Detection of Cell-Free, Liver-Specific mRNAs in Peripheral Blood from Rats with Hepatotoxicity: A Potential Toxicological Biomarker for Safety Evaluation

Makoto Miyamoto,¹ Mariko Yanai, Shingo Ookubo, Naoko Awasaki, Kenji Takami, and Ryoetsu Imai

Development Research Center, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Yodogawa-ku, Osaka 532-8686, Japan

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To verify the concept that cell-free organ/tissue–specific mRNAs leaking from drug-damaged organs/tissues into peripheral blood could be toxicological biomarkers for identification of the target organs of drug toxicity, we attempted to detect liver-specific mRNAs in peripheral blood from rats with chemical-induced hepatotoxicity. We selected α₁-microglobulin/bikunin precursor (Ambp) and albumin mRNAs as tentative liver-specific biomarkers and successfully detected them by reverse transcription (RT)-PCR in peripheral blood 24 h after β-galactosamine HCl (β-gal) or acetaminophen administration. Moreover, albumin mRNA was detected 2 h after β-gal administration, although plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were still unchanged. On the other hand, in peripheral blood from rat with bupivacaine HCl–induced skeletal muscle damage, neither Ambp nor albumin mRNA was detectable while plasma creatine kinase, ALT, and AST levels prominently increased 2 or 12 h after dosing. Furthermore, Ambp mRNA was also detectable in filtered plasma from rats with liver damage, indicating that cell-free Ambp mRNA can be present in peripheral blood. In conclusion, cell-free, liver-specific Ambp, and albumin mRNAs were detectable in peripheral blood from rats with chemical-induced liver damage. It is believed that the detection of cell-free organ/tissue–specific mRNA in peripheral blood is a promising approach in the survey of toxicological biomarkers.

Key Words: biomarker; cell free; hepatotoxicity; liver-specific mRNA; peripheral blood; rat.

Toxicological biomarkers play an essential role in drug development as indicators of adverse drug responses. The discovery of robust toxicological biomarkers that are applicable to both animals and humans avoids or minimizes risks that might occur in clinical trials by their use in preclinical safety assessment of novel compounds. Many clinical pathology biomarkers have already been identified and utilized for detecting target organ toxicity of drugs; however, unfortunately, most of them are not completely specific indicators of target organ toxicity. For instance, plasma or serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are well-established markers for liver damage, but they often arise upon extrapancreatic damage such as skeletal muscle disease (Nathwani et al., 2005). Therefore, discovery and vetting of new biomarkers that possess higher specificity for target organ toxicity of drugs are very much in demand for efficient drug development. In addition, one of the practical and desirable characteristics that enhance the appropriateness and usability of toxicological biomarkers is accessibility and noninvasiveness. In this regard, peripheral blood is considered to be one of the most ideal sources of new biomarkers.

RNA molecules are generally thought to be extremely labile in plasma/serum (Chan et al., 2003). However, Kopreski et al. (1999) reported that tumor-associated mRNA can be detected in the plasma/serum, and this result has led others to investigate circulating cell-free nucleic acids, including RNA, as a means of detecting and/or monitoring the status of a variety of cancers (Chen et al., 2000; Silva et al., 2002; Sueoka et al., 2005). In addition, the retina-specific rhodopsin mRNA has been reported to be increased in the peripheral blood of diabetic patients with preproliferative retinopathy (Hamaoui et al., 2004). These results indicate that organ/tissue-specific mRNAs that have leaked into the peripheral blood might reflect disease-induced damage of a particular organ. Furthermore, the recent development of comprehensive and high-throughput gene expression analysis technologies, such as DNA microarrays, has dramatically advanced our knowledge of organ/tissue–specific gene expression in various organs and tissues. These developments have attracted our interest in using cell-free organ/tissue–specific mRNAs in peripheral blood as new toxicological biomarkers to identify the target organs of drugs (see Fig. 1).

