Extracellular Matrix Regulation of Multidrug Resistance in Primary Monolayer Cultures of Adult Rat Hepatocytes

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Abstract

Previous studies reported that, in the absence of drug exposure, multidrug resistance, including resistance to Adriamycin (ADR), could develop in primary rat hepatocyte cultures (B. Carr, Proc. Am. Assoc. Cancer Res., 29:1158, 1988). However, the hepatocytes in that report were cultured on plastic without the benefit of an extracellular matrix (ECM). Because the ECM regulates hepatic gene expression, we have critically evaluated in primary cultures of rat hepatocytes how the ECM affects hepatic ADR resistance, the level of the drug efflux transporter associated with MDR, P-glycoprotein (pgp), and transport of a prototypical pgp substrate, vincristine. Hepatocytes cultured on type I collagen (Vitrogen) had greater resistance to ADR toxicity accompanied by parallel increases in the level of pgp mRNA, decreased drug accumulation, and enhanced drug efflux when compared with the hepatocytes maintained on the basement membrane matrix Matrigel. The development of ADR resistance coincided with the time course of increased pgp mRNA but was not coincident with the time course of expression of either the placental isozyme of glutathione S-transferase or P-450 reductase, proteins associated with MDR in some resistance models. Southern blot analysis revealed neither gross changes in pgp gene structure or gene copy number to account for the increase in pgp RNA levels for hepatocytes cultured on Vitrogen. ECM also regulated xenobiotic-inducible expression of hepatic pgp, since chemotherapeutic agents, including vincristine and colchicine, induced pgp mRNA exclusively in hepatocytes cultured on Vitrogen. The critical matrix proteins in Matrigel responsible for regulation of pgp were determined by the selective addition of its components to the culture environment. The presentation of the individual matrix elements as a rigid substratum to the hepatocyte did not decrease pgp mRNA. In contrast, the presentation to the same hepatocytes of either laminin or type IV collagen in a nonrigid state (solubly in the medium) selectively decreased hepatocellular pgp mRNA. We conclude that primary rat hepatocytes develop ADR resistance with time in culture due to increased expression of pgp and that ECM proteins represent endogenous physiological modulators of both basal and chemotherapeutically inducible expression of hepatic P-glycoprotein.

Introduction

Pgp, a plasma membrane protein, plays a major role in cellular detoxification by actively pumping xenobiotics out of cells (1). Not surprisingly, pgp is highly expressed in organs, such as liver, which are actively involved in xenobiotic detoxification (2). Moreover, xenobiotic exposure can increase the amount of pgp mRNA (3). For example, in normal rat liver, the level of pgp RNA increases in response to polycyclic aromatic hydrocarbon administration (4). This example of xenobiotically inducible expression of pgp most likely represents an important physiological step in xenobiotic detoxification, which permits the hepatocyte to adapt to local changes in its chemical environment. The phenomenon of increased pgp was first described as a response of cancer cells to chemotherapeutic agents (5). The increased amount of pgp led to resistance to the chemotherapeutic agents because the pump encoded by the pgp gene readily removed the chemicals from the cell. Furthermore, because pgp can transport many diverse compounds, cellular resistance developed to the toxic effects of many drugs in what has been termed “multidrug resistance” (6).

Despite the large body of data identifying chemical agents as modulators of pgp, the identification and regulation of pgp in normal tissues by intracellular and extracellular factors remain, with few exceptions (7), largely unexplored. Understanding the cellular cues involved in regulation of pgp in normal tissues may help explain why some organs, such as liver, constitutively express high levels of pgp and why neoplastic liver, in the absence of drug exposure, overexpresses pgp (6, 8). Clearly, it is difficult to define the factors which regulate hepatic pgp expression in vivo. However, a recent study found that by merely placing freshly isolated primary rat hepatocytes into culture, in the absence of drugs, a phenotype developed in these cells (within hours) (9) which was remarkably similar to the MDR phenotype described for cancer cells (6). Although it was suggested that multidrug resistance of these primary hepatocytes to ADR and other chemotherapeutic agents was an adaptive response to isolation and culture (9), the mechanisms responsible for MDR were not elucidated. Importantly, in this study, the primary rat hepatocytes were cultured

1 The abbreviations used are: pgp, P-glycoprotein; P170; Vitrogen; MDR, multi-drug resistance; GST-π, placentonal isozyme of glutathione S-transferase; ADR, Adriamycin; LDH, lactate dehydrogenase; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm sarcoma; cDNA, complementary DNA; PBS, phosphate-buffered saline.

