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Appearance of Differentiation Characteristics (Induction of Ah-Receptor-Dependent Genes) during Cultivation of Transformed Cell Clone K8 from Embryonic Rat Fibroblasts

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Abstract—The differentiation status of fibroblasts can be characterized by their ability to induce Ah-receptor-dependent genes. The ability to induce Ah-receptor-dependent genes encoding cytochrome P450 isoforms, Ah-receptor repressor, and NADPH-quinine oxidoreductase were studied in the transformed cell clone K8 obtained from immortalized embryonic rat fibroblasts by treatment with benzo(a)pyrene and in the parental clone F27. Treatment with benz(a)anthracene did not induce the genes in the transformed clone K8 on passages 4-14, but the induction was recorded in the transformed clone beginning from the 16th passage and later, whereas in F27 cells the induction was observed throughout the experiment. Induction levels of mRNA of the induction-regulating genes encoding the Ah-receptor and Ah receptor nuclear translocator were similar in F27 cells and in the transformed cell clone K8 in both early and late passages. Electrophoretic mobility shift assay showed that in clone K8 transmission of the induction signal was disturbed in the early passages before interaction of the activated Ah-receptor with the recognizing region of DNA. Possible mechanisms responsible for the absence of induction in the early passages in the transformed cells are discussed.

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Key words: cell transformation, cytochrome P450, Ah-receptor, enzyme induction

To realize their biological potential (toxic, mutagenic, transforming, etc.) most organic compounds polluting the environment require metabolic activation. This activation is catalyzed by the monooxygenase enzyme complex represented by the superfamily of cytochromes P450 (CYP). There are many CYP isoforms with different and sometimes overlapping substrate specificities and various mechanisms regulating the gene expression. CYP isoforms are divided into families based on the similarity of their amino acid sequences [1]. Organ- and species-specificity of different isoforms of the enzyme essentially determine the corresponding specificity of action of both carcinogens and toxic compounds that need metabolic activation. Many cytostatics are also metabolized (activated or deactivated depending on their structure) by CYP isoforms. The main pollutants in the environment, such as polycyclic aromatic hydrocarbons (PAH), are oxidized by isoforms CYP1A1 and CYP1B1. Expression of CYP is sensitive to various influences. Some xenobiotics that are substrates of the isoforms can induce CYP. Isoforms of family 1 can be induced by introduction of PAH, chlorinated dioxins, flavones, etc.

Upon entrance, the cell inducers interact in the cytosol with the Ah-receptor. The activated Ah-receptor is liberated from heat protein shock 90 and protein p23, which support its inactive state, and is transferred into the nucleus where it produces a triple complex with the Ah receptor nuclear translocator (ARNT). The triple complex is a transcription factor interacting with the recognizing sequence of DNA (the so-called xenobiotic responsive element (XRE)) [2, 3]. The genes containing XRE in their regulatory region are transcribed. XRE has been found in regulatory regions of many genes (including those encoding CYP family 1, isoforms of glutathione S-transferases, NADPH-quinone oxidoreductase (QOR), etc.) involved in metabolism of xenobiotics.

Abbreviations: ARNT, Ah receptor nuclear translocator; BA, benz(a)anthracene; BP, benzo(a)pyrene; CYP, cytochromes of the P450 family; PAH, polycyclic aromatic hydrocarbons; PCR, polymerase chain reaction; QOR, NADPH-quinone oxidoreductase; RT, reverse transcription; XRE, xenobiotic responsible element.

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The gene encoding the Ah-receptor repressor (AHRR) is also induced. Protein AHRR interacts with protein ARNT, producing an inactive complex that prevents formation of the triple complex of inducer–Ah-receptor–ARNT [4]. Thus, AHRR is responsible for reversed regulation of the induction caused by PAH and other ligands of the Ah-receptor.

In tumors the ratio of different CYP isoforms and their inducibility is changed compared to the homologous normal tissues [5]. Correspondingly, the sensitivity of tumor cells to xenobiotics including antitumor drugs metabolized by this system is also changed (as a rule reduced) compared to the normal homologous tissue. The mechanism of changes in CYP isoform expression in tumors is unclear, and the malignization stage of occurrence of these changes is also unknown. Elucidation of regulatory mechanisms responsible for changes in the expression of CYP isoforms in tumors would be promising for regulation of their levels in tumors and for increasing the effect of cytostatics. Moreover, such inducers of CYP as PAH or halogenated dioxins are promoters of carcinogenesis. It might be that the promoter-conditioned preferential growth of the initiated cells can be regulated through changes in the level of CYP or of proteins involved in transmission of the inducing signal.

