Identification of novel differentially expressed hepatic genes in cholesterol-fed rabbits by a non-targeted gene approach

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Abstract Several key genes involved in cholesterol metabolism are known to be directly regulated by cholesterol. The possible indirect effect, however, of increased levels of cellular cholesterol on gene expression and its possible role in cholesterol metabolism and atherosclerosis has not been thoroughly explored. In order to determine the overall effect of cholesterol on gene expression, we isolated differentially expressed genes from a PCR-based subtraction library prepared from the liver of chow-fed and cholesterol-fed rabbits. A total of nine up-regulated and four down-regulated cDNA fragments were isolated. As determined by Northern blot analysis, the expression of the isolated cDNAs began to change as early as the first week on the cholesterol-rich diet or as late as 4 weeks, which corresponded with hepatic cholesterol accumulation. Three of the cDNAs were identified by DNA sequence homology, whereas the remaining cDNAs had no significant homology match. CYP1A1, a cytochrome P450 isoenzyme, was found to be down-regulated in hepatocytes by cholesterol feeding. Osteopontin and Mac-2, which are produced by macrophages, were found to be up-regulated in Kupffer cells by cholesterol feeding. Overall these results demonstrate the usefulness of the subtraction library approach for identifying new candidate genes for exploring the pathogenesis of atherosclerosis.—**Remaley, A. T., U. K. Schumacher, H. R. Amouzadeh, H. B. Brewer, Jr., and J. M. Hoeg.** Identification of novel differentially expressed hepatic genes in cholesterol-fed rabbits by a non-targeted gene approach. *J. Lipid Res.* 1995. **36:** 308–314.

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Evidence for the role of cholesterol in the pathogenesis of atherosclerosis was first obtained over 80 years ago by feeding rabbits cholesterol-rich diets (1). It has now been established that cholesterol directly modulates the transcription of several genes involved in cholesterol metabolism (2, 3). Our knowledge, however, of cholesterol-mediated gene regulation is likely to be incomplete, as evidenced by our inability to define the molecular basis of several common forms of hypercholesterolemia. Furthermore, because cholesterol is a major component of cell membranes, alterations in the level of cholesterol affect the activity of numerous membrane-bound proteins (4), which has been proposed to play a role in the pathogenesis of atherosclerosis (5, 6). The pathophysiologically relevant changes in protein activity induced by excess cellular cholesterol, however, have not been identified, and whether these changes indirectly lead to homeostatic changes in gene expression has also not been thoroughly explored.

In order to fully understand the molecular basis of atherosclerosis, more information is needed on both the direct and indirect effects of cholesterol on gene expression. We describe the use of a non-targeted gene approach, namely a polymerase chain reaction (PCR)-based subtraction library (7), for assessing the overall effect of cholesterol on gene expression. Several novel differentially expressed hepatic genes were isolated by this method from a subtraction library prepared from the liver of chow-fed and cholesterol-fed rabbits.

MATERIALS AND METHODS

New Zealand White rabbits were fed ad libitum either standard NIH-09 rabbit chow or NIH-09 rabbit chow supplemented with 1.0% cholesterol (w/w), as previously described (8). Plasma and tissue lipid levels were determined enzymatically (9). Analysis for serum albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) were performed on a Monarch 2000 (Instrument Services Inc.), according to the manufacturer. Rabbit liver was fractionated into parenchymal and nonparenchymal cell

Abbreviations: PCR, polymerase chain reaction; CUR, cholesterol-up-regulated; CDR, cholesterol-down-regulated; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.

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fractions after collagenase perfusion by differential centrifugation (10). Nonparenchymal cells were further fractionated by centrifugal elutriation into Kupffer cells and endothelial cells (11). Cell fractions were estimated to be approximately 85% pure, as determined by morphologic assessment by light microscopy and peroxidase activity, as previously described (11, 12).

