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Induction of arginase II in livers of bile duct-ligated rats

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Abstract

Nitric oxide (NO) has been implicated in playing a role in liver cirrhosis, but the regulatory mechanisms are still unclear. As arginase shares a common substrate with NO synthase (NOS), the aim of this study was to investigate the expression of arginase I and II in cirrhotic liver. Liver cirrhosis was induced in rats by chronic bile duct ligation (BDL). Controls were sham-operated. Competitive polymerase chain reaction was performed to assay the expression of messenger RNA of arginase I and II. Protein expression was detected by immunohistochemistry and western-blotting. The level of arginine in plasma was lower in BDL rats, while the ornithine level in plasma was correspondingly higher ($r = -0.96$, $P < 0.0001$). Arginase I messenger RNA was reduced significantly in BDL rats (3.34 ± 0.32 vs. $1.32 \pm 0.21 \times 10^4$ attomole/μg of total RNA, sham vs. BDL, $P < 0.001$), as well as arginase I protein. In contrast, arginase II mRNA was induced in the livers of BDL rats, with negligible expression in controls (0.35 ± 0.11 vs. 3.64 ± 0.54 attomole/μg of total RNA, sham vs. BDL, $P < 0.001$). Arginase II protein was localized in some hepatocytes and hyperplastic bile ductular epithelial cells of cirrhotic livers but not in control livers. In conclusion, arginase II was induced in BDL livers, while the expression of arginase I was down-regulated. These data suggest that arginase I and II are regulated differently and may have different functions in the livers of BDL rats. Reduction of arginase I in BDL livers may be responsible for the lowering of arginine levels in the plasma, while induction of arginase II could be important in regulating NO synthesis as well as other important mechanisms involved in liver cirrhosis.

Keywords: Liver cirrhosis; Arginine metabolism; Arginase I and II; Gene and protein expression; Nitric oxide

1. Introduction

Arginine is one of the most versatile amino acids in animal cells. It serves as a precursor for the synthesis not only of proteins but also of NO, urea, polyamine, proline, glutamate, creatine, and agmatine [1]. Among the various enzymes that catalyse rate-controlling steps in arginine synthesis and catabolism, arginase (EC 3.5.3.1) has been recognised as one of the key enzymes. Thus, two isoenzymes of arginase, arginase I and II, which are biochemically and immunologically distinct, have been identified [2,3]. Arginase I, a cytosolic enzyme, is highly expressed in livers as a component of the urea cycle, and to a limited extent in a few other tissues [1]. It is expressed in coordination with the other urea cycle enzymes and is regulated by glucagon and glucocorticoids [4]. Its cDNA clones have been isolated from rat and human livers, and the structures of the rat and human genes have been determined. The human gene of arginase I is mapped to chromosome 6q23 [5]. In contrast, arginase II is a mitochondrial enzyme and is widely distributed in the tissues. It is found at high levels in the prostate and lower but still substantial levels are found in the kidneys, small intestine, mammary glands, and macrophages [3,6]. cDNA clones for arginase II have been isolated from mice, rats, and humans. In humans, the arginase II gene is located on chromosome 14q24.1-24.3 [7].

Liver cirrhosis, a leading cause of death in the world, is a chronic scarring process, the pathogenesis of which is not fully understood. Among many contributing factors, NO

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; mRNA, messenger RNA; cDNA, complementary DNA; RT, reverse transcription; PCR, polymerase chain reaction; BDL, bile duct ligation.

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has aroused much attention. Increased NO levels and enhanced vascular NOS activity in the plasma have been found in both patients and animals with cirrhosis [8,9]. In the liver itself, all three isoforms of NOS have been identified [10]. However, their expression patterns and regulatory mechanisms are still far from clear. Since arginase shares a common substrate, L-arginine, with NOS, any potential alteration of arginase expression could be important for NO and NOS regulation, as well as for the pathogenesis of liver cirrhosis. Therefore, in this study, we investigated the expressions of arginase I and II in cirrhotic livers, and discussed their pathophysiological implications.

2. Materials and methods

2.1. Induction of cirrhosis in rats

Liver cirrhosis was induced in male, 180–250 g, Sprague–Dawley rats by BDL as described previously [11,12]. Control rats received a sham operation in which the bile duct was gently manipulated, but not ligated or sectioned. At 22–26 days after the BDL, cirrhosis developed, as judged by a sudden increase of body weight, jaundice, and post-mortem histological examination. The rats of both groups were then killed. Blood was taken from the inferior vena cava and used for liver function tests and an amino acid assay. Livers were excised, washed with cold PBS, and snap-frozen in liquid nitrogen. The above-mentioned protocol was in accordance with our institutional guidelines, and all rats received humane care.