We have shown earlier that the treatment of immortalized embryonic fibroblasts with benzo(a)pyrene (BP) results in formation of transformed clones with different ability to induce CYP in response to inducer [6].

The purpose of the present work is to compare the constitutive and induced expression of the genes encoding the CYP1 family and QOR and also of the genes encoding the Ah-receptor, ARNT, and AHRR involved in their regulation in the culture of rat embryonic fibroblasts F27 immortalized by Rauscher's virus and in the transformed clone K8 obtained by treatment of F27 culture with BP. The inducibility, which was initially absent in clone K8, appeared during its cultivation. The expression of CYP and inducibility might be considered differentiation factors because they are not necessary for providing the cell viability and are organ-specific. The cultivation of tumor and transformed cells is usually associated with the loss of differentiation markers that characterizes tumor progression. The ability of the transformed clone to recover differentiation parameters during the cultivation shown by us suggests that in the transformed cells progression and dedifferentiation mechanisms can act alongside normalization processes. Elucidation of mechanisms of these processes can be a basis for a new approach for prevention and treatment of cancer.

MATERIALS AND METHODS

Cell cultures. The cells were immortalized by infection with Rauscher's virus as described in [7]. Pieces 1-mm and less in size from 16-18-day-old embryos of the rat line Fischer 344 were grown in RPMI medium supplemented with 20% fetal serum in an atmosphere of 5% CO_2 at 37°C until attachment of the cells to the support and appearance of the growth zone. The cells were treated with DEAE-dextran (20 μg/ml) for 20 min at room temperature, washed in RPMI medium, and then incubated for 45 min at 37°C with the viral concentrate with periodic shaking. Then the cells were supplemented with medium RPMI containing 2% fetal serum, and 12 h later the medium was changed for the full-value medium supplemented with 10% fetal serum.

Rauscher's virus was isolated from the culture fluid of the virus-producing cell line JLS-V9. The virus was concentrated by centrifugation and subsequent ultrafiltration. The presence of retrovirus in the culture was determined by the XC-test.

Transformed clones were obtained by treatment of F27 cells for 3 days with the carcinogen BP dissolved in DMSO at final concentration 0.15 μg/ml. Then the BP was washed off, the cells were passaged, and clones were obtained by planting cells from the transformation foci into semi-liquid agar. Isoforms of CYP1 were induced by exposition of the cells for 24 h to the presence of benz(a)anthracene (BA) dissolved in acetone at final concentration 5 μg/ml; to the control cells only acetone was added in the same volume (10 μl per ml medium).

RT PCR. For isolation of mRNA, TRI reagent (Sigma, USA) was used according to the producer's recommendations. The total RNA concentration was determined by the optical density at 260 nm. cDNA was prepared by reverse transcription (RT) with synthetic hexanucleotides. The reaction was performed at 42°C for 1 h using 4 μg of total RNA, and then the enzyme was inactivated for 5 sec at 94°C. The resulting cDNA was used for polymerase chain reaction (PCR). In all experiments, water was used as control instead of RNA. For all genes under study, PCR was performed as follows: denaturation at 94°C for 40 sec, annealing of primers (60°C, 10 sec), synthesis of the product (72°C, 10 sec), and exposition for 2 min at 72°C after the cycles. The number of cycles was varied from 25 to 30 depending on the gene under study. RNA was used as the control instead of cDNA. RT PCR was performed using reverse transcriptase, MuLV, and Taq-SE (SibEnzyme, Russia).

The primers (Table 1) and hexanucleotides were synthesized by Khelikon (Russia). Amounts of cDNA for amplification of specific genes were leveled by the amount of β-actin mRNA. The RT and amplification reactions were performed using a Tertsik apparatus (Russia). Products of RT PCR were separated by electrophoresis at 150 V in 2% agarose gel in TBE buffer. Electrophoregrams were subjected to densitometry, and the gels were analyzed using the Scion Image program, version 4.0.2.