Two PCR-based subtraction libraries (7) were produced from the livers of two adult male litter mates fed either a chow diet or a cholesterol diet for 4 weeks. Cholesterol-up-regulated (CUR) genes were identified from a library produced with the cholesterol-fed rabbit as the source of tracer mRNA and the chow-fed rabbit as the source of driver mRNA. The chow-fed rabbit was used as the source of tracer mRNA and the cholesterol-fed rabbit as the source of driver mRNA for the cholesterol-down-regulated (CDR) gene library. Poly(A) mRNA from rabbit livers was reverse transcribed and converted into cDNA (13). The cDNA was digested with Alu I and Rsa I, in order to produce cDNA fragments of approximately 500 base pairs, which are suitable in size for PCR amplification. cDNA fragments were ligated to synthetic DNA linkers, which later served as primers for PCR amplification. CUR cDNA fragments were ligated to the following phosphorylated linkers: 5'-CAGTCCGAATTCAA GCAAGAGCACA-3' and 5'-CTCTTGCTTGAATTCGG ACTG-3'. CDR cDNA fragments were ligated to the following phosphorylated linkers: 5'-GATCTTGGATCCAT AGGCTCCCTTG-3' and 5'-GGAGCCTATGGATCCAA GATC-3'. After hybridization of the tracer cDNA with a 10-fold excess of biotinylated driver cDNA (7), heteroduplexes formed between tracer cDNA and biotinylated driver cDNA were removed by streptavidin extraction (14). After six rounds of subtraction and PCR amplification, the subtracted cDNA fragments were ligated into pBluescript and transformed into *E. coli.* Nitrocellulose lifts of bacterial colonies were screened with ^{32}P-labeled subtracted cDNA as the probe. cDNA fragments were sequenced from both ends with sequenase (U. S. Biochemicals), using universal M13 reverse and forward primers. DNA homology searches of GenBank were done with BLAST software provided by the National Center for Biotechnology Information.

RESULTS

NZW rabbits fed a 1.0% cholesterol-supplemented diet quickly developed hypercholesterolemia (**Fig. 1A**). After 4 weeks on the cholesterol-rich diet, there was more than a 40-fold increase in plasma cholesterol. Greater than 90% of the plasma cholesterol when fractionated by Superose-6 chromatography and agarose gel electrophoresis was found to be in the form of the atherogenic β-VLDL particle (data not shown), as has been previously

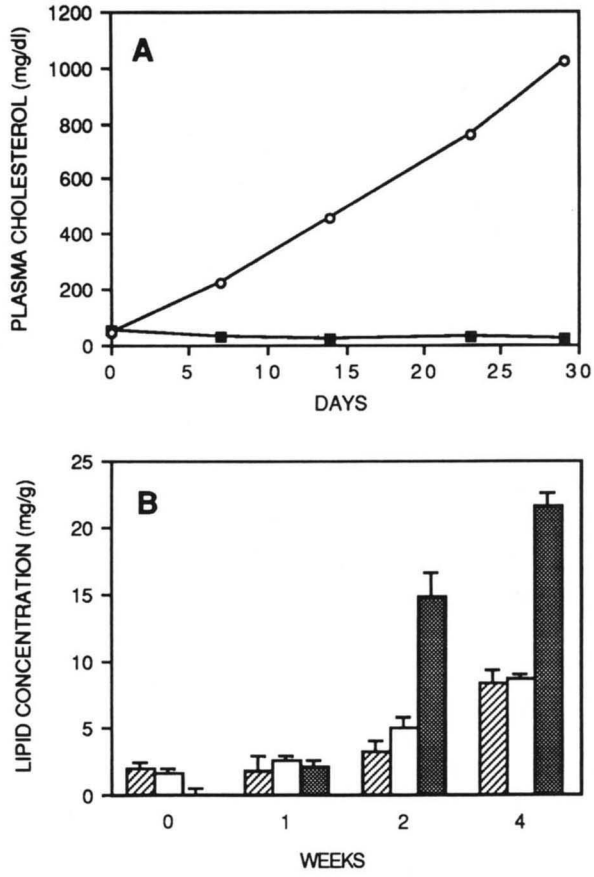

Fig. 1. Plasma cholesterol levels (panel A) of chow-fed (solid squares) and cholesterol-fed (open circles) rabbits. Hepatic lipid concentrations (panel B) of triglyceride (diagonally lined rectangles), free cholesterol (open rectangles), and cholesteryl esters (solid rectangles) for each week of cholesterol feeding. Results are shown as the mean 1 SD of three determinations.