In this study, to verify the concept that cell-free organ/tissue–specific mRNAs in peripheral blood could be toxicological biomarkers to identify the target organs of drugs, we attempted to detect liver-specific mRNAs by reverse transcription.
FIG. 1. Schematic concept of detection of cell-free organ/tissue–specific mRNAs in peripheral blood as toxicological biomarkers. Each organ/tissue is presumed to have specific mRNAs (orange, blue, red, and green lines for kidneys, heart, lungs, and liver, respectively). When a new drug, for which the toxicological target organs are unknown, is administered to animals or humans and causes damage to certain organs/tissues (e.g., heart and liver), organ/tissue–specific mRNAs from damaged organs/tissues may leak into the peripheral blood (blue and green lines in peripheral blood represent heart- and liver-specific mRNAs, respectively). If the existence of heart- and liver-specific mRNAs were detected in peripheral blood by an amplification technique of nucleic acids such as RT-PCR, it could be concluded that toxicological target organs of the drug are the heart and liver.

| MATERIALS AND METHODS |

**Animals and treatments.** Male and female Crl:CD(SD) rats (10–11 weeks old) were purchased from Charles River Laboratories Japan (Tokyo, Japan) and housed individually in hanging, stainless steel, wire-bottom cages in a temperature (20°C–26°C), light (12 h dark/light cycle), and humidity (40–80%)-controlled room. The rats had free access to a standard chow diet (CR-LPF; Oriental Yeast, Tokyo, Japan) and water throughout the studies. The studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and protocols were approved by our laboratory animal ethical committee.

For investigating gene expression of Ambp and albumin genes in normal rat organs/tissues, animals were sacrificed by exsanguination from the abdominal aorta under ether anesthesia, and a total of 42 organs and tissues (see Fig. 2) were collected within 30 min of euthanasia. All tissues except for bone marrow, femur, and whole blood were directly transferred into RNALater (Ambion, Austin, TX) and stored at 4°C until isolation of total RNA. Bone marrow was aspirated from the femur by a syringe with an injection needle and then transferred into QIAzol LS reagent (QIAGEN GmbH, Hilden, Germany), followed by storage at −80°C until isolation of total RNA. The femur was kept in RNALater for 1 h after removal of bone marrow, followed by the removal of surrounding skeletal muscles. Thereafter, the femur was frozen in liquid nitrogen and crushed with a Cryo-Press Frozen Cell Crusher (Microtec, Chiba, Japan) and stored at −80°C until use. Whole blood was collected with PAXgene tubes (PreAnalytIX, Hombrechtikon, Switzerland) for isolation of total RNA and was incubated at room temperature for 2 h before storing at −80°C until use.

**Total RNA isolation from rat tissues, whole blood, and plasma.** Total RNAs were isolated from about 50–200 mg each of collected tissues using an EZ1 RNA Universal Tissue Kit (QIAGEN) and from whole blood collected in PAXgene tubes using a PAXgene Blood RNA Kit (QIAGEN). Blood collected in EDTA tubes was centrifuged at 7500 × g for 10 min at 4°C to obtain plasma. The plasma was passed through a cellulose acetate filter membrane (pore size, 0.2 μm; Advantec Toyo Kaisha, Tokyo, Japan), and total RNA was extracted using an RNasy Mini Kit (QIAGEN). All procedures followed the manufacturer’s instructions (including the optional DNase treatment). RNA concentration was determined by absorbance at 260 nm using the NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples were stored at −80°C until assayed.

**RNA analysis by RT-PCR.** RT-PCR of Ambp, albumin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out on 200 ng of total RNA from organs/tissues, whole blood, and plasma. RNA was reverse transcribed using the ExScript RT reagent Kit (Takara, Shiga, Japan).

Primers used to amplify Ambp, albumin, and GAPDH were designed to span at least one intron of the genomic sequence to eliminate the problems associated with DNA contamination, and the primer pairs were as follows: Ambp, 5'-GCC TCA CTT TGA AGG CTG AC-3' (forward) and 5'-TAC TGG TCA CGC TGA TCT CG-3' (reverse); albumin, 5'-GAT GCC GTG AAA GAG AAA GC-3' (forward) and 5'-GCT GAC AGC ACT CCT TGT TG-3' (reverse); and GAPDH, 5'-GCT GCT GAG TAT GTC GTG GA-3' (forward) and 5'-CTA AGG TGG TGG TGC AG-3' (reverse). PCR was performed.
with a Bca BEST RNA PCR Kit (Takara) and the PCR Thermal Cycler Dice (Takara), and the PCR conditions were as follows: initial denaturation at 95°C for 5 min; 30 or 35 cycles of denaturation at 95°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 2 min; and final extension at 72°C for 5 min. The expected product sizes were as follows: Ambp, 207 bp; albumin, 196 bp; and GAPDH, 202 bp. After PCR amplification, PCR products were electrophoresed in 4% agarose gels, visualized by ethidium bromide staining, and photographed under UV illumination.