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on serum-coated plastic, a "substratum" which bears little resemblance to the ECM of the hepatocyte in vivo. This omission may have revealed an important regulator of hepatic pgp, since the ECM participates in the growth, development, and differentiation of the hepatocyte (10). Furthermore, the type and distribution of ECM proteins in the liver influence both the shape of the hepatocyte and "liver-specific" gene expression (11, 12). Indeed we (13) and others (14, 15) recently developed a new system for culture of hepatocytes on the reconstituted basement membrane, Matrigel. Unlike standard cultures of hepatocytes attached to serum-coated plastic or type I collagen, cultures on Matrigel appear to express numerous differentiated functions characteristic of the adult liver (13-15). For these reasons, we hypothesized that the ECM might play a critical role in regulation of pgp and that the cultured rat hepatocyte system would make it possible to define rigorously the role of the ECM in expression of hepatic pgp.

We now report that acquisition of ADR resistance in primary cultures of adult rat hepatocytes is associated with a dramatic increase in expression of pgp. In addition, we have found that individual components of the extracellular matrix dramatically influence hepatocellular expression of pgp. Moreover, our results show that both the chemical composition as well as biophysical properties of these ECM molecules participate in regulation of hepatocellular pgp gene expression.

Results

To explore the possibility that hepatocellular resistance to a specific chemotherapeutic agent is modulated by the ECM, we compared the time course of 180 μM ADR cytotoxicity (a concentration previously demonstrated to be toxic to freshly isolated rat hepatocytes) (16) in rat hepatocytes cultured on VIT or MG (Fig. 1A). Hepatocytes cultured on either matrix were equally sensitive to a 24-h exposure to 180 μM ADR at either 0-24 or 24-48 h in culture, as determined by leakage of LDH from the cell. In contrast, rat hepatocytes cultured on VIT and older than 72 h showed a significant increase in resistance to ADR (37% leaked LDH), compared to those cultured on MG (53% leaked LDH). Additional studies demonstrated that hepatocytes cultured on MG and exposed to ADR excluded less trypan blue dye (2-fold) (i.e., were more sensitive) than similarly treated hepatocytes cultured on VIT. As reported in the study by Carr (9), ADR resistance develops in the absence of drug exposure and increases with longer culture duration; however, we found that the rate of ADR resistance development much faster for hepatocytes cultured on VIT compared to MG.

To examine the specificity of the drug-resistant phenotype in hepatocytes cultured on VIT, we evaluated hepatocyte sensitivity to therapeutic agents known to be associated with the MDR phenotype (Fig. 1B). The hepatocytes were cultured for 72 h in the absence of drug, followed by a 24-h incubation with drug. As might be expected for a nonreplicating cell, the hepatocytes were remarkably resistant to methotrexate and podophyllotoxin. In contrast, hepatocytes were extremely sensitive to actinomycin D. The cytotoxicity of ADR increased as the concentration increased from 1 to 10 μM but only gradually increased between 10 and 90 μM. The hepatocytes were also remarkably resistant to VCR, demonstrating a dose-related increase in cytotoxicity (data not shown). The profound differences in susceptibility of the hepatocytes to the cytotoxic effects of actinomycin D compared to ADR (Fig. 1B) may reflect the form of pgp expressed by the hepatocytes. Another possibility may be that the hepatocyte can readily convert the ADR to the inactive aglycone (17).

To determine whether matrix-dependent sensitivity of hepatocytes to ADR was due to increased expression of the pgp drug transporter, we performed Northern blot analysis on total cellular RNA extracted from rat hepatocyte cultures at the indicated times (Fig. 2). The amount of pgp mRNA in freshly isolated hepatocytes that hybridized with the human cDNA to mdrl [pADR+ (18)] was barely detectable, consistent with the low level in vivo (4). In contrast, after culture for 24 h, expression of three pgp hybridizable mRNA transcripts (4.5, 5, and 6 kilobases) was increased regardless of the matrix, although the amount of pgp mRNA was much greater in hepatocytes cultured on VIT. Indeed, consistent with our mRNA results, a recent report by Fardel et al. (19) demonstrated that the amount of pgp protein (assayed by Western blot analysis) increased with time in culture in rat hepatocytes cultured on serum-coated plastic. In general, we have found that the rate of pgp mRNA accumulation (up to 5 days in culture) is at least 4-fold greater for hepatocytes cultured on serum-coated plastic, a "substratum" which bears little resemblance to the ECM of the hepatocyte in vivo. This omission may have revealed an important regulator of hepatic pgp, since the ECM participates in the growth, development, and differentiation of the hepatocyte (10). Furthermore, the type and distribution of ECM proteins in the liver influence both the shape of the hepatocyte and "liver-specific" gene expression (11, 12). Indeed we (13) and others (14, 15) recently developed a new system for culture of hepatocytes on the reconstituted basement membrane, Matrigel. Unlike standard cultures of hepatocytes attached to serum-coated plastic or type I collagen, cultures on Matrigel appear to express numerous differentiated functions characteristic of the adult liver (13-15). For these reasons, we hypothesized that the ECM might play a critical role in regulation of pgp and that the cultured rat hepatocyte system would make it possible to define rigorously the role of the ECM in expression of hepatic pgp.