reported in cholesterol-fed rabbits (15). In order to isolate genes that are not only directly but also indirectly regulated by the level of cellular cholesterol, rabbits were fed a cholesterol-rich diet for a sufficient period of time to allow for accumulation of free cholesterol in the liver. As can be seen in Fig. 1B, although cholesteryl esters steadily increased during the cholesterol feeding, free cholesterol did not significantly accumulate until 4 weeks. The 4-week-fed rabbits, therefore, were chosen to prepare the subtraction library because of the observed increase in free cholesterol and because it was before significant hepatic damage developed from the cholesterol feeding. As can be seen in **Fig. 2,** under low power, livers from rabbits cholesterol-fed for 4 weeks appeared histologically to be relatively normal (panel C) when compared to the chow-fed rabbit liver (panel A). There was no evidence in the cholesterol-fed rabbit liver of abnormal lobular architecture, inflammatory infiltrate, fibrosis, hepatic regeneration, or hepatic necrosis. The only significant abnormality, which was more apparent under higher

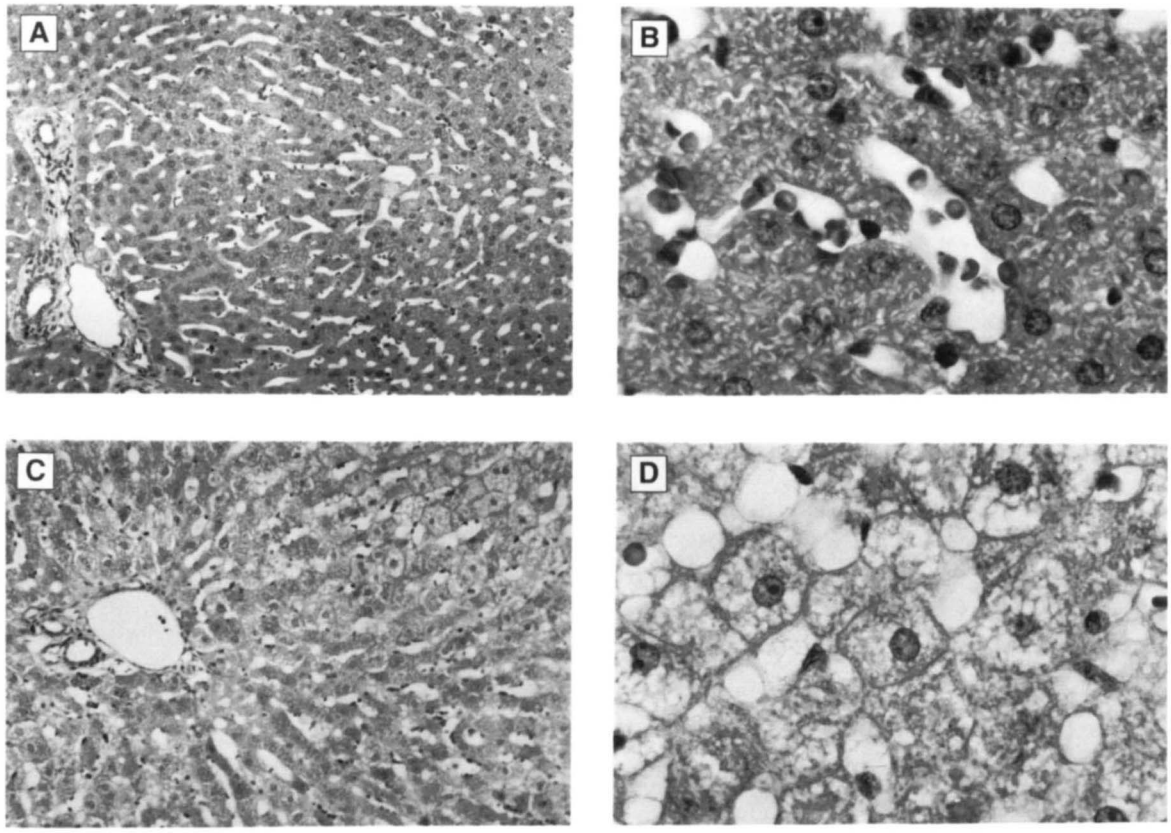

Fig. 2. Photomicrograph of hemotoxylin- and eosin-stained sections of chow-fed rabbit liver (100 ×; panel A), chow-fed rabbit liver (500 ×; panel B), cholesterol-fed rabbit liver (100 ×; panel C), and cholesterol-fed rabbit liver (500 ×; panel D).

magnification (panels B and D), was the presence of microvesicular steatosis, due to the intracellular accumulation of lipid. The absence of significant liver pathology was also confirmed by standard liver function tests (**Fig. 3**). The liver function tests either remained unchanged during the cholesterol feeding or increased slightly but remained within the normal range (16).

