of MH–22a and its clonal lines were also detected *in vivo* upon transplantation into the EAC and SCT (Fig. 2). To detect genetic variability *in vivo*, spectra of AF DNA in the EAC and SCT transplants were compared with the AF DNA spectra of the basic cell population of hepatocytes and clonal lines

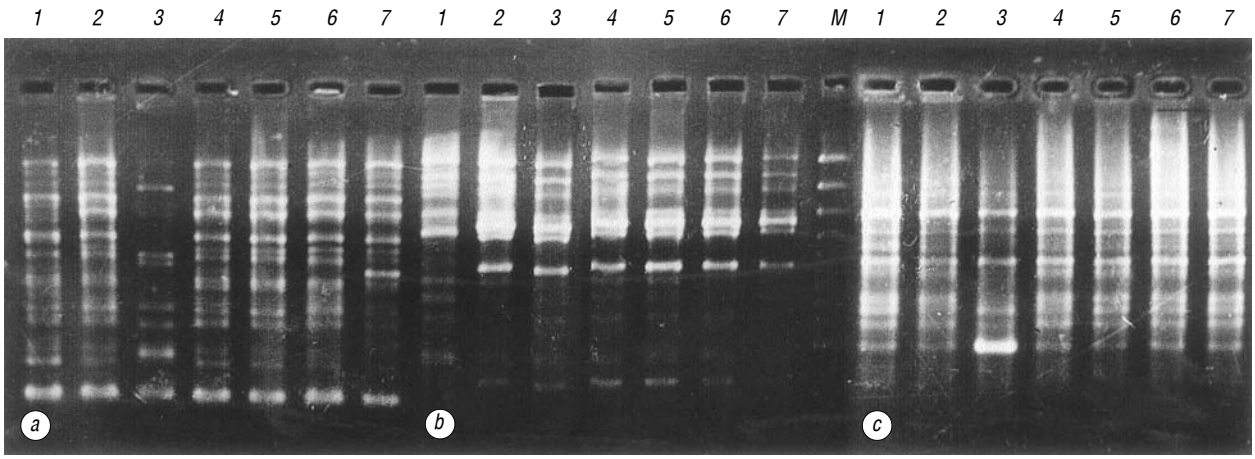

Fig.1. Electrophoregrams of AF DNA obtained from basic population of MH–22a cells and its clonal lines *in vitro*. *a* — primer 447; *b* — primer 452; *c* — primer 453; 1 — liver of C3HA mice; 2 — MH–22a; 3–7 — clonal lines: 3 — 22/4, 4 — 22/5, 5 — 22/6, 6 — 22/7 and 7 — 22/8, M — DNA ladder 100 bp (Fermentas)

in vitro. It was shown that upon those conditions genetic variability both in basic population of tumor hepatocytes and in clonal lines increased. The only exception was the clonal line 22/5, in which no changes were detected (see Table). It was revealed that the level of genetic rearrangements in the basic population and the clonal line 22/4 decreased upon the growth in EAC in comparison with that in SCT; didn't differ in two clonal lines, 22/5 and 22/8, and increased in the clonal line 22/6. Next, it was found that both the basic hepatocyte population and hepatocytes of three clonal lines (22/5, 22/7, and 22/8) were able to differentiate upon the growth in EAC. The signs of cytotypic differentiation (large cells, structured nuclei, the light eosinophilic cytoplasm, the small number of mitoses) (Fig. 3) and moderate signs of histotypic differentiation (formation of clusters of differentiated hepatocytes surrounded by thin collagen fibers) were observed.

