Brief Communications

Intralobular Distribution of CYP1A2 within Rat Liver during Postnatal Growth**

Mohammed Z. Allouh,[1] Zuhair M. Allouh* [2]

Abstract

The intralobular distribution of CYP1A2 in rat liver was investigated at three postnatal periods (neonatal, puberty, and adult) using an immunohistochemical technique. The histochemical observations were confirmed by determining the intensity of staining through measuring the mean gray value. A homogenous expression of CYP1A2 is revealed in neonatal period that changes through maturation to adulthood to become more focally distributed around the central vein. These findings demonstrate that the lobular distribution of CYP1A2 varies as a function of age. This variation can have a profound effect on the sensitivity of different regions within the hepatic lobule to CYP1A2 metabolite-induced hepatotoxicity.

Keywords: CYP1A2, Immunohistochemistry, Liver, Rat.

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Introduction

Cytochrome P450 (CYP) members are a large group of heme-thiolate enzymes that are responsible for most drugs and toxicants metabolism within the liver. [1] Their name is originated from the fact that they are colored cellular proteins (cyto=cell, chrome=color), with maximum light absorption around 450nm wavelength. The most common reaction catalyzed by CYP enzymes is a monooxygenase reaction which involves insertion of one oxygen atom into an organic substrate. [2] CYP superfamily includes over 2000 individual enzymes that represent species from all biological kingdoms. [3] Individual enzymes of this family are anticipated to be characterized by their specific temporal and spatial expression patterns. [4] However, common expression characteristics among several of these enzymes have been found in human beings. [1]

Cytochrome P450-1A2 (CYP1A2) is one of two CYP enzymes which belong to the 1A family, CYP1A1 and CYP1A2, which is expressed in most mammalian species. [5] However, it is the predominant form and comprises about 5% of the total hepatic CYP content. [6] CYP1A2 is considered a vital enzyme that mediates several important functions. It stimulates the metabolism of a wide variety of drugs and chemicals including caffeine [7] and phenacetin. [8] Nevertheless, it bioactivates several environmental pollutants and procarcinogens to cause hepatotoxicity or carcinogenicity. [9] CYP1A2 expression is, also, found to be significantly induced by cigarette smoking. [8]

1. Department of Anatomy, Faculty of Medicine, Jordan University of Science and Technology, Irbid, Jordan.
2. Department of Applied Sciences, Al-Huson University College, Al-Balqa' University, Al-Huson, Jordan.
**** Correspondence should be addressed to:***
Mohammed Z. Allouh
P. O. Box: 3030, Irbid 22110, Jordan
E-mail: m_allouh@just.edu.jo

** **Name and Location of Institution where the work was performed: Department of Anatomy and Cell Biology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.**

The hepatic intralobular distribution of CYP enzymes is considered of critical importance in determining the location and sensitivity of specific metabolite-induced hepatotoxicity or carcinogenicity. Some earlier studies had attempted to investigate the expression pattern of CYP enzymes, including CYP1A2, within the liver after exposure to certain chemicals. [10, 11] However, little information is available about the constitutive expression and distribution of CYP1A2 during postnatal growth.

Materials and Methods

Experimental Model and Tissue Samples Preparation

Sprague-Dawley male rats were used in this study. The rats were kept under controlled temperature of 22^{o}C ± 2^{o}C and 12 hour-light-dark cycle. Food and water were available *ad libitum.* The rats were allowed for at least one week acclimatization period before being decapitated by guillotine. Four animals were killed at each of the following ages; 1, 6, and 16 weeks postnatal. These ages are representing critical stages during rat growth. They are corresponding to the neonatal, puberty, and adult periods of the rat, respectively.

Rat livers were removed immediately after decapitation and rinsed in ice cold 0.9% NaCl. The livers were then cut into several block samples. Each sample was embedded in O.C.T. tissue tek compound (Sakura Finetek, Californis, USA) in a plastic mold. The samples containing molds were directly frozen in liquid nitrogen and stored at- 80^{o}C.

Cross-sections of 8 μm thickness were cut using a cryostat at -20^{o}C. Every two serial sections were picked up on a ProbeOn Plus microscopic slide (Fisher Scientific Ltd., Ontario, Canada). The reason for collecting two sections on each slide was to increase the possibility of choosing better fields for imaging. Serial slides bearing sections were labeled and stored at -20^{o}C freezer.

