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Cytochrome P-450 Family 1 in Rat Embryo Cell Culture Immortalized by Rausher Leukemia Virus

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Abstract—We studied comparative expression and activity of cytochrome P450 family 1 (CYP1) isoforms in rat embryo cells, both primary and immortalized by Rausher leukemia virus (RLV). In RLV-infected embryonal cells compared with the initial ones the expression levels of CYP1A1 and 1B1 mRNAs and benzo[a]pyrene (BP) hydroxylase activity were higher, regardless of their treatment with the CYP1 inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The sensitivity to BP and 7,12-dimethylbenzo[a]anthracene was higher in the cells immortalized with RLV. The expression level of mRNAs of induction-mediating proteins aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator was the same in both cell cultures tested. Higher sensitivity of cells immortalized with RLV compared with the initial embryo cells to transforming effect of BP, which was described previously, is possibly associated with elevated expression of CYP1 isoforms.

Key words: cytochrome P450, immortalization, Rausher virus

The great majority of organic compounds that are present in the environment require metabolic activation to express their biological potential (toxic, mutagenic, tumorigenic, etc.). The activation occurs due to the catalytic activity of monooxygenase enzyme complex involving cytochrome P450 (CYP). Many CYP isoforms have been identified, and these are characterized by different but in some cases overlapping substrate specificity and by different mechanisms controlling their expression. CYP isoforms are subdivided into several families by their amino acid sequence homology and similarity of regulatory mechanisms [1]. Different isoforms are species- and tissue-specific, which in turn determines the species- and tissue-specificity of the effect of carcinogens and toxic substances requiring the metabolic activation.

The CYP system is sensitive to various factors. Some xenobiotics induce expression of isoforms oxidizing them (substrate induction) [2]. CYP isoforms are also subjected to down-regulation of their expression. Indeed, interleukins [3] and reactive oxygen species [4] cause a repression of some CYP isoforms, whereas the activation of cAMP cascade leads to phosphorylation of CYP2 family isoforms with their subsequent degradation [5]. In tumors, the ratio between different CYP isoforms was found to be changed because of differently reduced expression of most isoforms [6]. So, tumor cells compared with normal homologous tissue cells are characterized by altered sensitivity to xenobiotics including antineoplastics metabolized by a given enzyme system. Studies seem to be promising in which a tumor is transformed with a gene encoding the CYP isoform metabolizing a distinct antitumor substance [7], thus acquiring higher sensitivity to the toxic effect of the drug.

Viruses play an important role in both the life and the evolution of organisms. New genetic material that a virus brings into a cell can undoubtedly lead to functional imponderables of both the cell and the whole organism. It is worth noting in illustration of viral influence on cell culture that fetal rat fibroblasts infected by Rausher leukemia virus (RLV) carrying no oncogene in its structure is characterized by a jump in sensitivity to transforming action of benzo[a]pyrene (BP) [8]. We have suggested that enhanced expression and activity of BP-metabolizing CYPs is one of the possible mechanisms of this phenomenon. The most effective BP-activating CYPs are members of family 1 [9]. This family comprises known isoforms such as CYP1A1, 1A2, and 1B1. The members of family 1 are prone to induction by substrates including BP [10]. Their induction leads to a dramatic increase in both mRNA and protein levels with corresponding enhancement of the active carcinogenic form production. Isoforms 1A1 and 1B1 are expressed in various cell types, whereas isoform 1A2 is hepatocyte-specific [11]. In the present study we investigated both native and RLV-infect-

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ed fetal rat fibroblast-like cell cultures for comparison of expression of CYP1 mRNAs, activity of the corresponding enzymes, and expression of mRNAs of aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT), the proteins involved in the induction signal transmission. We have found that the transfection with RLV elevates both constitutive and inducible expression of CYP1 mRNAs, enzymatic activity against BP, and BP toxicity with no change in the expression levels of AHR and ARNT mRNAs in the culture. These data are indicative of enhanced CYP mRNA expression and elevated level of CYP activities in the cell culture immortalized by RLV compared to the initial culture resulting in higher sensitivity of infected cells to transforming effect of carcinogens; they also indicate that some other factors of still unknown nature, which are possibly controlled by RLV, are involved together with the proteins AHR and ARNT in regulation of CYP1 isoform expression.

MATERIALS AND METHODS

Fetal fibroblast-like cells (F16) were prepared according to a standard protocol: 16-18-day-old embryos of Fisher 344 rats were hashed and washed free of blood. Cells were trypsin-released, washed free of trypsin, and plated into RPMI medium containing 20% fetal serum.

