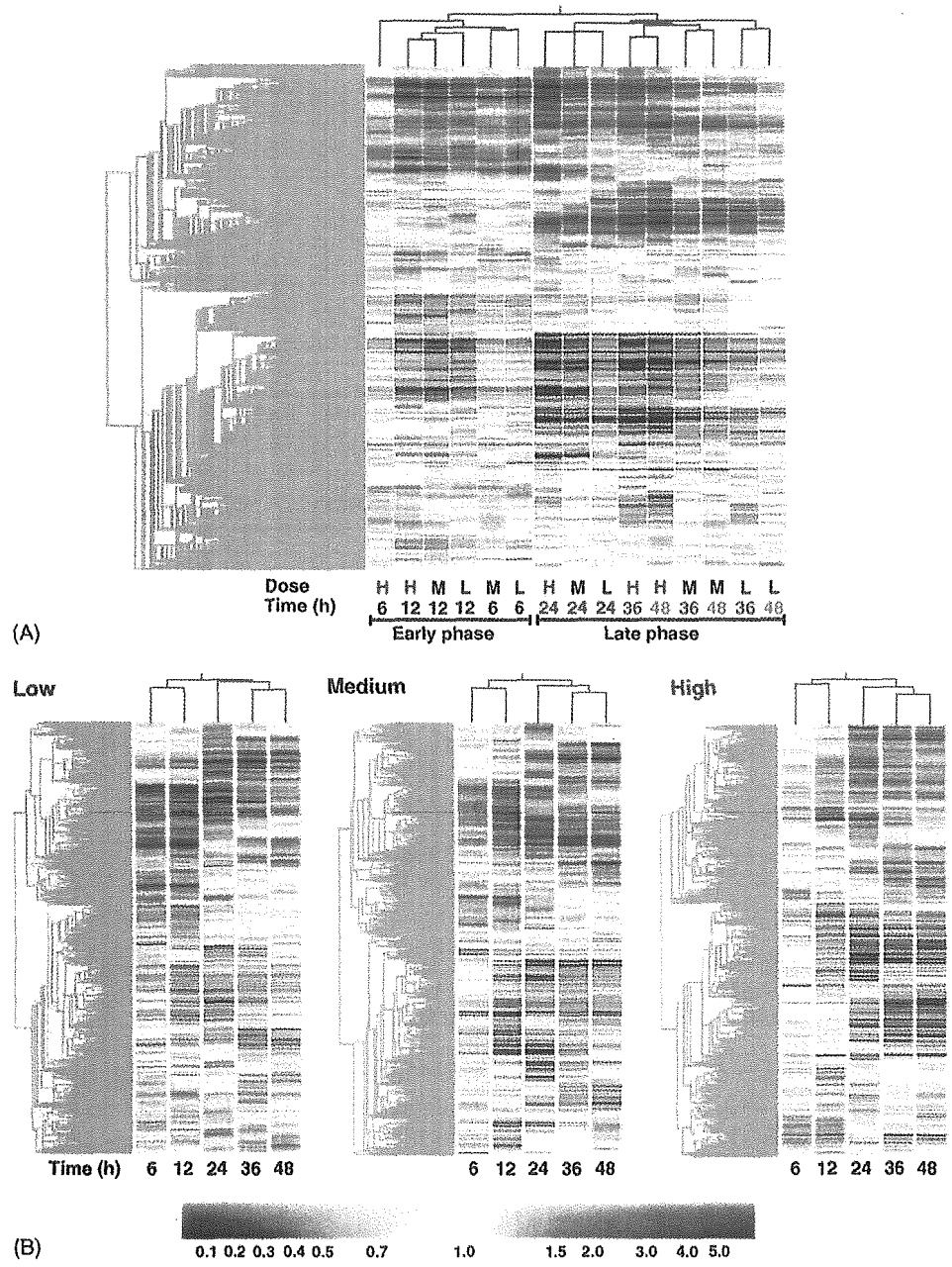

Fig. 2. Two-way hierarchical clustering analyses of hepatic gene expression profiles. Hierarchical clustering of all groups (A) and each dosage group (B). Data of 7978 genes are expressed in a color-coded matrix. Red- and blue-color in matrix indicate the expression levels of above and below the median, respectively. Dendrograms of each group (above the matrix, red lines) and gene (left of the matrix, green lines) represent the overall similarities in gene expression profiles. H: high dose (400 mg/kg); M: medium dose (150 mg/kg); L: low dose (50 mg/kg).

