Comparison of RAP-PCR analysis of gene expression in fresh and immortalised rat hepatocyte cell lines

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Abstract

Primary rat hepatocytes dedifferentiate rapidly losing the activities of the drug metabolising enzymes involved in the detoxification of xenobiotics in the liver. An alternative approach to using primary hepatocytes for toxicity testing is the development of immortalised hepatocyte cell lines via the transfection of primary hepatocytes with SV40 DNA. In order to assess the suitability of immortalised lines as an alternative to primary cell cultures we have used RNA arbitrarily primed polymerase chain reaction to compare gene expression in immortalised rat hepatocyte cell lines with that in primary rat hepatocytes. We have found that differences exist in the RNA transcripts between fresh and immortalised hepatocytes highlighting RNA arbitrarily primed polymerase chain reaction as a useful screening method for identifying immortalised lines which retain the most 'normal' phenotype in relation to the primary cells from which they originated.

Abbreviations: DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; RAP-PCR, RNA arbitrarily primed polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

Introduction

Human and animal cell cultures are being used in toxicity testing as a replacement for procedures involving animal testing. Primary cultures of rat hepatocytes, in particular, have been extensively used for studying xenobiotic metabolism and toxicity. However, there are drawbacks associated with primary hepatocytes in that they de-differentiate rapidly losing many liver specific functions, e.g. drug metabolising enzyme activity, within 24–48 h of culture (Grant et al., 1985). Amongst the most labile activities in primary cultures of hepatocytes are those of cytochrome P450 and the associated mixed function oxidase system, which are important in the metabolism and detoxification of xenobiotics in the liver. The total cytochrome P450 content has been found to decrease, and the relative proportion of cytochrome P450 isoenzymes to alter

markedly, in cultured hepatocytes with culturing time (Steward et al., 1985; Wortelboer et al., 1990).

As an alternative to primary hepatocyte cultures we have developed immortalised hepatocyte cell lines by transfecting primary hepatocytes with SV40 early region DNA (MacDonald et al., 1994; Yin et al., 1996). These lines are phenotypically more stable, retain many of the differentiated functions, and offer a readily available system for drug screening programmes, chronic toxicity studies and elucidation of the mechanisms of toxicity. However, different immortalised clones vary in the extent to which they retain the differentiated hepatocyte phenotype.

RNA fingerprinting using arbitrarily-primed polymerase chain reaction (RAP-PCR) provides a method for the detection of differential gene expression by comparing RNA transcripts between two or more populations (Liang and Pardee, 1995; McClelland et al., 1995). The process begins with first strand synthesis

initiated using an arbitrarily chosen primer that anneals and extends from sites contained within the RNA. Second strand synthesis proceeds in a similar manner during a single round of low stringency PCR after which the products serve as templates for subsequent high stringency PCR.

In this paper we have investigated the suitability of RAP-PCR as a screening technique for comparing the phenotype of immortalised liver cell lines with that of primary hepatocytes. The random amplification of RNA transcripts and their subsequent analysis on polyacrylamide sequencing gels provides RNA fingerprints of different cell populations which can be compared. In addition, we have examined the retention of drug metabolising enzyme activity in our immortalised lines using reverse transcription PCR (RT-PCR) to investigate the expression of cytochrome P450 isoform messenger RNA (mRNA).

Materials and methods

Cell culture. Hepatocytes, isolated from male Sprague Dawley rats by collagenase perfusion, were transfected with SV40 early region DNA by either calcium phosphate precipitation ('C' lines) or electroporation ('KC' lines) and cultured as described previously (MacDonald et al., 1994; Yin et al., 1996; MacDonald and Willett, 1997). Freshly isolated hepatocyte controls were used at 8 h post-perfusion. The HTC hepatoma tissue culture cell line was established from a Buffalo rat hepatoma Morris 7288C (Thompson, 1979) and was obtained from Professor J.D. Pitts (Glasgow).

RNA precipitation. Total RNA from freshly perfused hepatocytes and immortalised cell lines was prepared using the total RNA isolation reagent TRIzol (Life Technologies, Paisley). The cells were lysed directly by the addition of 1 ml TRIzol to a 75 cm² tissue culture flask (Corning). The homogenised samples were incubated at room temperature for 5 min and 0.3 ml of chloroform added. The tubes were vigorously shaken by hand for 15 s, incubated at room temperature for 15 min and subsequently centrifuged at 12000 g for 15 min at 4 °C. The resultant RNA containing aqueous phase was transferred to a fresh tube and precipitated by the addition of 0.625 ml isopropyl alcohol. The samples were incubated at room temperature for 10 min and centrifuged at 12000 g for 10 min at 4 °C. The resultant supernatant was washed once with 1.5 ml of

75% ethanol at 7500 g for 5 min at 4 °C. The RNA pellet was air dried and 30 µl of DEPC-treated distilled H₂O (0.05% v/v diethyl pyrocarbonate in water) added. The RNA was stored at -20 °C.