Table 1. Nucleotide sequences of primers used for PCR

Gene	Primer nucleotide sequence	Size of nucleotide pair amplicon
β-*Actin*	5'-TGCAGAAGGAGATTACT-GCC-3'	211
	5'-GCAGCTCAGTAACAGTC-CG-3'	
CYP1A1	5'-CCATGACCAGGAACTCT-GGG-3'	341
	5'-TCTGGTGAGCATCCAGG-ACA-3'	
CYP1B1	5'-ACCGCAAACTTCAGCAA-CTTC-3'	427
	5'-GTGTTGGCAGTGGTGG-CATG-3'	
AHRR	5'-CCC-ATC-AGA-TCC-TTT-GGA-TG-3'	160
	5'-AAA-GTC-AGC-ATC-CCT-CCT-TG-3'	
AHR	5'-TCCATGTAGCAGTGCCA-GG-3'	212
	5'-ATATCAGGAAGAGGCTG-GGC-3'	
ARNT	5'-GTCTCCCTCCCAGATGA-TGA-3'	218
	5'-AAGAGCTCCTGTGGCTG-GTA-3'	
QOR	5'-GGCTGGTTTGAGAGAG-TG-3'	459
	5'-GTCGGCTGGAATG-GACTTG-3'	

Binding of activated Ah-receptor with consensus oligonucleotide (electrophoretic mobility shift assay (EMSA)). The protein extract was prepared from nuclei as described earlier [8]. The binding was conducted by incubating the nuclear protein (10 μg) in 20 μl of the binding buffer (10 mM Hepes, pH 7.5, 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg poly(dIdC), and a consensus oligonucleotide labeled in the 5′-end with ^{32}P ((1-3)·10^5 cpm)).

Labeling was performed as recommended by the producer of the consensus oligonucleotides (Promega Corp., USA). Protein–oligonucleotide complexes were analyzed in 6% native polyacrylamide gel in 0.25-fold TBE buffer. The dried gel was exposed on Kodak film (USA) at –80°C for 1-7 days.

Immunoblotting. The proteins were separated by electrophoresis in 12.5% SDS-polyacrylamide gel, transferred onto an Immobilon-P membrane (Millipore Corporation, USA), and incubated with antibodies to CYP1B1 (Santa Cruz Biotechnology Inc., USA) for 1.5 h at room temperature. Peroxidase-conjugated donkey antibodies (Amersham Pharmacia Biotech, Sweden) were used as secondary antibodies. The specific signal was developed using the ECL system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Figure 1 and Table 2 present data on the constitutive and BA-induced level of mRNA of the Ah-receptor-dependent genes encoding the cytochrome isoforms P4501A1 and CYP1B1 and also AHRR and QOR in the rat embryonic cells immortalized with the Rauscher's virus (the culture F27) and in the clone K8 obtained from the F27 culture by treatment with BP. The constitutive level of mRNA with respect to β-actin was approximately the same in all of the cultures. The treatment with BA increased induction of the inducible genes in the immortalized cells F27 throughout the cultivation. But in clone K8 there were changes during the cultivation: no gene was induced in the early passages (4-14), whereas induction was recorded later (from the 16th to the 40th passage). The absence of induction in the early passages and its appearance in the further stages of clone K8 cultivation could be caused by changes in the expression of regulatory genes involved in transmission of the inducing signal: the Ah-receptor and ARNT. The expression of mRNA of these genes in all studied cells (F27 and early and late passages of K8) was approximately the same relative to β-actin (Fig. 2). Thus, the absence of induction in clone K8 in the early passages was not associated with disorders in expression of these genes. The treatment of cells with BA did not influence the expression of mRNA of the Ah-receptor and ARNT. This is in agreement with the conventional viewpoint that these genes are not induced by Ah-receptor ligands.

To assess the inducing effect of BA on the level of protein, we used the gene encoding CYP1B1 as a marker and determined the level of CYP1B1 induction by BA in the three cultures under study. In the immortalized F27 cells CYP1B1 induction was well pronounced, in clone K8 no CYP1B1 induction was recorded in the early passages, whereas in the late passages the protein amount markedly increased but the induction level was lower than in the immortalized F27 cells (Fig. 3). The Ah-receptor-dependent induction was essentially determined by the