2.2. Plasma amino acid assay and liver function tests

Heparinized plasma was used for the amino acid assay. Levels of amino acids in the plasma were quantified by high-performance liquid chromatography as described previously [13]. Liver function tests were performed on serum using routine clinical chemistry protocols for determining bilirubin, total protein, γ-glutamyltransferase, alanine and aspartate aminotransferases, and alkaline phosphatase levels.

2.3. Reverse transcription (RT)

Total RNA from liver tissue was extracted using TRIzol™ Reagent (Gibco BRL), according to the protocol of the manufacturer. The integrity of the RNA was checked by electrophoresis on a 1% formaldehyde agarose gel and verified by the presence of the 28 and 18S rRNA bands. RT was performed with oligo(dT) using SUPERSCRIPT™ II RNase H^- Reverse Transcriptase (Gibco BRL). First strand cDNA synthesis was performed at 42° for 1 hr. The reaction was stopped by heating the mixture at 95° for 10 min, and it was stored at −20° until further use.

2.4. Polymerase chain reaction (PCR)

PCR was done using a DNA amplification reagent kit (QIAGEN) with a Hybaid PCR express thermal cycler (Hybaid Limited). For arginase I, 5′-CTCTAAGGGACAGCCTCGAGGA-3′ was its sense primer and 5′-TGGGTTCACTTCCATGATATCTA-3′ its antisense primer. For arginase II, the primer sequences were 5′-ATCCGAGAAGCTGGCTTGCTGA-3′ for sense and 5′-TGGATCGGCCTCTTCCTCTT-3′ for antisense strands. Each PCR reaction contained 2 μL of RT product, 0.4 μmol of sense and antisense primers, 50 μmol of each dNTP, and 0.5 U of Taq polymerase in a final volume of 25 μL. The amplification procedure consisted of 30 cycles with the following parameters: denaturation at 94° for 30 s; primer annealing at 55° for 30 s; extension at 72° for 1 min. Each final PCR product was loaded on a 1.8% agarose gel, electrophoresed, and visualised by ethidium bromide staining. To confirm the authenticity of RT–PCR products of arginase I and II, the specific bands were sequenced using the dideoxy chain termination method.

2.5. Competitive PCR

To examine the levels of arginase I or II mRNA expression, an internal competitor DNA was first constructed. The competitor DNA, MIMIC, was a non-homologous DNA fragment derived from the v-*erb* B gene to which the pair of arginase I or II specific primer templates had been added. The MIMIC was constructed according to the protocol of the manufacturer of the PCR MIMIC™ construction kit (CLONTECH Laboratories Inc.). To determine the amount of arginase I or II MIMIC to be used in the PCR amplification, a preliminary experiment was done. A constant amount of arginase I or II cDNA was first amplified together with a 10-fold dilution series of its MIMIC. This reaction provided a fine-tuned 2-fold serial dilution of the MIMIC. The same amount of cDNA was then coamplified with the 2-fold MIMIC dilution series. The amount of MIMIC, which gave an optical density ratio of 1:1 with the target, was chosen for coamplification with the rest of the RT products. After PCR, the products were electrophoresed on an agarose gel, and the ratio of the optical density of the PCR product pairs (target vs. MIMIC) was determined, using the Kodak 1D imaging system (Eastman Kodak Co).

2.6. Immunohistochemical assay

Liver tissues from BDL and sham-operated rats, the same batch as used in the RT–PCR experiments, were fixed in 10% buffered neutral formalin solution, and then embedded in paraffin and sectioned. The sections were deparaffinized in xylene and hydrated through a graded series of alcohol to tap water. To detect expression of arginase I and II, each section was immunostained with

the RTU Vectastain Universal Elite ABC kit (Vector Laboratories Inc.). In brief, the sections were incubated with diluted primary antibodies at 4° overnight. For arginase I, it was a mouse monoclonal antibody (A59120, Transduction Laboratories) diluted 1:300 in PBS. This anti-arginase I has been used widely by various groups of researchers, and no evidence of cross-reactivity with arginase II has been reported [14,15]. For arginase II, we used a rabbit polyclonal antibody (raised by injecting a rabbit with the purified mature portion of recombinant human arginase II (residues 25–354)) diluted 1:400 in PBS. Anti-arginase II has been proven to be specific to arginase II [16,17]. After washing in PBS, the preparations were then incubated with a diluted biotinylated secondary antibody and ABC reagent for 30 min, respectively, in that order. Finally, the sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with Harris's hematoxylin. The negative controls were exposed to normal serum in place of the primary antibodies.