Poly(A) mRNA from the liver of rabbits that were either chow-fed or cholesterol-fed for 4 weeks was used to prepare an up-regulated and down-regulated subtraction library, as described in Materials and Methods. After six rounds of subtraction and PCR amplification, the subtraction of constitutively expressed cDNA transcripts shared between the tracer and driver pool of cDNA appeared to be complete, as evidenced by lack of significant cross-hybridization by Southern blots (data not shown). The two subtraction libraries were then screened with their corresponding subtracted ^{32}P-labeled cDNA, and approximately 10% of the 1000 colonies screened from both libraries were positive. Based on identical cDNA insert sizes, however, many of the positive colonies appeared to contain the same cDNA fragment. Redundant cDNAs were identified either by cross-hybridization on Southern blots or by alignment of DNA sequences. A total of nine unique cholesterol-up-regulated (CUR) cDNA fragments and four unique cholesterol-down-regulated (CDR) cDNA fragments were isolated (**Table 1**).

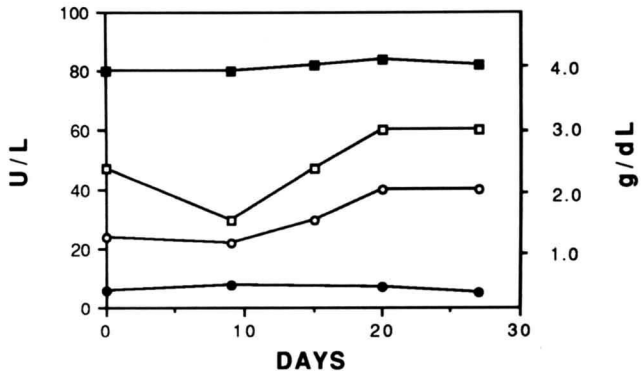

Fig. 3. Liver function tests of cholesterol-fed rabbits. Results of serum AST (□), ALT (○), GGT (●) and albumin (■) are shown during 4-week cholesterol feeding. Activity of serum enzymes are shown as U/L on the left Y-axis. Albumin concentration in g/dl is shown on the right Y-axis.

TABLE 1. Summary of differentially expressed cDNA fragment sizes and corresponding mRNA size in kilobases

Name	Fragment	mRNA
CUR1	0.56	2.4
CUR2	0.42	3.7
CUR3	0.41	4.4
CUR4	0.37	1.1
CUR5	0.48	1.4
CUR6	0.67	5.2
CUR7	0.32	3.9
CUR8	0.26	4.7
CUR9	0.55	4.7
CDR1	0.35	3.9
CDR2	0.39	3.3
CDR3	0.28	2.3
CDR4	0.32	2.3

The effect of cholesterol feeding on the expression of the isolated cDNAs at various times during the 4-week feeding period was examined by Northern blot analysis (**Fig. 4**). Each of the CUR and CDR genes responded to the cholesterol feeding as predicted; the CUR genes were up-regulated by cholesterol feeding, and the CDR genes were down-regulated by cholesterol feeding. In general, the CUR genes were not significantly expressed in chow-fed rabbit livers but were all up-regulated by the fourth week of cholesterol feeding. Although 4-week-fed rabbits were used to prepare the subtraction libraries, most of the differentially expressed genes began to change their expression within the second week of cholesterol feeding. The CUR4 gene responded the most quickly, with a significant increase in expression by the first week, which was before significant amounts of cholesterol accumulated in the liver (Fig. 1B). The expression of the other CUR genes began to change by the second week of cholesterol feeding, which corresponded to hepatic cholesterol accumulation (Fig. 1B). In contrast to the CUR genes, the CDR genes were expressed in the liver of the chow-fed rabbits but were almost completely suppressed by the fourth week of cholesterol feeding. CDR3 and CDR4 genes responded the earliest to cholesterol feeding, with greater than a 50% decrease in the level of expression by the first week. Similar to the late responding CUR genes, the expression of CDR1 and CDR2 began to change after 2 weeks.