Table. Genetic variability of MH–22a cells, their capacity to differentiate upon the growth in the EAC and to induce/undergo apoptosis upon coculti–vation with syngenic splenocytes

MH–22a cell line	Genetic variability of hepatocytes			Differentiation of hepatocytes grown in		Apoptosis revealed in		
	in vitro	*in vivo* SCT	*in vivo* EAC	SCT	EAC	hepatocytes CS	hepatocytes EP	Splenocytes EP
Basic population		++	+	–	+	+	+	+
Clonal lines:	+++	++	+	–	–	–	–	+
22/4								
22/5	–	–	–	–	+	+	+	+
22/6	–	++	+++	–	–	–	–	+
22/7	++	nd	++	nd	+	+	–	+
22/8	+	+	+	–	+	nd	+	+

In vitro — DNA polymorphism in clonal lines of tumor hepatocytes is estimated in comparison with DNA polymorphism of the basic population of MH–22a, taken as zero; *In vivo* — DNA polymorphism in tumor hepatocytes upon the growth in SCT and EAC is estimated in comparison with their DNA polymorphism upon the growth *in vitro*, taken as zero; nd — no determined.

Study of interinduction of apoptosis between tumor hepatocytes and syngenic splenocytes was carried out with the same clonal lines of hepatoma MH–22a (see Table). These experiments revealed that both the basic population of tumor hepatocytes and all studied clonal lines were able to induce apoptosis in splenocytes, although intensity of apoptosis in splenocytes was different. Meanwhile, splenocytes induced apoptosis in tumor hepatocytes only in the basic population and in three of five examined clonal lines, 22/5, 22/7, and 22/8 (see Table). It is to be noted that apoptosis in the clonal line 22/7 was detected only by the method of clonal survival, but not by EF. It is important that in clonal lines 22/4 and 22/6 negative results were obtained by the both methods for apoptosis detection used in present work. Clonal survival in these clonal lines was 99% (Fig. 4).

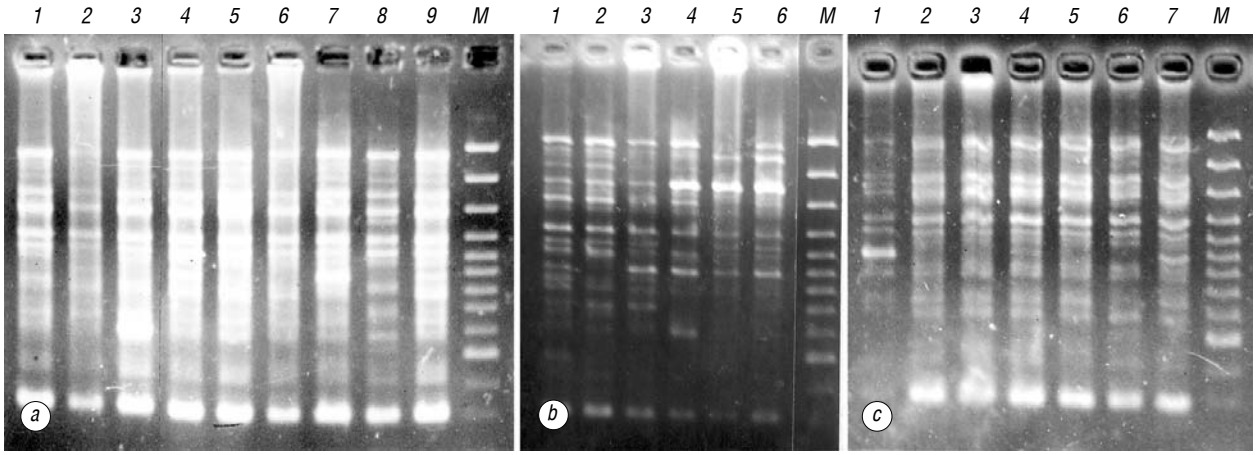

Fig. 2. Electrophoregrams of amplified DNA fragments obtained from cells of the basic population of MH–22a cells and its clonal lines after their growth *in vivo* in SCT and EAC with primer 447: *a* — basic population of MH–22a; 1 — basic population of MH–22a *in vitro*, 2 — SCT, 3–9 — EAC; *b* — clonal line 22/6; 1 — clonal line 22/6 *in vitro,* 2 — SCT, 3–6 — EAC; *c* — clonal line 22/8; 1 — clonal line 22/8 *in vitro*, 2 — SCT, 3–7 — EAC. M — DNA Ladder 100 bp (Fermentas)