We now report that acquisition of ADR resistance in primary cultures of adult rat hepatocytes is associated with a dramatic increase in expression of pgp. In addition, we have found that individual components of the extracellular matrix dramatically influence hepatocellular expression of pgp. Moreover, our results show that both the chemical composition as well as biophysical properties of these ECM molecules participate in regulation of hepatocellular pgp gene expression.

Fig. 1. Adriamycin sensitivity of hepatocytes cultured on Vitrogen or Matrigel (A) or sensitivity of hepatocytes cultured on Vitrogen to other cytotoxic agents (B): A, the hepatocytes were cultured in drug-free medium from plating. Twenty-four h prior to the harvest time, indicated as time in culture, 180 μM Adriamycin was added to the culture medium. B, hepatocytes were cultured in drug-free medium for 0-72 h and then treated with varying concentrations of the indicated drugs for a 24-h interval. Cytotoxicity was assessed by determining the ratio of the LDH in the medium to that remaining in the cells, as described in "Materials and Methods."
cytotes cultured on VIT compared to those on MG. Of note, the level of pA DR'-hybridizable mRNA in hepatocytes cultured on VIT was at least 2-fold greater than the level of pgp mRNA in the multidrug-resistant rat hepatocellular carcinoma (H-35) or human hepatoblastoma (HepG2) cell lines (Fig. 2).

To determine whether other phenotypic alterations frequently associated with the MDR phenotype could account for the increased ADR resistance in primary rat hepatocytes, we rehydrized the Northern blot with a cDNA specific for either the placental isozyme of glutathione-S-transferase (GST-π) or P-450 reductase (Fig. 2). GST-π mRNA, a phase II-conjugating enzyme whose overexpression can confer resistance to alkylating agents (20), was dramatically elevated as early as 24 h in culture on either matrix and remained elevated, but invariant, after the initial 24-h interval. In contrast, P-450 reductase mRNA (the enzyme responsible for the initiation of ADR-induced free radical DNA damage and presumably ADR cytotoxicity (21)), increased at 24 h and paradoxically increased further at 96 h, when ADR toxicity decreased. Consistent with the MDR phenotype, there was a loss in expression of constitutive cytochromes P-450 (22), as illustrated by P-450 2A1/2. It should be noted that 2A1/2 expression was totally absent from 96-h hepatocytes cultured on VIT. These findings show that other pheno-

typic alterations, frequently associated with the multidrug-resistant phenotype, i.e., decreased P-450 reductase or increased GST-π, do not account for the ADR resistance in hepatocyte cultures but suggest that increased pgp expression is the most likely candidate mediating this resistance.

The MDR phenotype is frequently associated with pgp gene amplification (18). Although we would not expect increased pgp gene copy in nonreplicating primary hepatocyte cultures, we used Southern blot analysis to probe for gross structural alterations in genomic DNA isolated from rat hepatocytes cultured on different matrices (VIT or MG) for various time intervals. The restriction pattern (and the amount) of EcoRI-digested hepatocyte genomic DNA that hybridized with the pA DR' cDNA was unchanged with either time in culture or matrix (data not shown). These data indicate that no gross structural changes in the pgp gene account for the overexpression of pgp mRNA seen in the resistant rat hepatocytes.

Next, we evaluated the kinetics of VCR transport, a typical pgp substrate (23), in hepatocytes cultured for 72 h on either VIT or MG (Table 1). At equivalent extracellular concentrations of VCR (50 nM), hepatocytes cultured on VIT had 3-4-fold lower drug levels than hepatocytes maintained on MG, an effect which was magnified by increased extracellular concentrations of VCR (200 nM). To evaluate whether the mechanism of decreased cellular VCR accumulation was due to accelerated efflux, hepatocytes cultured on either matrix were incubated with [3H]VCR to achieve similar intracellular concentrations (Table 1); extracellular drug of 50 nM and 200 nM for MG and VIT, respectively and then resuspended into drug-free medium for varying time intervals to determine the rate of [3H]VCR efflux. The rate constant for [3H]VCR efflux for hepatocytes cultured on VIT (K = 0.045 min⁻¹) was at least 3-fold greater (range, 3.4-5-fold; n = 3) than the rate constant for hepatocytes cultured upon MG (K = 0.013 min⁻¹). Moreover, the non-effluxable bound [3H]VCR was severalfold higher in the hepatocytes cultured on MG (data not shown). Finally, sodium azide-induced intracellular ATP depletion led to an increase in the steady-state level of intracellular [3H]VCR (Table 1), a result consistent with the requirement of ATP as an energy source by the pgp efflux pump (24). The over 3-fold increase in the rate of [3H]VCR removal from the hepatocytes cultured on VIT (Table 1) agrees with our densitometric determination of a 4-fold increase in pgp mRNA (Fig. 2) and is consistent with a role for pgp in this model of hepatic MDR.