The DNA sequences of the isolated cDNA fragments were analyzed by homology searches, and four of the differentially expressed cDNA fragments were identified (**Table 2**). CDR3 and CDR4 cDNA fragments represent two nonoverlapping regions of CYP1A1, a cytochrome P450 type oxidase (17). CUR4 was identified as Mac-2, a protein first described in macrophages (18). CUR5 was identified as osteopontin (19), a protein first purified from bone matrix (20). No significant homology matches were found for the remaining nine cDNA fragments. As we observed for CDR3 and CDR4 cDNA fragments, some of the unidentified cDNA fragments may also be nonoverlapping fragments from a single gene.

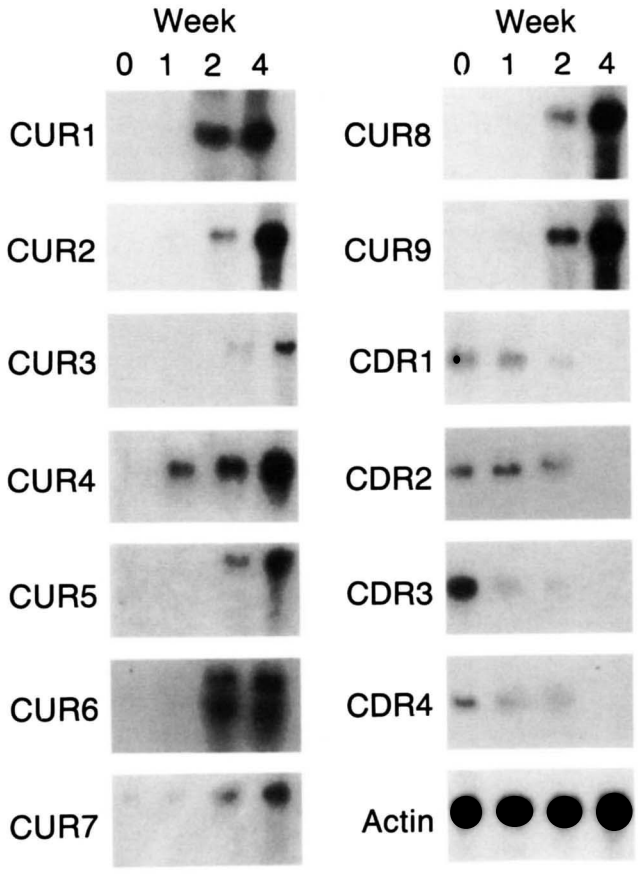

Fig. 4. Northern blot analysis of differentially expressed genes. Total RNA (10 μg) from rabbit liver was used to prepare Northern blots and probed with the indicated ^{32}P-labeled cDNA fragments. The number of weeks the rabbits were on the cholesterol-rich diet are indicated above each lane. Exposure times: Actin, CDR3, 4 (1 day), CUR1, 2, 4, 5, 8, 9, (3 days), CUR3, 7 and CDR1, 2 (5 days), CUR 6 (7 days).

Because Mac-2 and osteopontin have not been previously reported to be produced by hepatocytes, we examined whether the expression of these two genes was from nonparenchymal liver cells, which make up approximately 30% of hepatic cells (21). The livers of rabbits fed

TABLE 2. DNA sequence homology of differentially expressed genes

cDNA Fragment	Number b.p.	% Identity	Gene	Ref.
CDR3	174	100	Rabbit CYP1A1	17
CDR4	155	99	Rabbit CYP1A1	17
CUR4	143	85	Mouse Mac-2	18
CUR5	171	100	Rabbit osteopontin	19

Number b.p. refers to the number of bases sequenced and % Identity refers to the homology with the indicated gene.

either chow or cholesterol-supplemented chow for 4 weeks were perfused with collagenase and fractionated by differential centrifugation into parenchymal (hepatocytes) and nonparenchymal cells. Total RNA from each cell fraction was analyzed by Northern blot analysis (**Fig. 5**) for CYP1A1 (panel A), osteopontin (panel B), Mac-2 (panel C), and actin (panel D). Consistent with what has been previously observed (17), the CYP1A1 gene was primarily expressed in hepatocytes, and as we observed in Fig. 2, it was down-regulated by cholesterol feeding. In contrast, both Mac-2 and osteopontin were expressed in only nonparenchymal cells and were up-regulated by cholesterol feeding. Nonparenchymal cells primarily consist of endothelial cells and Kupffer cells (21), a macrophage-derived cell (12). In **Fig. 6,** the nonparenchymal cells of chow-fed and cholesterol-fed rabbit livers were further fractionated into endothelial cells and Kupffer cells and analyzed by Northern blot analysis for Mac-2 (panel A), osteopontin (panel B), and actin (panel C). mRNA of osteopontin and Mac-2 was primarily detected in Kupffer cells from cholesterol-fed rabbits and was up-regulated by cholesterol feeding, which is consistent with previous reports of Mac-2 and osteopontin expression by macrophages (18, 22).