To produce the cell line F27-RLV, the cells at passage 0 were infected with RLV by the method of Elliot et al. [12] with some modifications: Fisher 344 rat embryo islets no more than 1 mm in size were grown in RPMI medium supplied with 20% fetal serum in a CO_2 incubator (5% CO_2) until growth zones of attached cells were formed. Cells were maintained in 20 μg/ml DEAE-dextran for 20 min at room temperature, washed in RPMI medium, supplied with the virus concentrate with following incubation for 45 min at 37°C and recurrent agitation, and then maintained in RPMI medium containing 2% fetal serum, which was replaced after 12 h of incubation by the wholesome medium containing 10% fetal serum.

Rausher leukemia virus was prepared from the culture medium of virus-producing cells, line JLS-V9. The virus was concentrated by centrifugation followed by ultrafiltration. Production of the retrovirus in the culture was determined from the XC-test. XC cells (embryo rat cells transformed with Rous sarcoma virus) were added to the growing culture of transformed cells. When retrovirus was present in the culture, XC cells formed syncytia, 8-12 nuclei each, at day 2-3 of associated growth. Cells were fixed with methanol and stained with azure-eosin. The F16 and F27-RLV cells were morphologically indistinguishable.

mRNA was isolated using TRIzol (Gibco BRL Life Technologies, USA). Total RNA was determined from the absorption at 260 nm. Reverse transcription to produce cDNA was performed using a random-6N-primer. The amounts of cDNAs used for the amplification of individual genes were equalized by the level of β-actin mRNA. Primers and amplification protocols are given in the table.

Reverse transcripts from RT-PCR were separated by standard electrophoresis in 2% agarose gel in TBE (Tris-borate buffer containing 10.8 g/liter Tris, 5.5 g/liter boric acid, and 0.9 g/liter EDTA) at voltage of 150 V. CYP1 isoforms were induced with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the acetone solution of which was added to the cells at final concentration of 10^{-9} M for 24 h. CYP1 isoforms were functionally tested by their BP

Nucleotide sequences of PCR primers

Gene	Primers	Product length, bp	Reference
β-Actin	5'-TGCAGAAGGAGATTACTGCC-3' 5'-GCAGCTCAGTAACAGTCCG-3'	211	[22]
CYP1A1	5'-CCATGACCAGGAACTATGGG-3' 5'-TCTGGTGAGCATCCAGGACA-3'	341	[21]
CYP1B1	5'-ACCGCAAACTTCAGCAACTTC-3' 5'-GTGTTGGCAGTGGTGGCATG-3'	427	[23]
AHR	5'-TCCATGTAGCAGTGCCAGG-3' 5'-ATATCAGGAAGAGGCTGGGC-3'	212	[21]
ARNT	5'-GTCTCCCTCCCAGATGATGA-3' 5'-AAGAGCTCCTGTGGCTGGTA-3'	218	[21]

hydroxylase activity. Cells were disrupted by freeze–thawing, and BP hydroxylase activity was determined in lysates by fluorescence of 3-hydroxy-BP produced as described elsewhere [13]. Since sensitivity to the toxic effect of substances requiring enzymatic activation to develop their biological activity is an important functional feature of the monooxygenase enzyme system, we studied the toxicity of BP and 7,12-dimethylbenzo-[a]anthracene (DMBA) in the cultures F16 and F27-RLV using the MTT test [14]. This method is based on the ability of mitochondrial dehydrogenases to convert 3-(4,5-dimethylthiasol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a colored formazan, the amount of which detected by fluorimetry is in direct proportion to the cell amount in culture.

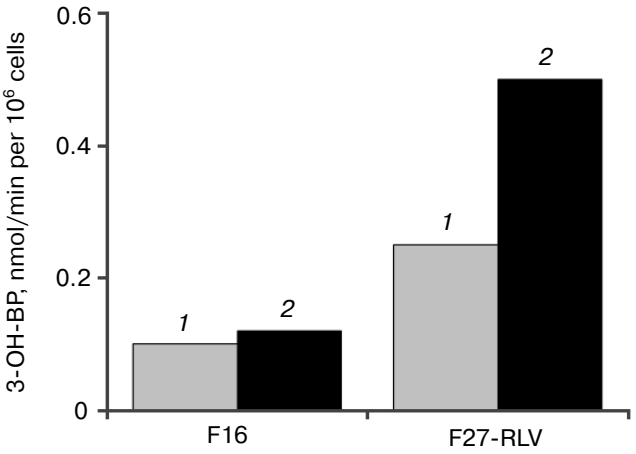

Fig. 2. Benzo[a]pyrene hydroxylase activity in the normal (F16) and RLV-infected (F27-RLV) fetal rat fibroblasts in absence (*1*) or presence (*2*) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