First strand synthesis. Total RNA was converted to cDNA using the Superscript First Strand Synthesis kit (Life Technologies, Paisley). Total RNA (5 µg) was mixed with either 1 µl of a 25 µM arbitrary primer stock A1 (Stratagene) (RAP-PCR) or 0.5 µg oligo(dT)₁₂₋₁₈ (RT-PCR) and the final volume increased to 12 µl with DEPC-treated distilled water. This RNA/primer mixture was incubated at 70 °C for 10 min and immediately placed on ice for at least 1 min. A reaction mixture (7 µl) containing 2 µl of 10 × PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 2 µl of 25 mM MgCl₂, 1 µl of 10 mM deoxynucleotide triphosphate (dNTP) mix (10 mM each dATP, dCTP, dGTP, dTTP) and 2 µl 0.1 M dithiothreitol (DTT) was added to each RNA mix and pulse spun. After incubation for 5 min at 42 °C, 1 µl (200 U) of superscript II RT was added to each tube and mixed. The samples were incubated at 42 °C for 50 min and the reaction terminated at 70 °C for 15 min. The reaction volume was made up to 100 µl by the addition of 80 µl DEPC-treated distilled H₂O and the cDNA stored at -20 °C until required or at 4 °C if used immediately.

RAP-PCR. RNA fingerprinting was carried out using a RAP-PCR kit (Stratagene). cDNA synthesis was performed as described above using an 18 mer arbitrary primer A1 (Stratagene). To a thin walled reaction tube 5 µl of 10 × reaction buffer, 3 µl of 25 mM MgCl₂, 28.75 µl of distilled H₂O, 0.1 µl of dNTP mix (25 mM of each dATP, dCTP, dTTP and dGTP), 0.2 µl of Taq DNA polymerase (5 U/µl; Pharmacia Biotech), 2 µl of 25 mM RAP-PCR primer A1, 10 µl of a 1:10 cDNA dilution and 1 µl of [α -³²P] dATP (10 µCi/ml) were added. The reaction was gently mixed, pulse spun and overlaid with 50 µl mineral oil. RAP-PCR was performed using the following cycling conditions: 1 cycle of 1 min at 94 °C, 5 min at 36 °C and 5 min at 72 °C; 40 cycles of 1 min at 94 °C, 2 min at 50 °C and 2 min at 72 °C and finally 1 cycle at 72 °C for 10 min. To analyse the product 5 µl of the above reaction was added to 5 µl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% Bromophenol blue and 0.05% xylene cyanol FF and the mixture heated to 80 °C for 2 min. The reaction was analysed by running 2.5 µl of the reaction mix in a 6% acrylamide sequencing gel.

RT-PCR. cDNA synthesis was performed as described using 0.5 μ g of oligo (dT)_{12–18}. A 10 mM primer mix specific for either 2B1, 2B2 or 3A1 isoforms of P450 was used (Omiecinski et al., 1990). To a thermowell tube (Costar) 5 μ l of each primer mix was added. A master mix of the following components, 5 μ l of 10 $\times$ NH₄ buffer, 4 μ l of 10 mM dNTP mix (10 mM of each dATP, dTTP, dCTP, dGTP), 2 μ l of 50 mM MgCl₂, 28.75 μ l distilled H₂O and 0.25 μ l Taq DNA polymerase (Pharmacia Biotech) was added to each reaction and pulse spun. To each tube 5 μ l of a 1:5 cDNA dilution was added and the total reaction overlaid with 50 μ l mineral oil. The reaction was pulse spun and placed in the thermocycler (Techne) to undergo a series of denaturation, annealing and elongation reactions as follows: 1 cycle at 93 °C for 4 min, 30 cycles at 93 °C for 1 min, 54 °C for 1.5 min and 72 °C for 1 min and 1 cycle at 72 °C for 5 min. The resultant PCR products were resolved on a 1.5% agarose DNA gel and visualised by ethidium bromide staining. A 0.125–23 Kb DNA sizing ladder was used.

Results and discussion

RAP-PCR was used to investigate differences between the RNA transcripts of freshly isolated and immortalised hepatocytes. As illustrated in Figure 1 RAP-PCR products were identified in the immortalised hepatocyte lines C3HIA and KCIIIB that were absent in 8 h post-perfusion hepatocytes.