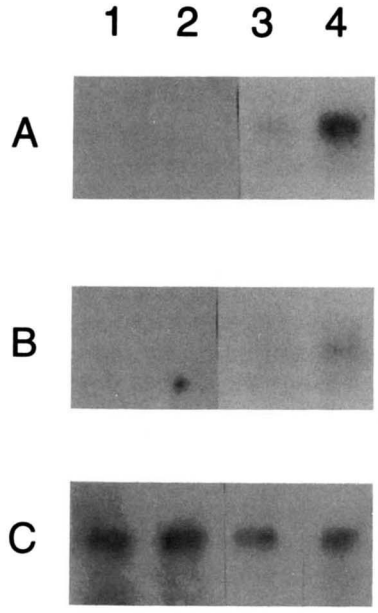

Fig. 6. Northern blot analysis of differentially expressed genes in endothelial cells and Kupffer cells. Total RNA (10 μg) prepared from chow-fed endothelial cells (lane 1), cholesterol-fed endothelial cells (lane 2), chow-fed Kupffer cells (lane 3), and cholesterol-fed Kupffer cells (lane 4) was used to prepare Northern blots and probed with Mac-2 (panel A), osteopontin (panel B), and β-actin (panel C) cDNA fragments.

DISCUSSION

By screening two subtraction libraries prepared from chow-fed and cholesterol-fed rabbit livers, we have isolated several differentially expressed cDNAs. The fact that the expression of these genes changed in response to the cholesterol feeding suggests that some of these genes may be relevant to cholesterol metabolism and atherosclerosis. Because rabbits were fed a cholesterol-rich diet for several weeks, the isolated genes potentially represent genes that are both directly and indirectly regulated by cholesterol. The expression of previously described genes that are directly regulated by cholesterol, such as the LDL-receptor, HMG-CoA reductase, and 7-α hydroxylase, changed relatively rapidly in response to a change in the level of cholesterol (2, 3). Of the differentially expressed genes shown in Fig. 4, the genes that changed the most acutely with cholesterol feeding, namely CUR4, CDR3, and CDR4, are the most likely to be directly regulated by cholesterol. By homology search (Table 2), however, none of the isolated cDNAs corresponded to any previously described genes that are directly regulated by cholesterol. This is probably the result of the bias in subtraction libraries for preferentially isolating the most abundant and most differentially expressed mRNAs. Rescreening the library after subtracting out the initially isolated cDNAs may in the future, reveal previously described cholesterol-regulated genes. Insights into the regulation of the isolated differentially expressed genes may also be revealed by analysis of their promoters for known sterol response elements (2, 3).

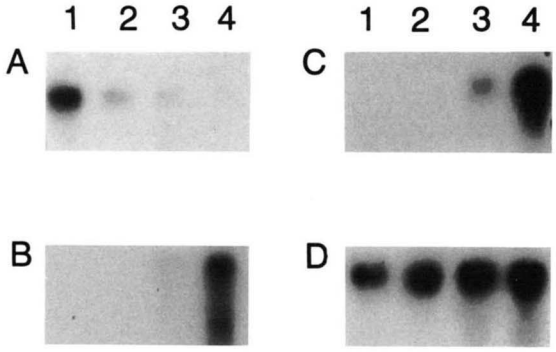

Fig. 5. Northern blot analysis of differentially expressed genes in parenchymal and nonparenchymal cells. Total RNA (10 μg) prepared from chow-fed parenchymal cells (lane 1), cholesterol-fed parenchymal cells (lane 2), chow-fed nonparenchymal cells (lane 3), and cholesterol-fed nonparenchymal cells (lane 4) was used to prepare Northern blots and probed with CYP1A1 (panel A), osteopontin (panel B), Mac-2 (panel C), and β-actin (panel D) cDNA fragments.