3.4. QT clustering analysis based on the dose of TA

QT clustering analysis was performed in order to estimate the major gene expression profiles based on the TA dosage (Fig. 3). In this process, we used GeneSpring QT clustering algorism. In all dosage groups, we selected the highest correlation coefficient that identified both the up- and the down-regulated types of cluster. The analysis setting for the minimal cluster size was 1000 genes and the minimal correlation coefficients of the 6, 12, 24, 36, and 48 h groups were 0.82, 0.92, 0.91, 0.88 and 0.86, respectively. The probe sets used in this clustering were the same as those used in the hierarchical clustering (7978 of 9936 genes). In the up-regulated

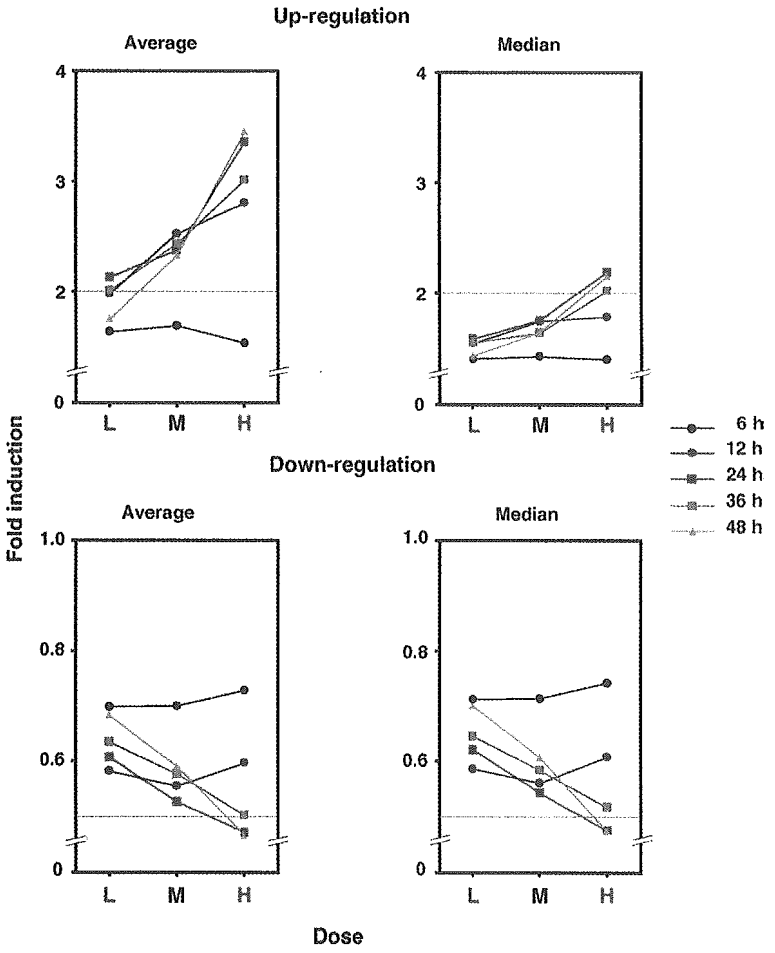

Fig. 3. QT clustering analyses based on the dose of TA. Groups at 6, 12, 24, 36, and 48 h after administration were evaluated and expressed as average (left) or median (right) values. Analysis settings are described in Section 3. Minimal correlation coefficients selected in this analysis were as follows: 6 h, 0.82; 12 h, 0.92; 24 h, 0.91; 36 h, 0.88; 48 h, 0.86. H: high dose; M: medium dose; L: low dose.

cluster, the gene numbers of each time group were as follows: 6 h, 1028; 12 h, 1010; 24 h, 1003; 36 h, 1008; 48 h, 1000. In the down-regulated cluster, the gene numbers of each time group were as follows: 6 h, 1903; 12 h, 1724; 24 h, 1546; 36 h, 1533; 48 h, 1513. The expression changes of these genes in each dosage group are shown as average and median values (Fig. 3). As a result, both the average and the median of the gene expression profiles before 12 h showed no difference between the three dosage groups. However, the gene expression profiles after 24 h of administration changed in a dose-dependent manner.