2.7. Western blots

Liver tissues were homogenized in a lysis buffer containing 20 mM HEPES (pH 7.4), 0.5% Triton X-100, 1 mM dithiothreitol, 10% glycerol, 10 μg/mL leupeptin, and 10 μg/mL aprotinin, and then centrifuged at 25,000 *g* for 30 min at 4°. Protein quantification of the supernatants was performed using the Bio-Rad protein assay (Bio-Rad). Samples (40 μg of total protein from each sample for arginase I, 100 μg of total protein from each sample for arginase II) were subjected to 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a Mini Trans-Blot cell (Bio-Rad). The membranes were then incubated with a 1:1000 dilution of anti-arginase I or a 1:800 dilution of anti-arginase II (the same antibodies used for the immunohistochemistry) for 2 hr. After washing, the membranes were incubated with a 1:1000 dilution of a horseradish peroxide-conjugated secondary antibody (Amersham Pharmacia Biotech). Immunodetection was performed with an ECL kit (Amersham Pharmacia Biotech) according to the protocol of the manufacturer.

2.8. Statistical analyses

Data were statistically analysed using Student's *t*-test for unpaired values. Probability values of <0.05 were taken to indicate statistical significance.

3. Results

3.1. General

Histological examination of the livers of BDL rats showed characteristic features of obstructive biliary cirrhosis. Diffuse micro-nodules had developed, accompanied by intensive ductular proliferation and inflammatory cell infiltration. Liver function tests on BDL rats indicated deranged and significantly abnormal liver function compared with the sham-operated rats (Table 1). An amino acid assay showed that the arginine level in the plasma of BDL rats was significantly lower than that in sham rats (156.8 ± 9.1 μM for sham vs. 37.0 ± 11.4 μM for BDL, $P < 0.0001$), while the plasma ornithine level was much higher in BDL rats than in sham rats (66.3 ± 3.8 μM for sham vs. 381.6 ± 26.9 μM for BDL, $P < 0.0001$). The alteration of plasma arginine was highly correlated with that of plasma ornithine ($r = -0.96$, $P < 0.0001$).

Table 1
Liver function test

	BDL ($N = 9$)	Sham ($N = 9$)
Bilirubin (μmol/L)	$119.4 \pm 35.7^{*}$	2.0 ± 0.0
Total protein (g/L)	$46.8 \pm 9.4^{*}$	57 ± 2.0
γ-Glutamyltransferase (U/L)	$94.1 \pm 65.3^{*}$	5.7 ± 1.6
Alanine aminotrasferase (U/L	$134.8 \pm 23.3^{*}$	91.9 ± 10.1
Aspartate aminotransferase (U/L)	$431.7 \pm 30.9^{*}$	74 ± 26.1
Alkaline phosphatase (U/L)	$464 \pm 33.3^{*}$	98 ± 15.9

Data are means ± SD.
* $P < 0.05$.

3.2. Arginase I and II mRNA expressions

To study arginase mRNA expression, competitive RT–PCR was performed. The integrity of total RNA extracted from liver tissues was confirmed by the presence of 28 and 18S rRNA bands in formaldehyde agarose gels. Fig. 1A demonstrates a characteristic result of the amplification product obtained from liver tissues using arginase I primers. An intense band at 796 bp, compatible with the predicted length of arginase I, was observed in samples from livers of sham and BDL rats. Fig. 2A shows a typical gel of PCR products obtained using arginase II primers. Distinct bands were identified at the predicted size of 596 bp in all liver tissues of BDL rats. However, only a very weak signal for arginase II was observed in a few sham rats. To demonstrate that the RT–RCR products had originated from mRNA instead of from genomic DNA, PCR using total RNA as the template was performed for both arginase I and arginase II primers, and no band was found in either case (data not shown). The authenticity of RT–PCR products was confirmed by direct sequencing. We found at least 99% homology of the arginase I and II RT–PCR products with the reported rat arginase I and II gene sequences, respectively [18,19].