Next, pgp inhibitors, verapamil and reserpine (25), were used to confirm that VCR efflux was accounted for

Table 1. Vincaeine transport parameters for hepatocytes cultured on either Vitrogen or Matrigel

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Extracellular VCR Accumulation (nM)</th>
<th>Steady-state Drug Level (pmol/mg Protein)</th>
<th>1 nM Sodium Azide</th>
<th>3H-VCR Efflux Rate Constant (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrogen</td>
<td>50</td>
<td>0.82</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Vitrogen</td>
<td>200</td>
<td>2.45 ± 0.47</td>
<td>19.3</td>
<td>0.046</td>
</tr>
<tr>
<td>Matrigel</td>
<td>50</td>
<td>3.1 ± 0.6</td>
<td>20.77</td>
<td>0.011</td>
</tr>
<tr>
<td>Matrigel</td>
<td>200</td>
<td>11.2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, not determined.

* Mean ± SE of the average value from 3 experiments performed on separate days.
by pgp in cultured rat hepatocytes. Addition of verapamil (20 μM) to the culture medium increased hepatocellular VCR accumulation to a level 2-fold above the untreated cultures (Fig. 3A). After loading the hepatocyte with [3H]VCR, we followed the efflux of intracellular [3H] by resuspension of the hepatocytes in fresh medium (Fig. 3B). The t1/2 for efflux of [3H]VCR from VIT-cultured hepatocytes was 17 min, whereas addition of either verapamil or reserpine decreased the rate of [3H]VCR efflux, as demonstrated by t1/2 values of 66 and 146 min, respectively. Hence, by employing pgp inhibitors, we confirmed that a functional pgp protein is expressed in cultured rat hepatocytes.

Since MDR cells typically show resistance to many anticancer drugs, including ADR, colchicine, and vincristine (6), we examined pgp mRNA expression after exposure of the hepatocytes to these agents. Treatment of hepatocytes with either ADR (data not shown) colchicine, cytochalasin D, or VCR (Fig. 4) dramatically induced the level of pgp mRNA, but only in hepatocytes cultured on VIT. Interestingly, griseofulvin and nocardazole produced only slight induction of pgp. In contrast, these same drug treatments appeared to actually suppress pgp mRNA in MG-cultured hepatocytes. Since integrin [a cell surface receptor for ECM proteins (26)] mRNA levels were not significantly changed, regardless of the drug treatment (except griseofulvin), it suggests that nonspecific membrane alterations by these drugs are not primary determinants of pgp induction. As we have previously reported (13), the levels of mRNA for the cytoskeleton proteins actin and tubulin are generally higher in the more flattened hepatocytes on VIT than in rounded cells on Matrigel. However, although cytoarchitecture can be disrupted by colchicine, cytochalasin D, and vincristine, no consistent matrix-specific drug-induced changes in the amount of the mRNA for the target enzymes, tubulin or actin (Fig. 4), were found, as judged by hybridization with specific cDNA probes. The finding that pgp is induced by antimitotic drugs, but only for those hepatocytes cultured on VIT, demonstrates that the ECM can also regulate xenobiologically inducible pgp expression.

In order to identify the specific component(s) of MG that was involved in regulating pgp, we cultured hepatocytes on substrata consisting of the individual components of MG at concentrations equivalent to those found in the MG matrix (Fig. 5). Despite the fact that MG is composed primarily of laminin and type IV collagen (27), incubation of hepatocytes on dishes precoated with rigid matrices of laminin, type IV collagen, or both laminin and type IV collagen did not prevent the increase in pgp mRNA expression. Indeed, the magnitude of expression of pgp mRNA on any of these three matrices was similar to the level of pgp mRNA in hepatocytes cultured on VIT or rat tail type I collagen or on serum-coated plastic. Because others have demonstrated that proteoglycans, such as those in MG, can maintain transcriptional gene expression in cultured hepatocytes (28), we tested their effect on pgp RNA levels. Since these negatively charged molecules are not suited as a matrix, we added them to the media (28). Addition of EHS purified dextran sulfate proteoglycan or heparin (Fig. 5) or carageenan or heparan sulfate (data not shown*) to the media of hepatocytes cultured on plastic failed to influence the amount of pgp mRNA.