RESULTS

The level of CYP1A1 and CYP1B1 isoform mRNAs in cell cultures of intact fetal rat fibroblasts (F16, passage 13) and the ones infected by RLV (F27-RLV, passage 26) are shown in Fig. 1. One can see that the constitutive level of CYP1A1 mRNA in the cells F16 is extremely low, whereas it is significant in the cells F27-RLV grown under the same conditions. Inducer (TCDD) increases the level of CYP1A1 mRNA both in F16 and F27-RLV cells. The expression level of CYP1B1, another member of CYP family 1, was found constitutive in both cell lines with higher level in F27-RLV cells. The inducer increases the level of CYP1B1 mRNA both in F16 and F27-RLV cells. The CYP1A2 mRNA is virtually not expressed and not determined by RT-PCR in both cell lines (data not shown). Our data are consistent with the observation of specific expression of CYP1A2 in hepatocytes [11]. The level of mRNAs of the proteins AHR and ARNT mediating the induction signal transmission to CYP1A1 and CYP1B1 was virtually equal in all of the cell clones tested (Fig. 1).

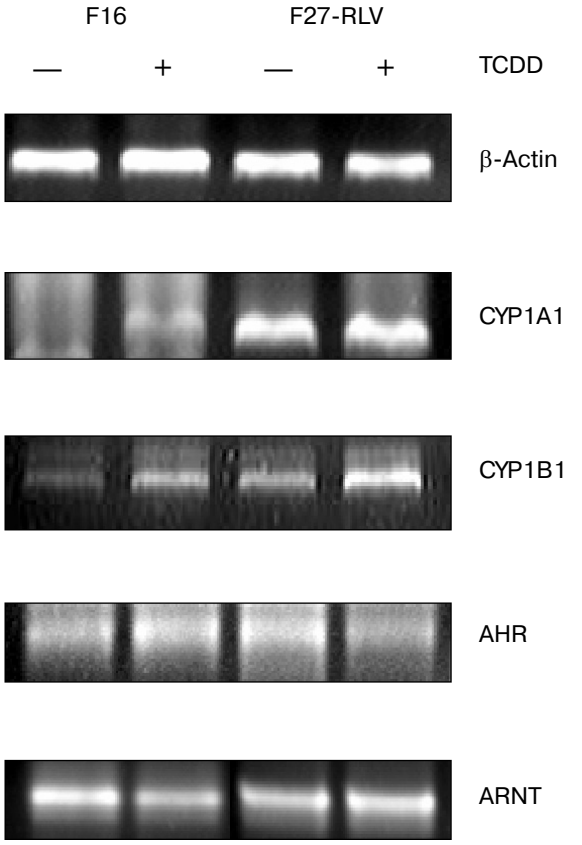

Fig. 1. Expression of β-actin gene, *CYP1A1*, *CYP1B1*, *AHR*, and *ARNT* determined by RT-PCR in fetal rat fibroblasts. TCDD (10^{-9} M) was used as the CYP1 inducer. The amounts of cDNAs taken for the amplification were equalized by the amount of β-actin mRNA.

Figure 2 demonstrates the levels of constitutive and TCDD-induced BP hydroxylase activities in the cells tested. In F16 cells, the constitutive level of the enzymatic activity is very low, whereas in F27-RLV cells this activity is more expressed. The induction degree (calculated as the ratio of TCDD-induced to the initial activity) was higher in F27-RLV cells compared with F16 cells, in which induction does not occur; these data are in agreement with the data on the CYP mRNA expression.

Sensitivity of the tested cultures to the toxic effects of BP and DMBA was determined from the MTT-test. Both BP and DMBA demonstrated high toxicity against F27-RLV cells, whereas the cells F16 were resistant to both substances throughout the range of concentrations applied (Fig. 3), which matches the mRNA levels (Fig. 1) and metabolic activities (Fig. 2) of CYP isoforms in these cells.