To examine the levels of arginase I and II mRNA expression in the different groups, competitive RT–PCR was carried out as described in Section 2. As shown in Fig. 1B, the level of arginase I mRNA expression in sham rats ($3.34 \pm 0.32 \times 10^4$ attomole/μg of total RNA) was more than 2-fold that expressed in BDL rats

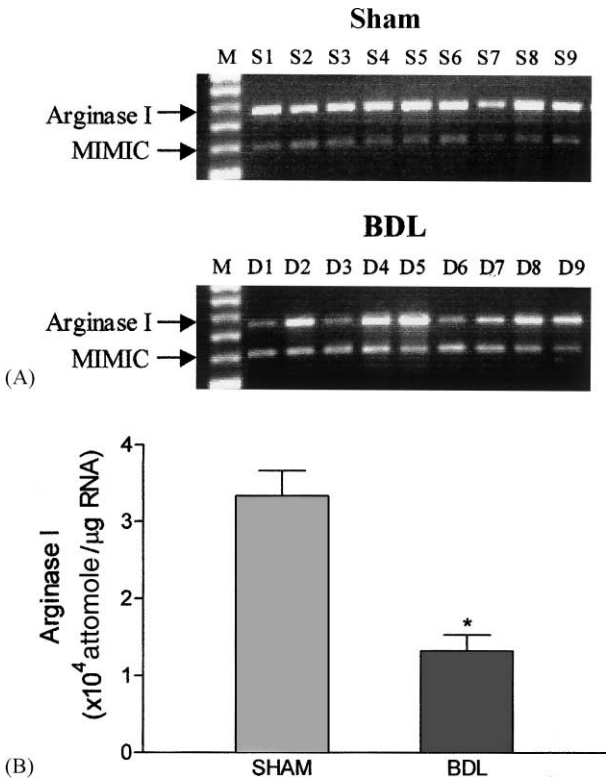


Fig. 1. Arginase I mRNA expression in livers. Total RNA was extracted from liver tissue, and mRNA was reverse transcribed and amplified with specific primers. (A) RT–PCR products with primers to arginase I. Lane: M, 100 bp molecular marker; S, sham; D, BDL; 1–9, individual rats used in each group. The 796 bp arginase I band and the 602 bp MIMIC band are indicated. (B) Quantitative analysis of mRNA expression in BDL rats vs. sham rats. Data are presented as means ± SEM. Key: (∗) $P < 0.001$ vs. sham.

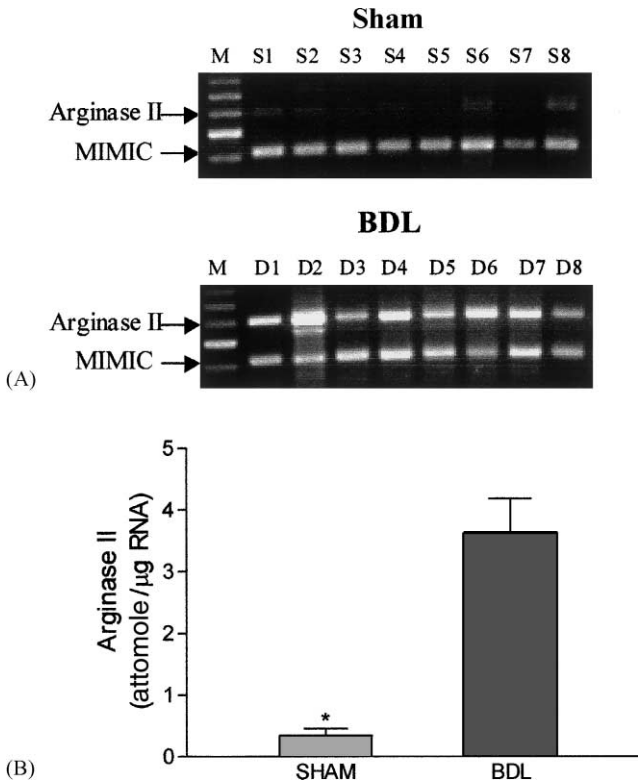


Fig. 2. Arginase II mRNA expression in livers. (A) Typical results of RT–PCR with primers to arginase II. Lane: M, 100 bp molecular marker; S, sham; D, BDL; 1–9, individual rats used in each group. The 596 bp arginase II band and its 418 bp MIMIC are indicated. (B) Graphical representation of arginase II mRNA expressed in livers of both BDL and sham rats. Data are presented as means ± SEM. Key: (∗) $P < 0.001$ vs. BDL.