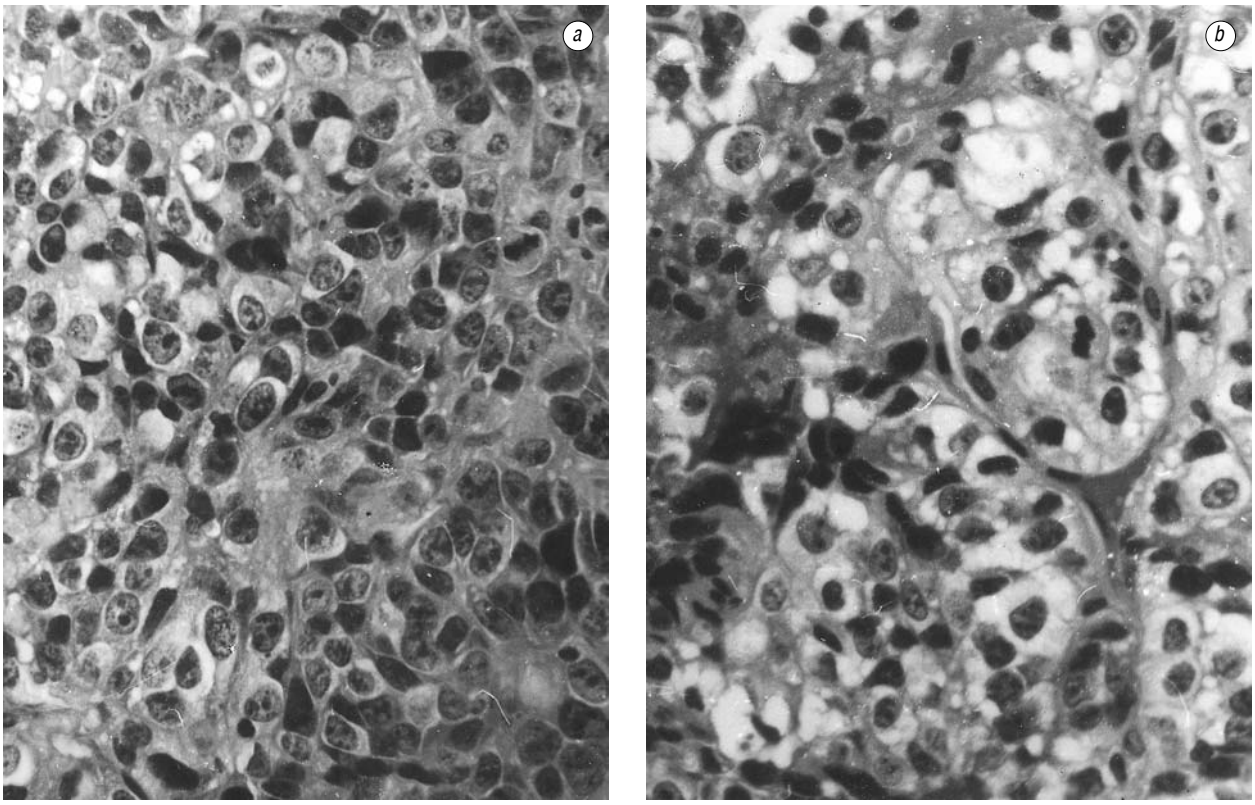

Fig. 3. Transplants of hepatoma MH–22a in SCT *(a)* and EAC *(b)*. Hematoxylin–eosin, x 400

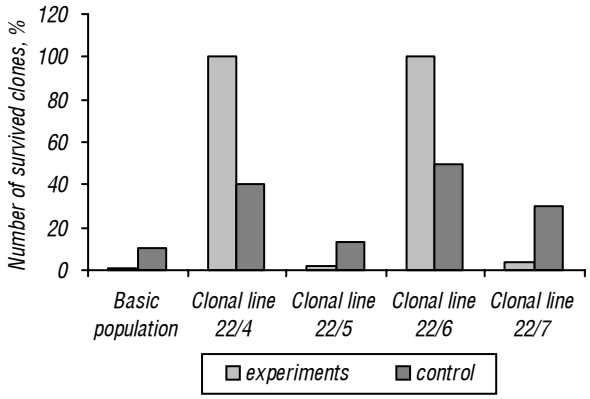

Fig. 4. Clonal survival MH–22a cells after coincubation with syngenic splenocytes