Using RT-PCR we demonstrated the presence of cytochrome P450 isoform mRNA in hepatocytes, 8 h post-perfusion (Figure 2). Clear specific PCR products for the isoforms cytochrome 2B1, 2B2 and 3A1 were identified with respective sizes of 109, 163 and 203 base pairs as described previously (Omiecinski et al., 1990). In contrast, however, using the same P450 isoenzyme specific primers, larger PCR products were identified in both the calcium phosphate transfected and electroporated immortalised hepatocyte cells. Figure 3 illustrates the results obtained for the electroporated hepatocyte line KCIVA, and similar results were obtained for the calcium phosphate transfected lines. Cytochrome 2B1 and 2B2 specific bands were found to be approximately 10 times larger than that expected with the cytochrome 3A1 specific product approximately double the expected size. However, analysis of the sequence of the amplified bands showed that the level of homology with the P450 primers was low

Figure 1. RNA fingerprint of freshly isolated hepatocytes (lane 1), HTC (lane 2) and the immortalised cell lines C3HIA (lane 3) and KCIIIB (lane 4). Arrows indicate bands identified in the immortalised lines which were absent in primary hepatocytes.

and thus we concluded that the P450 mRNAs are not expressed in the immortalised hepatocyte lines.

RAP-PCR and differential display, which employs oligo (dT) in the first strand synthesis, have been used in different biological systems to detect variation in gene expression. In our study RAP-PCR identified differences in the RNA transcripts obtained from our immortalised and freshly isolated hepatocyte populations indicating differences in gene expression in our immortalised lines. Frueh et al. (1997) used differential display to demonstrate that phenobarbitone treatment of chick embryo liver cells significantly modulated the expression of more than fifty different genes a large

Figure 2. cDNA from freshly isolated hepatocytes was amplified with primers specific for lactate dehydrogenase (lane 2), CYP2B1 (lane 3), CYP2B2 (lane 4) and CYP3A1 (lane 5). Lanes 1 and 6 contain a DNA sizing ladder.

Figure 3. Comparison of 2B1, 2B2 and 3A1 specific products identified for freshly isolated (lanes 2–4) and immortalised hepatocytes (lanes 6–16). Lanes 6, 7 and 8 show PCR products for CYP2B1, CYP2B2 and CYP3A1 primers respectively, identified in the KCIVA (passage 6). Lanes 10–12 are products for KCIVA at passage 7 for CYP2B1, CYP2B2 and CYP3A1 primers. Lanes 14–16 show PCR products for CYP2B1, CYP2B2 and CYP3A1, respectively, identified in the immortalised cell line KCIVA (passage 11).

fraction of which were negatively regulated by drug treatment. Previously, Ralph et al. (1993) used RAP fingerprinting to identify transcripts from mink lung epithelial cells whose steady state levels were regulated by transforming growth factor β . They found three products which displayed differential regulation and concluded that RAP fingerprinting should be able to identify many of the genes that change in steady state expression during the cell cycle.

RNA fingerprinting has also been used in the analysis of gene expression patterns in diseased tissues where genes were identified whose expression was

upregulated with time in hearts undergoing chronic rejection (Utans et al., 1994). Guimaraes et al. (1995) found differential display to be capable of identifying very low abundance mRNAs when comparing the gene expression patterns of different cell populations including hematopoietic cells. In addition to identifying differentially expressed genes, RNA fingerprinting has been adapted for the direct and rapid cloning of displayed fragments including bands that give faint signals on displays (Lee, 1996).

As result of the differences found in our RNA transcripts we decided to examine our populations of hepatocytes to see if they varied at the level of drug metabolising enzyme expression. We could find no evidence for the presence of mRNA for CYP 2B1, 2B2 or 3A1 in the immortalised cells, although they were expressed in the freshly isolated cells. CYP 2B1 and 2B2 are the major phenobarbitone inducible P450 isoenzymes in rat liver, but the majority of cell lines, including hepatoma cells, do not express them either constitutively or after exposure to phenobarbitone (Waxman and Azaroff, 1992). Hepatocyte cell cultures progressively lose the ability to synthesise cytochrome P450 with culturing time (Waxman and Azaroff, 1992), and, in fact, the ability to respond to phenobarbitone induction by an increase in CYP 2B1/2 activities has been proposed as a sensitive and stringent marker for differentiated function (Guzelian et al., 1989). Padgham and colleagues (Padgham et al., 1993, 1994) described a general decline in cytochrome expression in hepatocytes within 2–3 h of isolation. They found the expression of CYP 3A1 mRNA to be particularly labile, becoming almost undetectable within 4 h of culture, whereas expression of CYP 2B1/2 mRNAs was temporarily stable. It would appear that the primers available to us for our study have proven to be those of particularly labile P450 isoenzymes. Thus, although it was not possible to maintain 2B1, 2B2 or 3A1 expression in the immortalised cells, there may be expression of other more stable isoenzymes such as CYP 1A2 (Padgham et al., 1993).

Conclusions

In conclusion, therefore, we have demonstrated differences in the RNA transcripts between freshly isolated and immortalised hepatocytes suggesting the occurrence of differences in gene expression in our immortalised lines. Overall this study supports the use of

RAP-PCR as a useful screening method for identifying those immortalised lines which retain the most 'normal' phenotype in relation to primary hepatocytes.

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