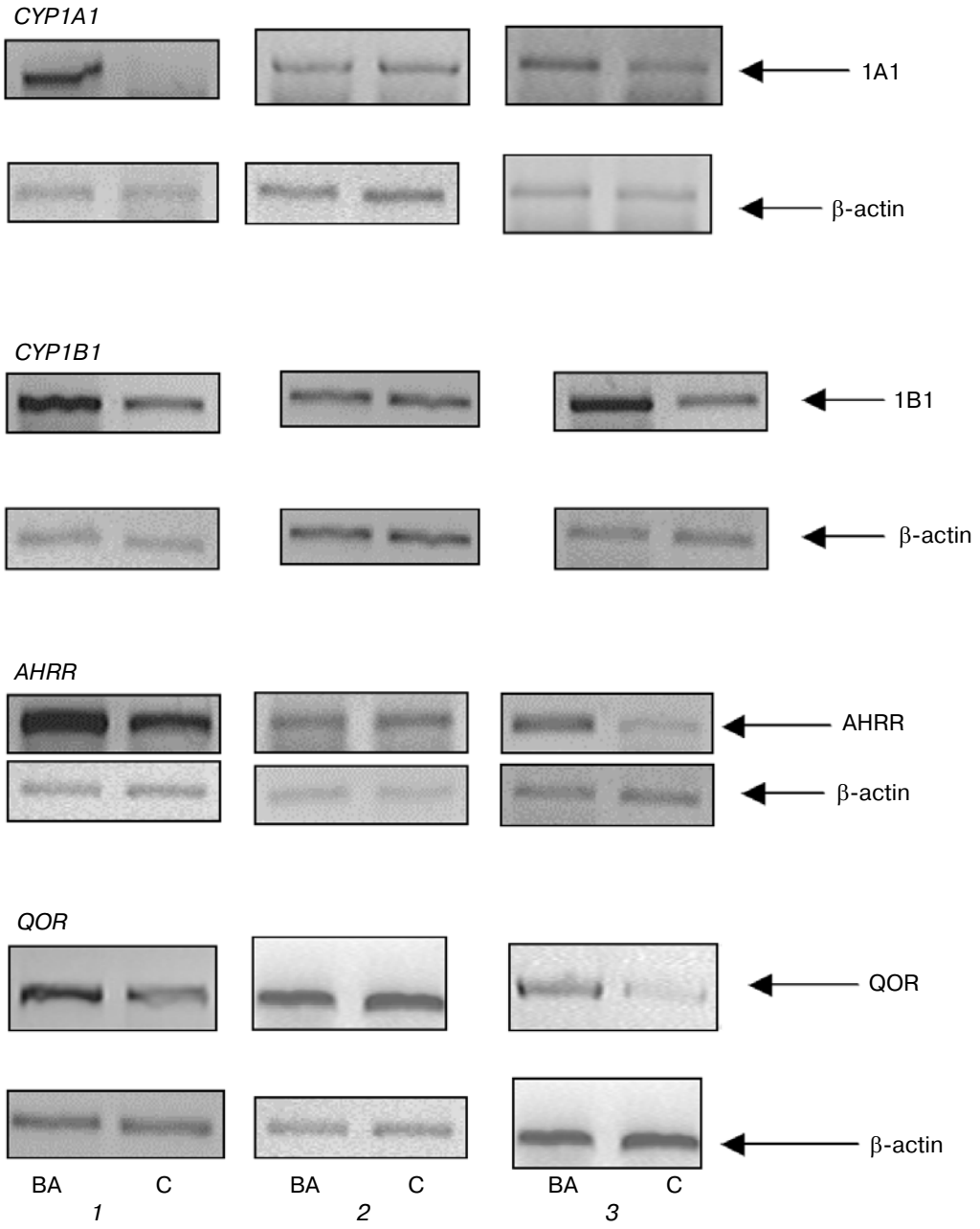

Fig. 1. Expression of mRNAs of genes encoding CYP1A1, CYP1B1, AHRR, and QOR in F27 clone (*1*) and in K8 clone in early (*2*) and late (*3*) passages in control (C) and BA-treated cells (5 μg/ml for 24 h).

Table 2. Induction level of mRNA of genes encoding CYP1A1, CYP1B1, AHRR, and QOR in F27 and K8 in early and late passages determined by ratio of BA/β-actin and control/β-actin bands from Fig. 1

Cells	*CYP1A1*	*CYP1B1*	*AHRR*	*QOR*
F27	6.50	3.60	4.30	2.60
K8, early passages	1.00	0.95	0.95	0.90
K8, late passages	2.30	3.90	3.90	2.45