3.5. QT clustering analysis based on the time after TA-administration

QT clustering analysis was performed in order to estimate the major gene expression profiles based on the time after TA-administration (Fig. 4). In the low-limited correlation analysis, we selected the highest correlation coefficient that identified the up- and down-regulated type of clusters. The low-limited analysis setting for the minimal cluster size was 1000 genes and the minimal correlation coefficients of the low-, medium- and high-dose groups were 0.68, 0.58, and 0.47, respectively. The probe sets used in this clustering were the same as those used in the hierarchical clustering (7978 of 9936 genes). In the up-regulated type cluster, the low-, medium- and high-dose groups contained 1021, 1003 and 1000 genes, respectively (Fig. 4A). The down-regulated type cluster of the low-, medium- and high-dose groups contained 2129, 2050 and 1606 genes, respectively (Fig. 4B). In the high-limited correlation analysis, we selected the highest correlation coefficient that identified only one dominant cluster (Fig. 4C). The high-limited correlation analysis setting for the minimal cluster size was 1000 genes and

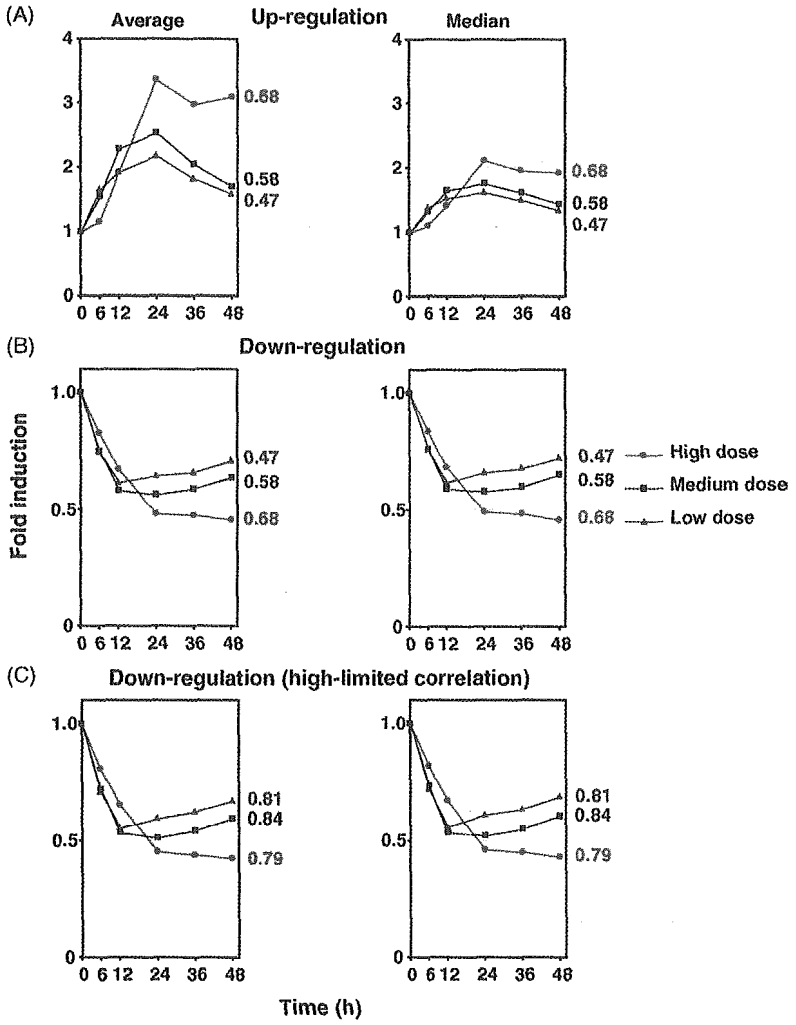

Fig. 4. QT clustering analyses based on the time after TA-administration. Groups at 6, 12, 24, 36, and 48 h after administration were evaluated with low (A and B)- and high (C)-limited correlation coefficients. Analysis settings are described in Section 3. Data are expressed as average or median values of these clusters. The minimal correlation coefficients selected in this analysis were as follows: low-limited correlation: high dose, 0.68; medium dose, 0.58; low dose, 0.47 (A and B). High-limited correlation: high dose, 0.79; medium dose, 0.84; low Dose, 0.81 (C).