The majority of the isolated genes did not change until after several weeks of cholesterol feeding (Fig. 4), which corresponded to hepatic cholesterol accumulation (Fig. 1B). This suggests that these genes are likely to be indirectly regulated by cholesterol, perhaps by secondary cellular changes that occur in response to high intracellu-

lar levels of cholesterol (4). Not all genes, however, that respond indirectly or relatively slowly to cholesterol feeding are necessarily unimportant in cholesterol metabolism or atherosclerosis. For instance, the mRNA levels of cholesteryl ester transfer protein (CETP) and several apolipoproteins do not change in rabbits until after several weeks of a cholesterol-rich diet (23–26). Similarly, some of the late responding genes shown in Fig. 4 may be responding to the gradual increase in plasma cholesterol from the cholesterol feeding (Fig. 1A) and may also be involved in extracellular cholesterol metabolism. Alternatively, some of these genes may be involved in intracellular cholesterol esterification and storage. Prolonged cholesterol feeding of rabbits can produce hepatic damage (27) and may also potentially result in changes in gene expression that are unrelated to cholesterol metabolism and atherosclerosis. However, after 4 weeks of cholesterol feeding, we did not observe, either histologic (Fig. 2) or biochemical (Fig. 3) evidence for significant liver damage. Furthermore, some of the genes potentially affected by hepatic damage may, nevertheless, still be relevant to the pathogenesis of atherosclerosis. The cytotoxicity of excess cellular cholesterol has been proposed to be the result of the effect of cholesterol on the activity of membrane-bound proteins (4–6). Alterations in protein activity may lead to homeostatic changes in gene expression that may be similar between the cells of the liver and the vessel wall.

The identification of two hepatic differentially expressed genes as osteopontin and CYP1A1 (Table 2), which have been previously shown to undergo a change in gene expression in vascular cells during atherogenesis (28, 29), supports the use of the subtraction library of the liver for identifying genes that are generally relevant to atherosclerosis. CYP1A1 mRNA has been detected in smooth muscle cells of the vessel wall and is up-regulated in proliferating smooth muscle cells of atherosclerotic plaques (28). Interestingly, in several animal models of atherosclerosis, induction of the CYP1A1 gene by polyaromatic hydrocarbons has been shown to directly correlate with the degree of atherosclerosis (30, 31). An exact role, however, for CYP1A1 in atherosclerosis or cholesterol metabolism has not been described, but many other cytochrome P450 isoenzymes are involved in oxidation of cholesterol or cholesterol precursors during the biosynthesis (32), catabolism (33), and steroid hormone conversion (33) of cholesterol. Oxidative stress by an unknown mechanism has been shown to down-regulate the CYP1A1 gene (34). The down-regulation that we observed of CYP1A1 may be a compensatory response to the high levels of cholesterol and cholesterol oxidation products in the liver of cholesterol-fed rabbits.

Osteopontin, although first described in bone (20), has also been detected in proliferating smooth muscle cells and in macrophages of atherosclerotic plaques but not in normal vessels (29, 35). Based on its function in bone, osteopontin has been postulated to promote calcification of atherosclerotic lesions (29, 35). In addition, it has recently been shown to inhibit the induction of NO synthetase, which regulates vascular tone (36). Our data on the cholesterol-loaded Kupffer cells would suggest that the cholesterol that accumulates in macrophages of atherosclerotic plaques could be a stimulus for increased osteopontin production by macrophages. Another interesting possibility is that both osteopontin and Mac-2, the other identified differentially expressed gene (Table 2), can function as adhesion proteins. Osteopontin has a RGD binding site and has been shown to facilitate the adhesion of osteoblasts (37). Mac-2, a galactose binding lectin, is the major non-integrin laminin binding protein on the surface of macrophages (38). The up-regulation of these two proteins by cholesterol could facilitate monocyte adherence or macrophage infiltration into atherosclerotic plaques, which is believed to be one of the early events in atherosclerosis (39, 40). These data also suggest that Kupffer cells, which readily accumulate cholesterol in cholesterol-fed rabbits (41) and are easily purified in large numbers (12), may be a useful model system to study in vivo gene expression in macrophage foam cells.

In summary, we have isolated several novel differentially expressed hepatic genes from cholesterol-fed rabbits. The identity of three of the genes has been determined and suggests several potential mechanisms for their involvement in the pathogenesis of atherosclerosis. Clues to the function of the unidentified genes may be revealed by sequencing full length cDNAs and identifying functional motifs. Overall, these results establish the usefulness of the PCR-based subtraction library approach for identifying novel differentially expressed genes and in providing new insights into the pathogenesis of atherosclerosis.

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