It has recently been demonstrated by ourselves (29) and others (30, 31) that the biophysical state of the ECM molecule can dramatically influence its effect on hepatic gene expression. Therefore, we added “soluble” MG (MG dissolved in media to a final concentration identical to MG matrix) to hepatocytes cultured on VIT or plastic and found that it suppressed the increase in pgp mRNA expression (Fig. 5). To identify the specific active component in soluble MG, we cultured rat hepatocytes in the absence of matrix elements on serum-coated plastic for 72 h and then exposed the cells for a 24-h interval to media containing soluble matrix components. Fig. 6A demonstrates that a short exposure (24 h) to soluble laminin, the major component of MG, had only a slight effect on pgp mRNA expression. This preparation of laminin was active, however, because it permitted the same hepatocytes to retain inducible P-450 gene expres-

*J. D. Schuetz and E. G. Schuetz, unpublished observations.
sion (data not shown\(^1\)). In contrast, treatment of hepatocytes with soluble type IV collagen almost completely suppressed the increase in pgp RNA. The rehybridization of this blot (Fig. 6A) with a cDNA for the liver-specific gene, albumin, confirms that the effect of type IV collagen was specific for pgp expression. Moreover, the suppression of pgp by soluble type IV collagen was specific since pgp expression was less changed by soluble type I collagen. In a separate experiment, we found that a longer duration of treatment with either soluble laminin or MG [but not type I collagen (data not shown)] suppressed pgp mRNA to levels which are actually less than MG alone (Fig. 6B). These results demonstrate that the major components of the basement membrane MG, type IV collagen and laminin, are the active effector molecules responsible for decreasing pgp mRNA expression in cultured rat hepatocytes.

Discussion
Our results demonstrate that the molecular mechanism accounting for ADR resistance in normal adult rat hepatocytes placed into primary culture is upexpression of pgp. Moreover, it is clear from these studies that the ECM can modulate hepatocellular pgp mRNA expression and ADR toxicity. Indeed, hepatocytes cultured on type I collagen (VIT) showed greater resistance to ADR (Fig. 1), accompanied by significantly higher levels of pgp mRNA (Fig. 2), and decreased drug accumulation (Table 1) due, most likely, to the 3-4.5-fold increase in the rate of drug efflux compared to hepatocytes cultured on the basement membrane matrix, MG. Our systematic analysis of the individual elements in MG demonstrates that, in the hepatocyte culture environment, laminin and type IV collagen are critical MG components that regulate hepatocellular pgp expression. Moreover, we also show that the biochemical composition of the ECM is not the sole regulator of hepatic pgp, but gross changes in biophysical structure of the ECM (rigid versus soluble) control hepatic pgp expression as well. Indeed, matrices comprised of rigid substrata of either laminin or type IV collagen were unable to decrease expression of pgp mRNA in cultured hepatocytes, whereas hepatocytes cultured on the less rigid gel, MG, or addition of either laminin or type IV collagen in a nonrigid soluble state to the media decreased hepatic pgp mRNA. Taken together, our results demonstrate that ECM molecules are endogenous physiological regulators of hepatic pgp gene expression.
The biological potency of the individual components in the ECM is increasingly appreciated as these proteins [e.g., laminin (30)] are found to be indispensable for the regulation of hepatocyte gene expression (13). The first clue that the ECM regulated hepatic pgp was our finding that the type of culture matrix could profoundly influence hepatic ADR toxicity. Subsequently, we found that matrix selectively modulated pgp RNA levels. The fact that hepatocytes cultured on MG had lower pgp levels (expression close to in vivo levels) compared to those cells cultured on VIT led us to survey the components of MG for candidates which might regulate pgp. We selected laminin because it plays a critical role in regulation of hepatic albumin gene expression (30, 31) and drug resistance in small cell lung cancer lines (32). Like MG, laminin alone [added solubly to the media (Fig. 6B)] could suppress hepatic pgp gene expression; however, laminin required an exposure of 72 h to suppress the level of pgp, whereas MG suppressed pgp more rapidly (less than 24 h). This finding prompted us to examine the next major component in MG, type IV collagen. Because soluble type IV collagen had no effect on the expression of albumin mRNA in hepatocytes cultured on type I collagen (30), we were surprised to find that soluble type IV collagen rapidly suppressed pgp RNA expression. Indeed, the suppression of pgp by the type IV collagen molecule was strikingly selective, because expression of neither albumin (Fig. 6A) nor integrin mRNAs (data not shown) was changed by type IV collagen. Moreover, soluble heparin sulfate proteoglycans, which are specific mediators of hepatocellular gene expression (28), were without effect on pgp mRNA (Fig. 5).