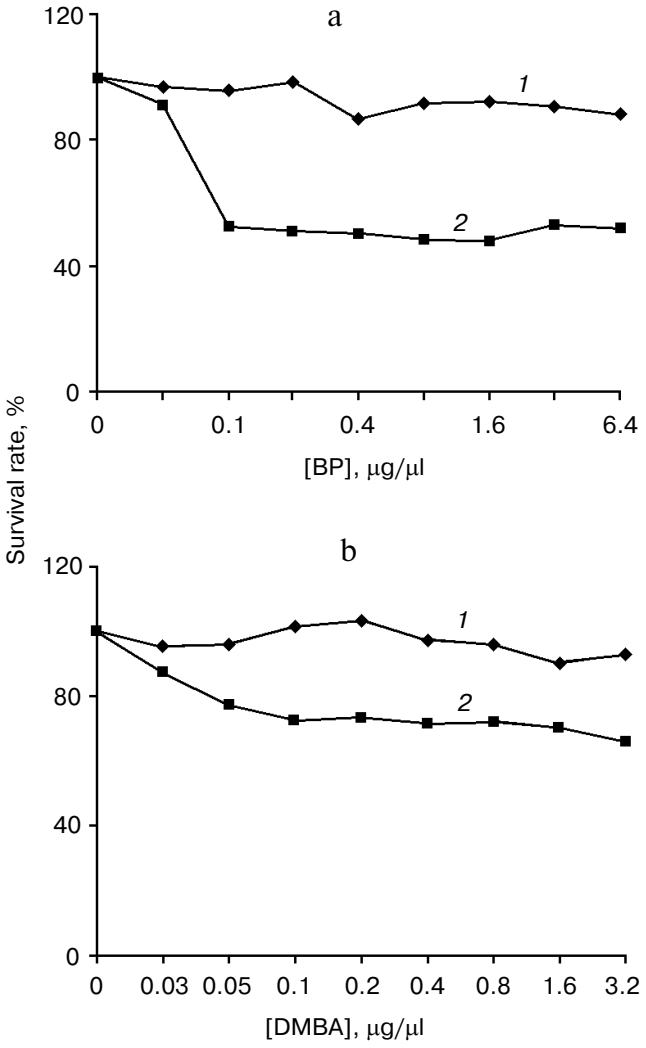

Fig. 3. Effect of benzo[a]pyrene (BP) (a) and 7,12-dimethylbenz[a]anthracene (DMBA) (b) on the survivability (% of control) of the fetal rat fibroblasts F16 (*1*) and F27-RLV (*2*) determined from the MTT-test.

DISCUSSION

The primary culture of fetal rat cells has low sensitivity to the transforming effect of carcinogens such as BP [8]. However, the cells infected by RLV (which does not contain any oncogene and can immortalize cells without affecting their morphology rather than transform them) acquire high sensitivity to the carcinogens added afterwards and undergo transformation [8]. A possible mechanism of this effect of RLV embodies changes in expression of xenobiotic-metabolizing enzymes, primarily CYP. Major CYP isoforms metabolizing polycyclic aromatic hydrocarbons including BP are members of family 1. These are precisely the isoforms whose alterations in expression and activity we studied.

Our data suggest increased expression of both constitutive and TCDD-induced pool of CYP1A1 and CYP1B1 isoforms in cell culture infected by RLV (and carrying the virus in persistent form) compared with the initial cell culture. Correspondingly, the RLV-infected cells compared with non-infected ones oxidize BP more effectively and are more sensitive to indirect toxic agents (Figs. 2 and 3). Note that the level of mRNAs encoding the induction-mediating proteins AHR and ARNT was the same in both cultures (Fig. 1) suggesting that (an)other still unknown factor(s) controlled by RLV may be involved, together with these known proteins, in expression of CYP1 isoforms.

Few studies have attempted to determine the condition of CYP isoforms in viral infection. Blood serum of patients suffering from respiratory virus infection suppresses the level of CYP3A6, but not the level of CYP1A1 in the culture of hepatocytes [15]. In mice injected with Coxsackie virus B3 both constitutive and inducible expression of CYP1A1 decrease [16]. In contrast, the level of CYP1A1 protein remains unchanged in liver when mice are infected by hepatitis B virus [17]. In hepatocyte culture infected by smallpox virus the level of phenobarbital-induced expression of CYP1A1 mRNA did not change, whereas the expression level of CYP2B1 mRNA decreased [18]. Expression levels of both CYP1A1 mRNA and protein decreased in various human epithelial cells infected by human papilloma virus E6/E7 [19]. Contrariwise, keratinocytes immortalized by simian virus SV40 demonstrated increased expression of CYP1A1 and CYP1B1 mRNAs [19]. So different virus types, such as SV40 and RLV, cause enhanced expression of CYP1 isoforms, thus suggesting that this phenomenon is not virus-specific but is a result of cell immortalization.

The enhanced expression of CYP1 mRNAs we observed in our experiments might not be a true enhancement, but maintenance of the expression level through passages. It is known that various types of normal cells lose monooxygenase activity and CYP expression up to complete disappearance [20]. Alternatively, tumor cells retain the level of CYP through passages. These observations suggest that the increase in expression of CYP isoforms is connected to enzyme stabilization through a series of passages and results from immortalization of the cells rather than from their becoming malignant or transformed.

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