the minimal correlation coefficients of the low-, medium- and high-dose groups were 0.79, 0.84 and 0.81, respectively (Fig. 4C). In the down-regulated type cluster, the low-, medium- and high-dose groups contained 1016, 1002 and 1016 genes, respectively (Fig. 4C). Both the average and median values of the QT clustering and the expression profiles of almost all dosage groups demonstrated peak values at 24 h after administration, which was the same as the maximal toxic time. Independently of the extent of toxicity, the major gene expression profiles based on the time after TA-administration showed almost the same pattern. However, the changes of the up- and down-regulated genes occurred in a dose-dependent manner.

3.6. *Toxicity marker analysis*

Previously, in rats we identified potential hepatotoxic marker genes from five hepatotoxicants (acetaminophen, bromobenzene, carbon tetrachloride, dimethylnitrosamine and TA) by using cDNA microarray [16]. The up- and down-regulated groups consisted of nine and seven genes, respectively (Fig. 5). In the present study, changes in the expression of potential hepatotoxic marker genes were demonstrated. As shown in Fig. 5, the maximal up- or down-regulated time points in almost all the individual gene expression profiles reflected the maximal toxic time. In addition, the individual gene expression profiles of each dosage showed similar patterns. In

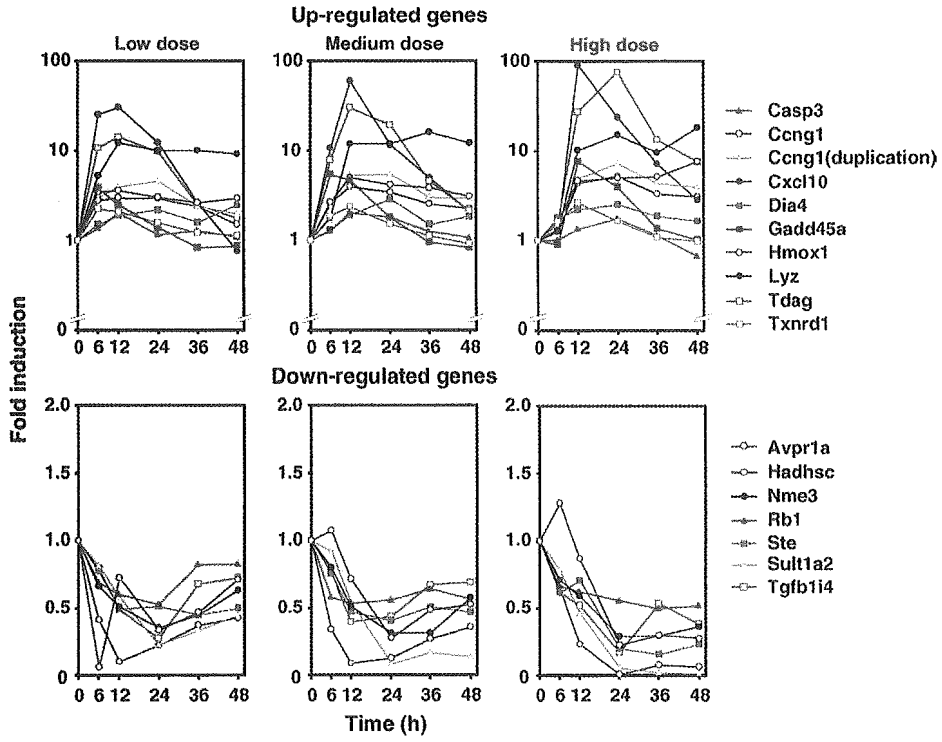

Fig. 5. Gene expression profiles of the selected genes. The up-regulated group consisted of nine genes and the down-regulated group seven genes. Total RNA samples from four rats were pooled and used for this analysis. Symbols, accession numbers and gene names are as follows: Casp3, NM_012922, caspase 3, apoptosis related cysteine protease (ICE-like cysteine protease) mRNA; Ccng1, NM_012923, cyclin G1 mRNA; Cxcl10, U22520, interferon inducible protein 10 (IP-10) mRNA, complete cds; Dia4, NM_017000, diaphorase (NADH/NADPH) mRNA; Gadd45a, NM_024127, growth arrest and DNA-damage-inducible 45 alpha mRNA; Hmox1, NM_012580, heme oxygenase (decycling) 1 mRNA; Lyz, NM_012771, lysozyme mRNA; Tdag, NM_017180, T-cell death associated gene mRNA; Txnrd1, NM_031614, thioredoxin reductase 1 mRNA; Avpr1a, Z11690, mRNA for V1a arginine vasopressin receptor; Hadhsc, NM_057186, L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain mRNA; Nme3, NM_053507, expressed in non-metastatic cells 3, protein (nucleoside diphosphate kinase) mRNA; Rb1, D25233, mRNA for retinoblastoma protein, partial sequence; Ste, NM_012883, sulfotransferase, estrogen preferring mRNA; Sult1a2, NM_031732, sulfotransferase family 1A, member 2 mRNA; Tgfb1i4, NM_013043, transforming growth factor beta 1-induced transcript 4 mRNA.