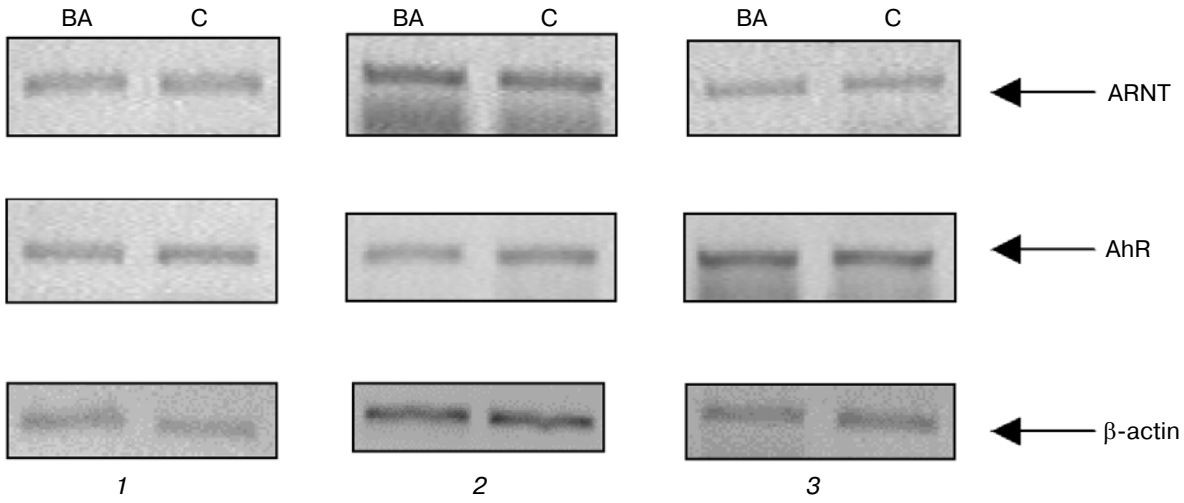

Fig. 2. Levels of Ah-receptor and ARNT mRNAs in culture of immortalized F27 cells (*1*) and in K8 clone in early (*2*) and late (*3*) passages in control (C) and BA-treated (5 μg/ml) cells.

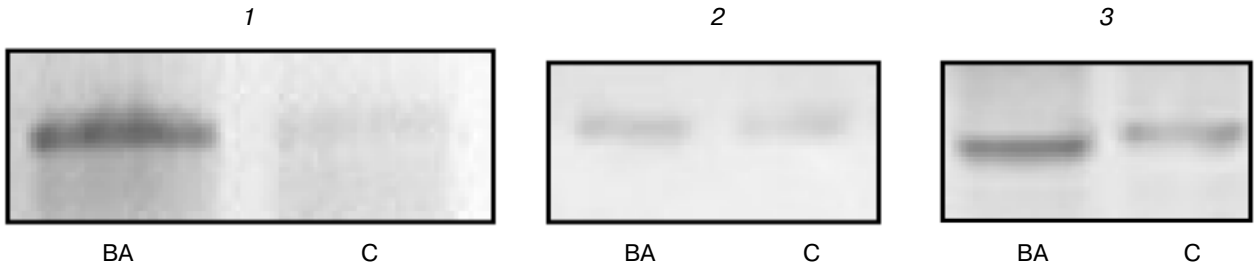

Fig. 3. Western blot analysis of protein CYP1B1 in F27 cells (*1*) and in K8 clone in early (*2*) and late (*3*) passages in control (C) and in BA-treated (5 μg/ml) cells.

binding efficiency of the activated complex of Ah-receptor–ARNT–ligand with the regulatory region in the DNA molecule (the XRE-region). To elucidate whether the absence of induction in the early passages of clone K8 was caused by a disturbed binding with the DNA recognizing region, we studied this binding using the EMSA method. The nuclear protein fraction was isolated from the cells, and the binding with a consensus oligonucleotide containing the XRE-sequence was analyzed.

Figure 4 shows that 1 h after the addition of BA into the medium the binding is sharply increased in the F27 clone cells. In late passages of clone K8 the binding was somewhat lower than in F27 cells, whereas in early passages of the K8 clone the binding of the nuclear protein from both BA-treated and untreated cells was similar. Thus, the absence of binding with XRE in the K8 clone seemed to be caused by the absence of CYP1A1, CYP1B1, QOR, and AHRR induction.

Thus, the absence of induction in the early passages in the transformed clone K8 was caused by events that preceded the binding of the Ah-receptor–ARNT–ligand complex with the recognizing region of DNA. Such events could be represented by the absence of interaction of the BA with the Ah-receptor, by impossibility of detachment of proteins (HSP90 and/or p23) supporting the inactive state of the Ah-receptor in the absence of ligand from the Ah-receptor–ligand complex, by absence of phosphorylation of the Ah-receptor–ligand complex by protein kinase C, and by absence of interaction of the Ah-receptor–ligand complex with protein ARNT.