Why does MG permit cultured hepatocytes to maintain pgp mRNA close to in vivo levels, whereas hepatocytes cultured on VIT (or serum-coated plastic) overexpress pgp mRNA? The mechanism might involve the differential retention/activation of specific cell surface receptors (e.g., integrins) which bind ECM proteins (26) in VIT versus MG-cultured hepatocytes. The differential expression of the integrin proteins could then activate different intracellular transduction pathways, ultimately resulting in alterations in pgp gene expression (26). An alternative hypothesis suggests that ECM regulates hepatic gene expression by regulation of cell shape (11). The concept of cell shape as a determinant of pgp hepatocellular expression seems less likely because pgp mRNA was suppressed in both rounded hepatocytes cultured on MG matrix and flattened hepatocytes cultured in medium supplemented with soluble ECM components. Finally, since it has been demonstrated that agents which induce differentiation can, in some cell types, decrease expression of pgp and MDR (33), an alternative possibility is that MG regulates hepatic pgp by maintaining a more differentiated hepatic phenotype.

Although the hepatocyte in vivo does not rest on a basement membrane of “Matrigel,” the biochemical composition of MG imitates the composition and biophysical conformation of some of the ECM molecules.
contacted by the hepatocyte in vivo. Given the dramatic effects that in vitro alterations in ECM molecules have on pgg gene expression in cultured hepatocytes, we would predict that changes in vivo in the composition and/or biophysical state of the ECM [such as the extensive remodeling of the ECM (particularly the increased amount and rigidity of type I collagen)] characteristic of hepatic cirrhotic and fibrotic disease states might facilitate the development of hepatocytes with the MDR phenotype. Indeed, a dramatic induction in pgg gene expression in rat cholestasis, a cirrhotic disease state characterized by extensive disorganization in ECM deposition (34), is consistent with this idea. Similarly, it is intriguing that hepatocellular carcinomas (with extensively remodeled ECM) are frequently multidrug resistant prior to any drug exposure (24). Our results suggest that increased pgg expression in these disease states may be linked to the underlying alterations in the composition and/or biophysical conformation of the collagens and glycoproteins in the ECM.

Our results show that ECM proteins are also important in the chemotherapeutically inducible expression of hepatic pgg. Specifically, those hepatocytes cultured on type I collagen readily responded to treatments with chemotherapeutic agents by increasing the steady-state level of pgg mRNA, whereas the increase in pgg mRNA by those same agents in hepatocytes cultured on Matrigel was minimal (Fig. 5). These results suggest that not only do the composition and biophysical structure of the hepatic ECM regulate constitutive expression of hepatic pgg, but that drug induction of hepatic pgg is regulated by the ECM as well.

Whereas other studies of rat liver pgg show a single hybridizable pgg mRNA transcript (3, 4, 35–37), we detect three distinct pgg mRNA transcripts hybridizable with the human pADR1 cDNA (Fig. 5). This finding suggests that, like the mouse (38), rats may also express three distinct pgg gene products. Whether the three mRNA transcripts represent unique pgg gene products is unknown. Silverman et al. (39) have recently isolated a rat cDNA for rat mdr1b, and we have isolated a rat cDNA for mdr2.5. However, a rat cDNA homologous to mdr1a remains to be identified. Alternatively, some hepatocellular pgg mRNA transcripts may represent mRNAs with extended 3′-polyadenylation signals, alternate polyadenylation signals, or variations in the length of the 5′-untranslated leader sequence. However, at this point, it is clear that all mRNA transcripts hybridizable to pADR1 appear to be coordinately regulated by the ECM.

Induction of pgg mRNA(s) in primary rat hepatocyte cultures by colchicine (Fig. 5) stands in contrast to the inability of this same agent to induce pgg mRNA in the replicating rat liver cell line H-35 (40). It is possible that the discrepancies between our results reflect pharmacological variations, such as treatment duration or dose, or, alternatively, differential hybridization of unique rodent pgg mRNAs, since the two studies used two different cDNAs to human pgg. Reanalysis of these same rodent liver pgg mRNA transcripts utilizing cDNAs and oligonucleotides unique to the individual rat pgg members might reveal whether the differences between the cell lines in xenobiotic regulation of pgg mRNA are due to differential expression of unique pgg family members or some other factor unique to replicating cell lines. Finally, the apparent differential xenobiologically inducible expression of pgg in primary cultures of hepatocytes versus in replicating rat liver cell lines suggests that care must be taken in identifying pgg regulatory factors in replicating hepatocyte lines and extrapolating these results to regulation of pgg in normal quiescent rat liver.