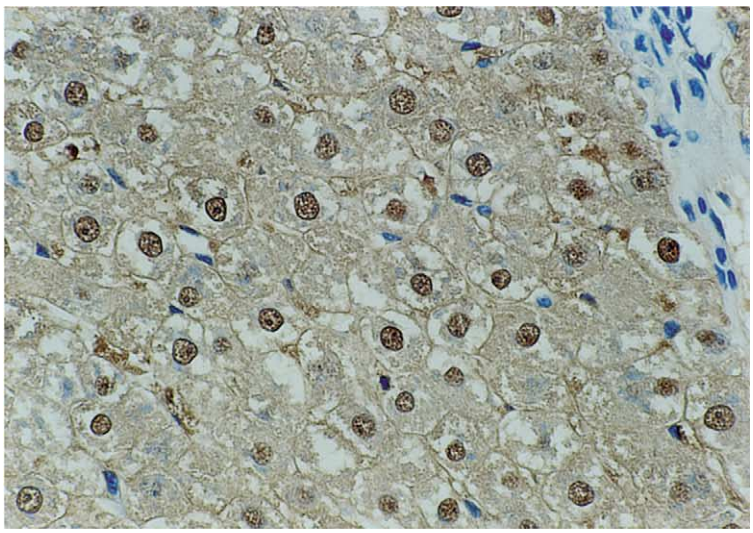
($1.32 \pm 0.21 \times 10^4$ attomole/μg of total RNA, $P < 0.001$ vs. sham). This result indicates that arginase I mRNA expression decreased significantly in BDL rats. However, the expression of arginase II mRNA in BDL rats (3.64 ± 0.54 attomole/μg of total RNA) was induced by about 10-fold, compared with that expressed in sham rats (0.35 ± 0.11 attomole/μg of total RNA) (Fig. 2B).

3.3. Protein expression of arginase I

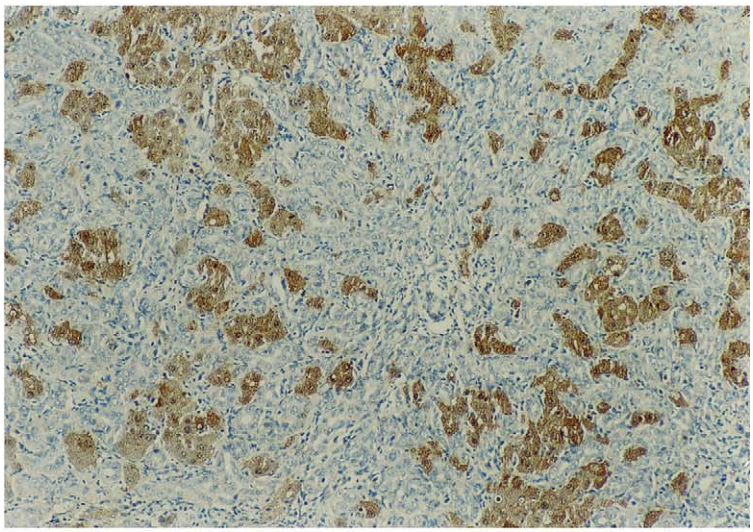
As shown in Fig. 3, immunoreactivity to the anti-arginase I antibody was observed in hepatocytes. In sham rats, arginase I was detected in nearly every hepatocyte (Fig. 3A). Staining was slightly more intense in the periportal zone and was less intense in the pericentral region. In contrast, only a portion of the remaining or regenerated hepatocytes was immunostained for arginase I in BDL rats (Fig. 3B,C). The positive staining granules were fine and localised diffusely in the cytoplasm of the hepatocyte. In addition, some nuclei were also stained with the arginase I antibody. However, the reason for this is unclear. Non-parenchymal cells, such as sinusoidal cells and bile ductular epithelial cells, were negative for arginase I in both BDL and sham rats. To confirm the results of this morphological study, western-blotting was performed. As seen in Fig. 5A, arginase I was detected in both BDL and sham rat livers. However, the expression of arginase I was much lower in the livers of BDL rats than sham rats. These data, together with the RT–PCR results, indicated that arginase I expression was decreased in BDL rats.