The presence of the constitutive expression and of approximately the same expression of the inducible gene mRNAs in the induction-lacking system (early passages of clone K8) and in the induction-possessing cells indicated that the constitutive expression of these genes was realized by a mechanism different from that operating during the induction. Note that the constitutive expression of CYP1A1 was not recorded in cells lacking the Ah-receptor. The insertion into such cells of the Ah-receptor gene induced the constitutive expression of CYP1A1 [9].

The absence of induction concurrently with expression of the Ah-receptor and ARNT proteins was

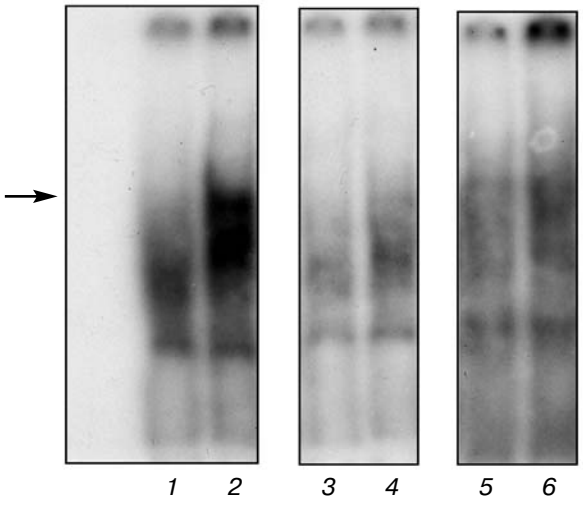

Fig. 4. Binding of activated Ah-receptor–ARNT complex with consensus oligonucleotide: *1*) control F27 cells; *2*) F27 cells after incubation with BA (5 μg/ml for 24 h); *3*) control K8 clone cells, early passage; *4*) K8 clone cells, early passage after incubation with BA (5 μg/ml for 24 h); *5*) control K8 clone cells, late passage; *6*) K8 clone cells, late passage after incubation with BA (5 μg/ml for 24 h). The arrow indicates binding of the Ah-receptor–ligand–ARNT complex with the recognizing region of the oligonucleotide.

described earlier. In most cases, it was associated with methylation of CpG-islands in the XRE region that prevented the binding of the Ah-receptor–ligand–ARNT complex with XRE [10, 11]. The induction of CYP1A1 was also absent in mammary cell subline MCF-7 subjected to selection in the presence of adriamycin. The absence of induction was not associated with the loss of expression of the Ah-receptor and ARNT proteins. The cause of the absence of induction is unknown, but it is not due to methylation of CpG-islands in the regulatory region of the CYP1A1-encoding gene [12]. The available findings are insufficient to conclude which of the above-mentioned factors is responsible for the absence of induction in the early passages of clone K8.

At present, the cause of changes in clone K8 inducibility during cultivation is unclear: whether they are due to arising of a new cell clone that supplanted the clone incapable of inducing or due to other events unassociated with "clone competition" during cell cultivation.

Appearance of differentiation markers in tumor or in transformed cells under some influences has been described earlier. Changes in the microenvironment of such cells are the most effective differentiation factor. There is a classical example of differentiation of mouse teratoma cells injected into the animal's blastocyst [13]. There are also other data indicating that expression of differentiation markers in tumor cells depends on the cell microenvironment. Thus, hepatoma cells transplanted subcutaneously did not express CYP isoforms and these enzymes were not induced in them, but upon transplantation into the liver these cells started expressing CYP isoforms and injection of inducers increased the expression level [14-16]. Expression of some CYP isoforms, in particular of the CYP1B1 isoforms in tumors, is higher than in the homologous normal tissues [17]. The possibility of using this finding in chemotherapy is discussed in work [18]. Expression of the minor isoform CYP2A6 in hepatomas is higher than in normal liver [19, 20]. However, the increased expression of some differentiation parameters is an initially inherent feature of the tumor cells and is not increased upon transplantation. The ability of a transformed clone for spontaneous differentiation during cultivation without an external influence (injection of differentiation factors, changes in cell microenvironment, etc.) is a unique observation, and we have not found a description of such a phenomenon in the literature.

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