RESULTS

Confirmation of Liver Specificity of Candidate Target mRNAs

Based on the human reference database for gene expression analysis (RefExA, http://157.82.78.238/refexa/main_search.jsp) and published reports (Lindqvist et al., 1992; Salier et al., 1993), we selected the Ambp gene encoding β2-microglobulin and bikunin precursor, which is generally considered to be expressed exclusively and at high levels in normal liver. In addition, we selected the albumin gene, one of the most well-known liver-specific genes. The GAPDH gene was used as a reference for RT-PCR. Using the RT-PCR assay, we investigated whether Ambp or albumin mRNAs were detectable in a total of 42 organs and tissues that are routinely examined in preclinical toxicity studies. Representative results are shown in Figure 2. The migration of the Ambp amplicon was in agreement with its expected size, that is, 207 bp (lane 1). Ambp mRNA was detected readily in the liver but was undetectable in any other organs/tissues examined in this study. The migration of the albumin amplicon was in agreement with its expected size, that is, 196 bp (lane 1). Albumin mRNA was strongly expressed in the liver as expected; however, slight to faint signals were also detectable in lung, stomach, duodenum, thymus, pancreas, aorta, esophagus, thyroid gland, mammary gland, trachea, testis, brown adipose tissue, white adipose tissue, and uterus, though signal intensities in these organs/tissues were obviously weaker than that in the liver. GAPDH mRNA was detected at almost the same level among all organs/tissues examined. From the above results, Ambp and albumin mRNAs were confirmed to be liver specific.

Detection of Ambp and Albumin mRNAs in Peripheral Blood from Rats with Liver Damage

To obtain rat models of chemical-induced hepatotoxicity, we selected two frequently used hepatotoxic compounds, D-gal and APAP, which induce hepatotoxicity through different
mechanisms (El-Mofty et al., 1975; James et al., 2003). Both D-gal and APAP caused prominent increases in plasma ALT and AST levels 24 h after dosing (Fig. 3A). Representative sections of the liver from control and chemical-treated rats 24 h after dosing are shown in Figure 4. Histopathologically, degeneration and single-cell necrosis of hepatocytes and inflammatory cell infiltration were observed in D-gal–treated rats, and centrilobular necrosis of hepatocytes and inflammatory cell infiltration were noted in APAP-treated rats. Ambp and albumin mRNAs were detected in peripheral blood from

![Graphical representation](https://academic.oup.com/toxsci/article-abstract/106/2/538/1739111)

**Fig. 3.** Detection of Ambp and albumin mRNAs in peripheral blood from rats with chemical-induced hepatotoxicity. (A) Shows 24 h after administration of either D-gal, 800 mg/kg, or APAP, 1500 mg/kg. MC solution, 0.5% (wt/vol). (B) Shows 2 h after administration of D-gal, 800 mg/kg. (C) Ambp gene expressions were examined in nine major organs/tissues 24 h after administration of APAP, 1500 mg/kg. Panels show representative results from one animal. The tissues examined were liver (lane 1), kidney (lane 2), heart (lane 3), lung (lane 4), spleen (lane 5), stomach (lane 6), testis (lane 7), skeletal muscle (lane 8), and intestine (lane 9). The lower panel shows the detection of GAPDH mRNA as a reference.
all animals receiving d-gal or APAP 24 h after dosing but were undetectable in peripheral blood from control animals receiving saline or 0.5% (wt/vol) MC solution under this experimental condition (Fig. 3A). One rat (animal no. 3) treated with APAP showed lower plasma ALT and AST levels and fewer histopathological changes in the liver than the other APAP-treated rats; however, both Ambp and albumin mRNA signals were detected (Fig. 3A). Similar results were obtained from three experiments conducted independently (data not shown).

Next, we examined whether Ambp and albumin mRNAs were detectable in peripheral blood in the early stage of hepatic damage. Albumin mRNA signals were detected in peripheral blood 2 h after d-gal administration while plasma ALT and AST levels were unchanged (Fig. 3B). At this time point, Ambp mRNA signals were undetectable under this experimental condition (Fig. 3B).

It is known that APAP causes toxicological changes in the extrahepatic organs/tissues, that is, kidney and heart (Thomas, 1993). To confirm that Ambp gene expression could not be ectopically induced in the extrahepatic organs/tissues by APAP administration, we investigated liver specificity of Ambp mRNA in a total of nine major organs/tissues from rats administered APAP (Fig. 3C). Ambp mRNA was detected clearly in the liver and faintly in lung but was undetectable in any other organs/tissues examined; however, it did not seem to reflect the general induction of Ambp gene expression because the signal was not observed uniformly in all rats examined. GAPDH mRNA was detected at almost the same level in all organs/tissues examined.