3.4. Expression of arginase II protein

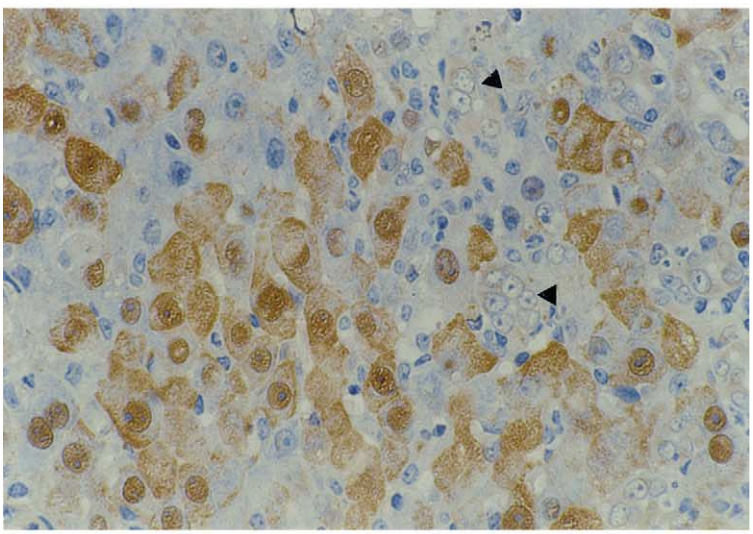
Morphologically, immunoreactivity with anti-arginase II antibody was clearly observed in some hepatocytes (Fig. 4B and C) and in some hyperplastic bile ductular epithelial cells (Fig. 4D) in BDL rats. In the positively stained cells, small particulate structures were localised in the cytoplasm, reflecting the mitochondrial localisation of arginase II. However, arginase II immunostaining was absent in liver sections of sham rats (Fig. 4A). These results are in agreement with those of western blot analysis (Fig. 5B), in which a protein of 35 kDa, recognized as arginase II, was detected only in the livers of BDL rats, while no such expression was found in sham rats. These results were compatible with those of the RT–PCR experiments and thus indicated that arginase II was induced in the livers of BDL rats at both mRNA and protein levels.



(A)



(B)



(C)

Fig. 3. Localisation of arginase I in liver tissue. Liver tissues were stained with anti-arginase I monoclonal antibody by the ABC method. (A) Tissue from sham rats: almost all of the hepatocytes were positively stained, whereas non-parenchymal cells and bile ductules were negative. (B and C) BDL rat tissue: only some remaining or regenerated hepatocytes were stained. The positive particles were fine and localised diffusely in the cytoplasm. Arrowheads in panel C point to degenerating hepatocytes. (Panels A and C, original magnification 400×; panel B, original magnification 100×.)