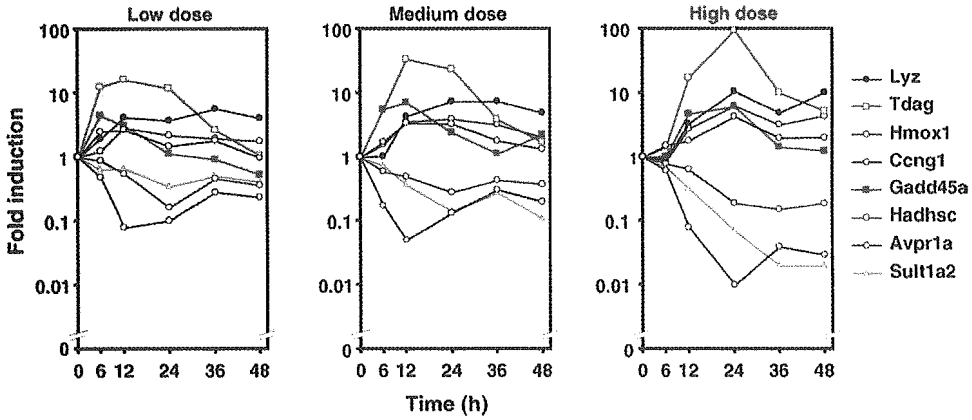

Fig. 6. Real-time RT-PCR analysis of the expression of the representative genes. Total RNA samples from four rats were pooled and used for real-time RT-PCR analysis. This figure shows the representative five up-regulated and three down-regulated genes as shown in Fig. 5. Official gene names are described in Fig. 5.

order to confirm the gene expression changes found in the DNA microarray analysis, real-time RT-PCR analysis was performed in eight representative genes (Fig. 6). The expression profiles and extent of these eight gene expressions were almost the same as those of the DNA microarray.

4. Discussion

Gene expression changes have been used to provide specific mechanistic information concerning the mode of action of toxicants. Toxicogenomics is an approach that applies microarray technology to toxicological evaluation paradigms. Toxicogenomic gene expression studies have been facilitated by the recent development of high-density microarrays.

In our previous study [16], the relationship between the hepatic gene expression profiles and hepatotoxicity was investigated in rats using in five typical hepatotoxicants. The mRNA of TA (400 mg/kg weight) administered rats was analyzed. Finally, 17 possible potential hepatotoxicity markers were proposed. In the present study, in order to evaluate the gene expression for hepatotoxicity in detail, we evaluated the effects of three dosages of TA, which causes zone-3 necrosis [17], by using a 9936 gene-spotted microarray slide. The dosage was selected according to a previous report [17]. The high-, medium-, low-dose administration was expected to cause severe toxicity, low toxicity and no toxicity, respectively. In this study, we compared between the extent of toxicity and the changes of gene expression in detail.

Previously, the effects of 300, 400 or 500 mg/kg weight of TA-administration in rats were reported [5,11,16]. The maximal point of TA toxicity estimated from the serum AST and ALT activities was 24 h after the administration. The present results were the same as those of previous reports. In the present study, the unconjugated bilirubin was not increased by TA-administration but the conjugated bilirubin was increased at 24 h by the high dosage of TA. These data confirmed that the toxicity model of TA-administration was successfully conducted in the present study. Additionally, the toxic time points of each dosage were estimated as 24 h.