Our studies indicate a remarkable correlation between the enhanced pgg mRNA level and the increased rate of VCR efflux in cultured hepatocytes (Table 1). This finding is noteworthy because in some instances decreased drug accumulation is secondary to decreased drug uptake. However, a discrepancy does exist between the small degree of protection from ADR toxicity and the large changes in VCR transport. An explanation for this discrepancy could be that either the high concentration of ADR or duration of exposure saturated the available pgg, which led to ADR accumulation and increased cytotoxicity. Another possibility for the difference between VCR transport kinetics and ADR cytotoxicity is that the pgg expressed in rat hepatocytes has different affinities for these drugs. This would be supported by the different pattern of toxicity for ADR, actinomycin D, and colchicine. It is unlikely that GST-π or P-450 reductase is contributing to ADR resistance, since the time course for alterations in expression of these mRNAs did not correlate with the time course of ADR resistance or type of matrix (Fig. 2). Similarly, because primary hepatocytes are nonreplicating, it is unlikely that alterations in topoisomerase II, a target site in the action of ADR and a known site of ADR resistance (41), plays a protective role in hepatocellular cytotoxicity. However, we cannot exclude the possibility that expression of the microsomal glycosidases (17), which cleave ADR to the inactive aglycone, changes with time in culture or are regulated by ECM proteins. Another possibility is that P-450 2A1/2 may have an undefined role in the activation of ADR, since it declines in both MG- and VIT-cultured hepatocytes.

Finally, although we have not directly determined whether transcriptional (we have found that the RNA polymerase inhibitor novobiocin prevents the VIT-induced increase in pgg) or posttranscriptional mechanisms regulate the response of pgg mRNA to ECM, the system of primary cultures of rat hepatocytes provides an attractive system to rigorously investigate how both ECM and xenobiotics separately or together regulate this important detoxification protein.

Materials and Methods

Plasmids. The cDNA clones to rat β-actin and β-tubulin and albumin were obtained from Dr. Lola Reid (Albert Einstein Medical Center, Bronx, NY), and cDNAs to Pgp [designated pADR1 (18)] and GST-π (20) were obtained from Dr. Ken Cowan (National Cancer Institute, NIH, Bethesda, MD). Mouse integrin β1 (pMIN78) was obtained from Dr. R. O. Hynes (Massachusetts Institute of Technology, Cambridge, MA); P-450 reductase was from Dr. Charles Kasper (42) (University of Wisconsin, Madison, WI); P-450 2A1/2 was from Dr. Frank Gonzalez (43) (NIH, Bethesda, MD).

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1 J. D. Schuetz and E. G. Schuetz, manuscript in preparation.
Animals. Male Sprague-Dawley rats from Dominion Laboratories (Dublin, VA), weighing 200–210 g, were the donor animals used for preparing hepatocyte cultures. They were housed as pairs in wire-bottomed cages with access to food and water. C3H/HeJ female mice (approximately 10 weeks old) from Charles River Breeding Laboratories were used for passage of the EHS tumor (13).

Preparation of Hepatocytes. Hepatocytes were prepared as reported in detail previously (13) by perfusion of the liver in situ with a calcium-free buffer followed by a solution of 0.036% collagenase (Collagen Corp., Palo Alto, CA). A solution of 0.036% collagenase (Collagen Corp., Palo Alto, CA) in our standard serum-free culture medium, which is a modification of Waymouth MB-752 medium, containing insulin (0.157 μM) as the only hormone. The freshly isolated hepatocytes (3.5 x 10⁶) in a total volume of 3.0 ml of culture medium were placed into 60-mm culture dishes coated with either VIT or MG (see below) and were incubated at 35°C in a humidified atmosphere of air containing 5% CO₂. Culture media were renewed every 24 h.

MG/VIT. Hepatocytes were inoculated into 60-mm dishes coated with either 50 μl (50 μg) of VIT (a commercially available type I collagen obtained from Collagen Corp., Palo Alto, CA), prepared as described by the manufacturer, or 0.1 ml of undiluted MG (0.1 mg). MG was prepared from EHS tumor as described by Kleinman et al. (27). Immediately before its use, MG was warmed to 4°C, and then 100 μl of undiluted MG were spread onto 60-mm dishes and warmed to 35°C for 30 min to allow the gelation to be completed. Laminin purified from EHS tumor was a gift from Dr. Hynda Kleinman (NIH, Bethesda, MD). Mouse type IV collagen isolated from EHS tumor was supplied by GibCO/BRL (Gaithersburg, MD) or Collaborative Research Products (Bedford, MA).