Undetectable Ambp and Albumin mRNAs after Skeletal Muscle Injury

It is well known that plasma ALT and AST levels can increase not only by hepatic injury but also by extrahepatic ones, for instance, skeletal muscle damage. We examined whether Ambp and albumin mRNAs were detectable in peripheral blood from rats with skeletal muscle damage by im BPVC injection (Nosaka, 1996). By blood chemistry, plasma CK levels prominently increased 2 h after injection, indicating the occurrence of skeletal muscle damage but recovered to basal levels 12 h after injection. In contrast, plasma ALT and AST levels clearly increased 12 h after injection (Fig. 5).
these conditions, neither Ambp nor albumin mRNA was detectable in peripheral blood at any time point examined (Fig. 5). All animals receiving BPVC showed severe myonecrosis histopathologically (Fig. 5) but no abnormalities in the liver (data not shown).

Detection of Cell-free Ambp mRNA

We used PAXgene tubes to collect blood samples because they allow mRNAs in whole blood to be stabilized immediately after collection. However, this method does not separate mRNAs in plasma fraction from ones in blood cell fraction. To investigate the mode of existence of mRNAs in the peripheral blood, the plasma that was passed through a 0.2-µm filter prior to RNA extraction was obtained. This size filter should be sufficient to exclude any intact cells that might have contaminated the plasma. In all rats treated with d-gal, Ambp mRNA was detected in whole blood (Fig. 6, upper panel) 24 h after administration and was also detectable in the filtered plasma (Fig. 6, lower panel). This indicates that cell-free Ambp mRNA is present in whole blood from rats with liver damage. The Ambp mRNA signal intensity from almost all filtered samples was reduced slightly.

DISCUSSION

Generally, RNA is unstable and highly susceptible to degradation by RNases. Thus, it has been commonly assumed that cell-free RNA cannot survive in the peripheral blood. However, Kopreski et al. (1999) reported that tyrosinase mRNA is detectable in serum from patients with malignant melanoma. In addition, Hamaoui et al. (2004) found that the retina-specific rhodopsin mRNA increased in the peripheral blood of diabetic patients with preproliferative retinopathy. In the present study, we sought to verify the concept that cell-free organ/tissue–specific mRNAs in peripheral blood are available as toxicological biomarkers for identification of the target organs of drug toxicity. We attempted to detect liver-specific
mRNAs in peripheral blood from rats with hepatotoxicity by RT-PCR and obtained successful results.

Selecting Ambp and albumin mRNAs as tentative hepatotoxic markers, we examined whether they were detectable in peripheral blood from rats administered two different mechanism-based hepatotoxic compounds, d-gal and APAP. Both mRNAs were detected in peripheral blood from all animals 24 h after dosing with the chemicals. Hepatic damage was confirmed by blood chemistry and histopathological examination of the liver.

Moreover, albumin mRNA was detectable in peripheral blood 2 h after d-gal administration at which time plasma ALT and AST levels were still unchanged, and no histopathological abnormalities were found in the liver. These results dispel the idea that liver-specific mRNAs could be detected in peripheral blood only when hepatic damage was extremely severe. Further, these results may indicate that albumin mRNA is a more sensitive indicator than plasma ALT and AST levels in this experimental condition. On the other hand, Ambp mRNA was not detected in peripheral blood 2 h after d-gal administration. This could be attributed to higher intrinsic expression of the albumin gene in liver as compared with the Ambp gene. This suggests that organ/tissue specificity and expression levels in target organs/tissues are key factors for the selection of candidate mRNAs as toxicological biomarkers. Selection of mRNAs with complete organ/tissue specificity is the most desirable strategy. Because the recent advance of comprehensive gene expression analysis tools has allowed the systematic accumulation of data on organ/tissue–specific genes (Ge et al., 2005), information to select candidate target mRNAs is easily obtained. Nevertheless, more difficult may be finding candidate mRNAs that have complete organ or tissue specificity. As noted in the case of albumin mRNA, evaluation with several marker mRNAs would be valuable to assess accurate results and to avoid false-positive results.