Hierarchical clustering was performed to examine the gene expression data derived from the microarray analysis. All groups were extensively sorted into the early phase (6 and 12 h) or in the late phase (24, 36 and 48 h). In the early phase, the clusters were sorted in a time-dependent manner. Previously, Bulera et al. [18], reported in a comparison between low dose (40 mg/kg) and high dose (500 mg/kg) at early times (low dose: 3 and 6 h; high dose: 8 h) after the administration in rats using a microarray method, reported that all three groups were sorted into a the similar cluster. This result was similar to that of early phase in the present experiment (Fig. 2A). On the other hand, the late phase cluster was clustered in a dose-dependent manner (Fig. 2A). Therefore, we report here that the hierarchical clustering analysis demonstrated that the gene expression patterns were changed dose independently in the early phase of hepatotoxicity, but in dose dependently in the late phase of hepatotoxicity. Furthermore, hierarchical clustering at different dosages showed the same result as shown in Fig. 2B. The hierarchical distance between the early and the late phase were separated in a dose-dependent manner as shown by the dendrograms (Fig. 2B), suggesting that there was a certain relationship between the extent of hepatotoxicity and the gene expression pattern.

QT clustering based on the time after TA-administration was performed. This method can estimate the majority of gene expression profiles as previously reported [16]. As shown in Fig. 3, this result confirmed our finding from the hierarchical clustering analysis. Moreover, the hierarchical clustering and QT clustering based on the time after TA-administration also suggest that there was a dose dependency in the extent of the gene expression changes after the hepatotoxicity appeared.

QT clustering based on the TA dosage was also performed (Fig. 4). The profiles of both the average and median values from the QT clustering reflected the changes in biochemical markers. This result confirmed those of our previous study [16]. Furthermore, the present study indicated that the changes of the gene expression in the QT clustering became distinct in a dose-dependent manner. Taking these results into consideration, in this study, we demonstrated that the hepatic gene expression profiles are independent of the TA dosage and reflect the changes in the serum biochemical markers.

Category classification of the genes affected by TA-administration showed that the numbers of down-regulated genes were greater than those of the up-regulated genes, as reported previously [16].

Bulera et al. [18] reported the gene expression data after 24 h of TA-administration (500 mg/kg). In their study, expression changes were analyzed in 1600 genes and they showed that 133 genes were up-regulated and 163 genes were down-regulated. The microarray slides used in the present study spotted 49 of their 133 up-regulated genes (36.8%) and 68 of their 163 down-regulated genes (41.7%). In total, 117 of the 296 genes (39.5%) were spotted. In the present study, 39 of the 49 genes (79.6%) were up-regulated and 64 of the 68 genes (94.1%) were down-regulated (data not shown).

For example, FMO1, which metabolizes TA to a toxic metabolite, was down-regulated (<0.1-fold). In a total of 103 of 117 genes (88.0%), the expressions were similar to those reported by Bulera et al. [18].

In the previous study, using five traditional hepatotoxicants including TA we performed simultaneous measurements using other microarray slides (Rat Drug Response Chip, Hitachi) [16]. Most of the genes showed similar expression profiles to those of the present study. The expression profiles were confirmed in eight representative genes by real-time RT-PCR, which showed almost the same profile as that of the DNA microarray. This result suggested that our candidate genes could be sensitive markers for hepatotoxicity. However, the relationship between these genes was not clarified.

In summary, the present study demonstrated that there are distinct gene expression differences between pretoxic- and toxic-periods (Fig. 2), there is a dose dependency in the extent of the major gene expression changes after toxicity appears (Fig. 3), the major gene expression profiles reflect the biochemical marker activities (Fig. 4), and the candidate genes identified in our previous microarray analysis could be used as sensitive markers for hepatotoxicity (Fig. 5). In conclusion, the potential toxic effects appearing as gene expression changes are independent of the dosage of TA. The major gene expression profiles estimated by QT clustering analysis would be a sensitive marker for predicting potential hepatotoxicity.

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