Northern Blot Analysis. Total RNA was isolated from cells pooled after 2–4 weeks culture. The RNA was extracted with guanidine isothiocyanate and subjected to ethidium bromide staining. Only RNA that had a gel mobility equivalent to 28S and 18S rRNA were transferred for hybridization analysis. The membrane sheets were prehybridized and hybridized, and the signal intensity was determined by autoradiography and densitometry as described (13).

Southern Blot Analysis. Rat DNA was isolated from liver, and 10 μg were digested with restriction endonucleases and resolved by electrophoresis in a 0.8% agarose gel. The DNA was denatured, neutralized, and capillary transferred onto a Nytran filter. The filter was air dried, baked in vacuo, and prehybridized at 42°C for at least 30 min in 50 mM sodium phosphate, pH 6.5, containing 50% formamide, 5X standard saline citrate (0.15 M NaCl and 0.015 M trisodium citrate), 5X Denhardt's solution, 0.1% sodium dodecyl sulfate, and 100 μg/ml single-stranded DNA. The filters were hybridized as above with the pADR cDNA probe radioactivity labeled by nick-translation with the use of [³²P]dCTP and a kit from Bethesda Research Laboratories (specific activity at least 10⁸ cpm/μg DNA). Hybridized bands were visualized autoradiographically.

Cellular Uptake and Efflux of [³H]Vincristine. Drug uptake was measured in primary rat hepatocytes cultured for 72 h in Waymouth MB-752 medium on MG or VIT by an adaptation of the techniques described previously (44, 45). Briefly, to initiate uptake, radioisotopic [³H]-vincristine (71 Ci/mmol; Amersham), specific activity adjusted to 1000–2000 dpm/pmole, was added to rat hepatocytes in culture media at 37°C. Net drug uptake was terminated by rapid aspiration of the media and washing three times with 3 ml of ice-cold PBS. This procedure removed 99.8% of the extracellular radioactivity. To account for background surface adsorption of [³H]VCR, triplicate plates containing only matrix and media were incubated at 0°C in ice-cold drug-containing medium. This surface-adsorbed drug was subtracted from each experimental time point. After washing, the hepatocytes were solubilized by incubation with 1 ml of 1 N NaOH at 37°C for 1 h. Cell lysate was withdrawn and neutralized with HCl, and the amount of radioactivity was determined on duplicate aliquots by scintillation spectrophotometry. To normalize each plate, the protein content of the cell lysate was determined (46), and the result was expressed as pmol of VCR per mg protein. To measure the effect of verapamil on cellular uptake of [³H]VCR, hepatocytes being labeled with [³H]VCR were coincubated with verapamil (1–20 μM). The efflux of [³H]VCR was determined over a 1.5-h interval after an initial 2-h preloading to steady-state drug level (see Table 1). Following the 2-h incubation with [³H]VCR, the cells were washed three times with 3 ml of ice-cold PBS. The pgp inhibitors, reserpine or verapamil, were added to the cultures during the last 15 min of [³H]VCR preloading. To initiate drug efflux, warm media (37°C) containing either reserpine, verapamil, or no drug were added to each culture dish. Samples were collected at various intervals by rapidly removing the media and washing with ice-cold PBS. VCR efflux rate constants were determined by linear interpolation from the regression line determined from the drug efflux curve, as has been previously described (44, 45).

Lactic Acid Dehydrogenase Activity and Cell Toxicity. LDH activities were determined in media samples and cell homogenates essentially as described (47). Briefly, media were removed from plates of cells and centrifuged to remove cell debris. The hepatocyte monolayers were washed one time with PBS, and the cells were scraped into 1 ml 50 mM Tris-HCl, pH 7.4, and lyzed by sonication. One ml of reaction mixture (1 μM pyruvate and 0.1 mM NADH in 50 mM Tris-HCl, pH 7.4) was pipetted into a cuvet, either cell lysate (5 ml) or media sample (100 μl) was added, and the reaction was allowed to proceed for 1–2 min at room temperature in a spectrophotometer (Beckman DU-65) set to 340 nm. Lactate dehydrogenase activities were calculated from the slopes using an extinction coefficient of 6.2 mM⁻¹cm⁻¹ for NADH and were expressed as percentage leakage from the cells. Additional studies measured trypan blue dye exclusion in hepatocytes as follows: the medium was aspirated from the hepatocytes followed by the addition of 0.5% trypan blue in PBS. After a 5-min incubation period at room temperature, the dye was aspirated, and the number of dye-excluding cells was determined by inspection with an inverted microscope. The fraction of dye-excluding...
cells in a field was compared to untreated controls and expressed as a percentage value.

References