DISCUSSION

It is well known that in cancerous tissues tumor progression, dream state of cells or spontaneous regression of tumor may occur [15, 16, 20]. Among the factors that determine the fate of tumor cells is the state of their genome. The intensive investigation of apoptosis at tumorigenesis has shown that its role on different stages of the disease beginning from the appearance of the first atypical cells is unquestionably important, but it is far from being clear yet. In particular, peculiarities of the interinduction of apoptosis between cancer cells and lymphocytes are poorly studied [10, 14].

The study of the heterogeneity of the hepatoma cell line MH–22a by clonal analysis has allowed to suppose the possible correlation between the genetic variability, capability for differentiation at the growth in EAC, ability to induce apoptosis in splenocytes by tumor hepatocytes, and the resistance of tumor hepatocytes to splenocyte–induced apoptosis. The data obtained may be summarized in the next way:

Clonal lines 22/4 and 22/6 are characterized by high level of genetic variability; lack of differentiation at growth in EAC; resistance to the splenocyte–induced apoptosis; capability for the apoptosis induction in splenocytes.

Basic population, clonal lines 22/5, 22/7, and 22/8 are characterized by low level of genetic variability; capability for differentiation at growth in EAC; interinduction of apoptosis between tumor hepatocytes and splenocytes.

The data presented point to the general role of genetic variability level among studied features of tumor cells. The DNA–polymorphism studied by RAPD–PCR did not allow reveal any definite disturbances in the tumor cell genome; however, it was used as a non–specific probe that might characterize rearrangements in genome occupying some hundreds or thousands of nucleotides [17]. The greater efficiency of the RAPD–PCR method applied in this study than B1–PCR used in our previous experiments might be possibly explained by a higher conservatism and stability of the genome sites, in which the B1 repeated elements of mouse were placed [3]. Hence, it may be concluded that even non–specific genome disturbances affected such biological characters of tumor cells, as their capabilities for differentiation at the growth in the EAC and interinduction of apoptosis between tumor cells and splenocytes.

Similar results were reported in [6] and demonstrated the heterogeneity of clonal lines in the genome rearrangements; it was suggested that the accumulation of the genome aberrations could be linked, directly or indirectly, to tumor progression.

In another work [7], hypersensitivity to radiation–induced non–apoptotic and apoptotic death was established in cell lines from patients with the chromosome instability syndrome. Lymphoblastic cell lines from these patients were characterized by long–term cell cycle arrest, increased apoptosis, and by the decrease of clonogenicity after irradiation in comparison with normal tissues. Study of the Hodgkin—Reed—Sternberg cell line L1236, with the use of cytogenetic analysis, did not reveal in the cells any specific chromosomal aberrations, whereas use of panels of microsatellite loci allowed to find the regions with loss of heterozygocity in sites of chromosomes 6p, 9q, and 17p [19].

The ability of tumors to suppress components of immune system, in particular, the capacity of tumor cells to influence lymphocyte apoptosis, is of special importance [1, 8, 11, 12]. We showed that the tumor hepatocyte population may be heterogeneous in abilities to induce apoptosis in splenocytes and to undergo splenocyte–induced apoptosis [3, 18]. The heterogeneity in genetic variability of the studied population of tumor hepatocytes revealed both B1– and RAPD–PCR is of great interest, too. We demonstrated that high DNA–polymorphism in hepatocytes revealed by RAPD–PCR correlate with capability of tumor hepatocytes for differentiation upon the growth in EAC. We hypothesize that genetic rearrangements in the heterogeneous tumor cell population may lead to the appearance of the clones that, on one hand, are more stable to the lymphocyte–induced apoptosis, while, on the other hand, become more aggressive to lymphocytes.

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