The plasma levels of conventional markers for hepatotoxicity, ALT and AST, can rise as a result of extrahepatic damage, for instance, skeletal muscle damage (Nathwani et al., 2005), making the evaluation of ALT and AST changes problematic. On the other hand, we confirmed that neither Ambp nor albumin mRNA was detectable in skeletal muscle under our experimental conditions (Fig. 2). Therefore, Ambp and albumin mRNAs ought to be absent in peripheral blood from rats with skeletal muscle damage. In fact, we demonstrated that neither mRNA was detectable at any time point examined, although increased plasma ALT and AST levels were noted 12 h after im bupivacaine administration. Interestingly, plasma CK levels, a marker for skeletal muscle damage, sharply increased 2 h after bupivacaine administration but recovered to basal levels 12 h after administration because of its short half-life in plasma (Nosaka, 1996). In this case, if only conventional blood chemistry is performed 12 h after bupivacaine administration, it would be difficult to conclude that skeletal muscle was damaged. However, if liver-specific mRNAs in peripheral blood were evaluated concurrently, it would be possible to exclude liver damage. It is clear that measuring ALT and AST levels is much faster and more convenient than assaying for liver-specific mRNAs at the present time. However, combining the results of the two together may overcome the lower organ specificity experienced with ALT and AST as markers of liver damage.

Ambp mRNA was detectable even in plasma that was passed through a 0.2-μm filter, demonstrating that cell-free mRNAs exist in peripheral blood from rats with liver damage. Ambp mRNA signal intensity was slightly reduced after filtration (Fig. 6) due to RNA degradation that resulted from increased sample handling (Ng et al., 2002). While mRNAs may be a more fragile biomarker, it is reasonable to use the system (e.g., PAXgene tube) specifically designed for the collection and stabilization of RNA from whole blood when collecting material for the assay.

Although exogenously purified RNA is degraded in seconds after its addition to plasma, endogenous cell-free mRNA is stable in peripheral blood (Tsui et al., 2002). Hasselmann et al. (2001) reported that circulating cell-free mRNA can be protected from degradation by being contained in particles such as apoptotic bodies. Additionally, several findings have indicated that extracellular RNA might be protected from serum RNases by proteins or proteolipid complexes (Rosi et al., 1988; Wieczorek et al., 1985, 1987). It is not fully understood, however, how plasma and serum mRNA is protected from RNases. Therefore, to understand the nature of these mRNAs will be one challenge for the future.

Safety biomarkers are most useful when they predict rather than report damage. In this regard, the detection of cell-free organ/tissue–specific mRNAs in peripheral blood seems insufficient for predicting toxicity because cell death (including both apoptosis and necrosis) in the target organs/tissues is considered to be the source of the mRNA (Chan et al., 2003). However, safety biomarkers that report damage are thought to be valuable if they are used for preventing expansion of damage or permitting cessation of treatment and subsequent recovery (Duffy et al., 2007). Additionally, cell-free organ/tissue–specific mRNAs in the peripheral blood have several characteristics that satisfy the requirements for good biomarkers. First, mRNAs are accessible and noninvasive because they can be easily obtained from peripheral blood. Second, the...
assay methods theoretically can be established with ease and in a short time if the nucleotide sequence of the target mRNA is already known. Third, if the mRNA is expressed similarly between rodent and human and if it shows similar organ/tissue specificity, the assay methods could be readily applied to both species. These characteristics are beneficial in transferring the technology from nonclinical studies to clinical trials. In contrast, assays such as immunoassays and Western blot analysis often have limited application due to a lack of cross-species reactivity of the antibodies.

In this study, we used the RT-PCR assay to detect Ambp and albumin mRNAs because RT-PCR is extremely sensitive. However, it is difficult to obtain accurate quantitative values because of its methodological limitation. It is essential for a good biomarker to be reliable, accurate, and reproducible. Therefore, to verify the practicality of cell-free organ/tissue-specific mRNAs in peripheral blood as new toxicological biomarkers, one of the critical challenges will be quantification of target mRNAs.

In conclusion, cell-free, liver-specific Ambp mRNA was detectable in peripheral blood from rats with chemical-induced hepatotoxicity. Use of mRNAs as biomarkers is most likely detectable in peripheral blood from rats with chemical-induced hepatotoxicity. Use of mRNAs as biomarkers is most likely detectable in peripheral blood from rats with chemical-induced hepatotoxicity. Use of mRNAs as biomarkers is most likely detectable in peripheral blood from rats with chemical-induced hepatotoxicity. Use of mRNAs as biomarkers is most likely detectable in peripheral blood from rats with chemical-induced hepatotoxicity.

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