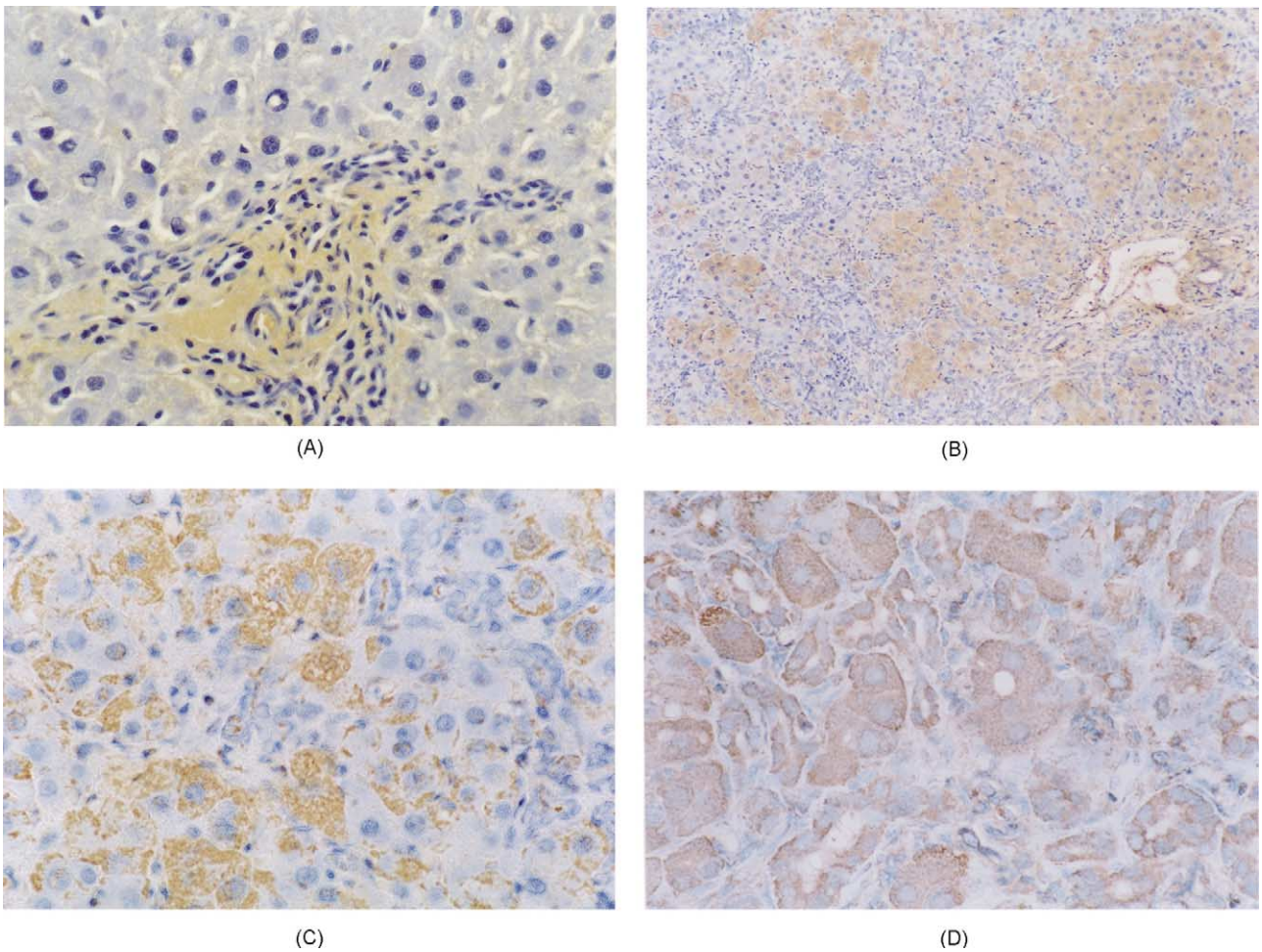


Fig. 4. Immunostaining of arginase II in liver tissues. Liver tissues were stained with anti-arginase II polyclonal antibody by the ABC method. (A) Sham rat tissue: no cell was positively stained. (B–D) BDL rat tissue: arginase II immunoreactivity was found in some remaining or regenerated hepatocytes (B and C) and also in some bile ductular epithelial cells (D). (Panels A, C, and D, original magnification 400×; panel B, original magnification 100×.)

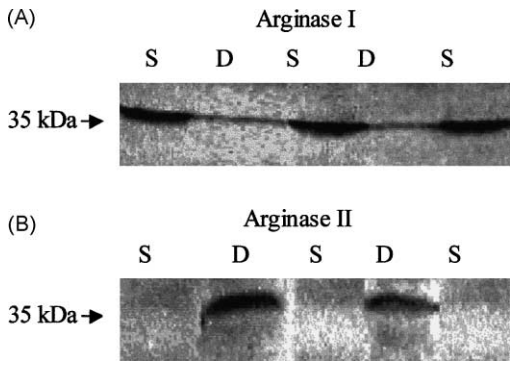


Fig. 5. Western blot analysis of arginase I and II in liver tissues. The proteins in the liver tissue extracts were resolved by 12% SDS-PAGE and then were transferred to a nitrocellulose membrane. Panel A: arginase I; panel B: arginase II; D: BDL rats; S: sham rats. The data illustrated are representative of at least three separate experiments.

4. Discussion

In this study, we found that arginase I was significantly down-regulated in BDL livers at both mRNA and protein levels. In accord with previous reports by other groups [16,20], we found abundant arginase I mRNA in livers of normal rats and diffuse staining of arginase I protein in nearly all hepatocytes. However, in BDL rats, the expression of arginase I was reduced significantly. As indicated by quantitative RT–PCR analysis, the arginase I mRNA level in BDL rats was less than half of that detected in sham rats. Correspondingly, arginase I protein was also decreased, and it was identified only in part of the remaining or regenerated hepatocytes in the cirrhotic livers.

Between the two isoforms of arginase that catalyse L-arginine to L-ornithine, arginase I is best known as a component of the urea cycle within hepatocytes and involved primarily in the detoxification of ammonia and urea synthesis [20]. Its expression has been studied in situations such as liver injury, trauma, and sepsis, during which the plasma was found to have low arginine levels but high arginase activity. Release of hepatic arginase into the plasma had been suggested to account for these changes of plasma arginine and arginase [21,22]. In fact, the release of arginase from the liver into the plasma has been regarded as a reliable indicator of hepatocellular damage in humans and animals [23,24]. In this study, we

found a dramatic drop of plasma arginine levels in the BDL rats. Correspondingly, plasma ornithine levels were higher in BDL rats than in sham rats ($r = -0.96$, $P < 0.0001$). These data suggest that high amounts of arginase are released by destroyed hepatocytes during the process of cirrhosis leading to the conversion of plasma arginine to ornithine. As supported by liver function test results, the liver function of BDL rats was impaired. This is one possible explanation for the observed down-regulation of arginase I in the BDL livers. However, it may also be possible that during the development of cirrhosis, hepatocytes which are undergoing tubular transformation may have lost their ability to express arginase I.

Recently, interest in arginase and arginine metabolism has been growing with the isolation and cloning of arginase II. Unlike arginase I, arginase II is localised within the mitochondrial matrix and is distributed in a wide range of tissues [1,20]. Different subcellular locations of the arginases may direct its product, ornithine, to different end products and thus lead to different functions.

The most important findings of this study are that, for the first time, arginase II was found to be induced significantly in the cirrhotic liver at both mRNA and protein levels. By RT–PCR techniques, we identified clear arginase II mRNA expression in BDL cirrhotic rats, whereas no convincing expression was seen in sham rats. Arginase II protein was induced and localised in some hepatocytes as well as in some hyperplastic bile ductular epithelial cells of cirrhotic livers. In contrast, no positive staining granules were found in sham rats. This indicated that arginase II was not induced by the surgery itself. As other researchers have shown that lipopolysaccharide can induce arginase II expression [25–27], inflammation may be one of the factors that lead to the induction of arginase II. However, exactly what accounts for the induction of arginase II in the cirrhotic liver still requires further study. Since the expression level of arginase II is quite low compared with that of arginase I, and the two isoforms have similar kinetic characteristics [28], it is not likely that arginase II will compensate for the loss of arginase I. Induction of arginase II may have other important roles in the pathogenesis of liver cirrhosis. Arginase II could regulate NOS activity and NO production, as arginase II could compete with NOS for the sole substrate, L-arginine. Gotoh *et al.* [7] and Wang *et al.* [31] found that arginase II could be coinduced with inducible NOS (iNOS) in murine macrophages, and arginase II could down-regulate NO production and prevent NO-mediated apoptosis [29–31]. As mentioned earlier, in liver cirrhosis, arginase I may be released into the bloodstream by damaged hepatocytes, thus decreasing plasma arginine levels. At the same time, the induction of arginase II may consume the intracellular pool of L-arginine. By directly making less substrate available for NOS, arginase II may down-regulate the NO production in hepatocytes where iNOS could be induced. Arginase II may also play a role in the regulation of proline and polyamine synthesis [32]. Ornithine produced by arginase II is the precursor of proline, polyamines, glutamate, and other related substances [1]. Polyamines are essential for cell proliferation and differentiation. Cells that are deficient in arginase cannot proliferate in serum-free medium unless ornithine or polyamines are provided [33,34]. Proline is a substrate for collagen synthesis [35]. Since increased collagen synthesis and hepatocyte regeneration are two of the key events in liver cirrhosis, arginase II may be indirectly involved in the pathogenesis of liver cirrhosis by regulating the levels of proline and polyamines.

In conclusion, we found that arginase I expression was decreased in liver cirrhosis induced by BDL, whereas arginase II was induced at both mRNA and protein levels. These results suggest that arginase I and II have different functions and are regulated differently in liver cirrhosis. The induction of arginase II could be important in that it may regulate NO production as well as be responsible for the biosynthesis of proline and polyamines in liver cirrhosis. Further studies are warranted to directly elucidate the regulatory pathways of both arginase I and arginase II in liver diseases.

Acknowledgments

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