Immunohistochemical Technique

Labeled slides were removed from the freezer and air dried for 15 minutes. Sections were fixed with 4% formaldehyde for five minutes and followed by two 5-minute washes in phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2). Slides were then placed in 3% hydrogen peroxide in methanol for five minutes followed by two to five minute washes in PBS. After that, the sections were treated for 30 minutes with at least 200 μl of blocking solution, which consisted of 5mM Ethylenediaminetetraacetic Acid (EDTA) in PBS, 5% goat serum and 1% bovine serum albumin. Blocking solution was drained from each slide onto a paper towel. After that, sheep anti-rat CYP1A2 polyclonal antibody (Chemicon International, California, USA) was used at a dilution of 1:100 in blocking solution. This antibody has no cross-reactivity with other CYP enzymes, especially CYP1A1. Using western analysis, the antibody only recognizes a single protein with a molecular weight of 54 kDa, which corresponds to CYP1A2 protein. The diluted antibody was applied over the sections on the slides for overnight at 4^{o}C in fridge. One slide on each experimental set served as a negative control by applying the blocking solution only over it, without adding the primary antibody.

Next morning, the slides were washed twice in fresh PBS solution for five minutes per wash. Then biotinylated anti-sheep secondary antibody (Chemicon International, California, USA) was applied over the sections on each slide for 40 minutes at room temperature, followed by two to five minute washes in fresh PBS solution. After that, 150 μl of Avidin ABC kit (Vector Laboratories Inc., California, USA) diluted in PBS was applied over the sections which were then kept in the dark for 60 minutes. Slides were then washed in phosphate buffer solution (PB; 0.02 M sodium phosphate buffer, pH 7.2) twice for five minutes each. To obtain a specific color reaction visible under the bright-field microscope, slides were immersed in 0.05% diaminebenzidine in PB for five minutes. This was followed by a five minute wash in PB, and then a five minute wash in PBS. Finally, the slides were mounted

with cover slips using Geltol mounting medium (Thermo Shandon, Pennsylvania, USA) and left for 20 minutes to harden before being examined under the microscope.

Image Capturing and Data Collection

Images were captured from each slide using a Zeiss Axioskop 20 microscope (Carl Zeiss GmbH, Jena, Germany), and a Sony S70 digital still camera (Sony Corporation, Tokyo, Japan). The differences in intensity of staining were measured and compared among three regions within the hepatic lobule: around the central vein (perivenous), around the portal triad (periportal), and the region in between (midzonal). Scion imaging program (developed by U.S. National Institute of Health and Image J is the current version available) was used to measure the staining intensity from the captured images [12]. The images were first converted to gray scale and the mean gray value was then measured for thirty contiguous hepatocytes in each of the perivenous, midzonal and periportal regions of the lobule.

The mean gray value measurements were used to indicate the intensity of staining by CYP1A2 antibody. Black and white were automatically assigned optical density values of 0 and 255, respectively, at the ends of an arithmetic linear regression ($r^2 = 1.00$). Images from the negative control slides were used to determine the maximum mean gray value of no staining, since they are not labeled by the primary antibody. Mean gray value measurements that represented the perivenous hepatocytes were taken from hepatocytes located in the first three rows around the central hepatic vein, while measurements represented the periportal hepatocytes were taken from hepatocytes located in the first three rows around the portal triad. Measurements of the midzonal hepatocytes were obtained from hepatocytes located more than four rows away from either central vein or portal triad.

Statistical Analysis

After applying the Levene test to determine the homogeneity of variance, data were evaluated at 5% level of significance by one way analysis of variance (ANOVA) or nonparametric Kurskal-Wallis H-test. If a significant difference was found ($P<0.05$), then either Scheffe's post-hoc analysis test or nonparametric Mann-Whitney U-test was applied to examine the statistical differences among groups within the same age. All statistical tests were performed using SPSS program (standard version 13.0, SPSS Inc., Illinois, USA).

Results

Immunohistochemical Staining

Intralobular distribution of CYP1A2 in neonatal, pubertal, and adult rat livers was revealed by performing the immunohistochemical analysis. The staining for CYP1A2 appeared within most hepatocytes throughout the liver lobule at the age of 1 week (Figure 1.A). At the age of 6 weeks, the staining intensity was reduced within the lobule and appeared to be repressed toward the region of the central vein (Figure 1.B). At the age of 16 weeks, the staining was limited to a small strap of hepatocytes (1-2 cell rows) around the central vein only (Figure 1.C). These observations indicate that as the expression of CYP1A2 is more homogenous within the lobule during the neonatal period, it tends to repress toward the perivenous region with age until it becomes concentrated in a small area around the central hepatic vein in adulthood.

Mean Gray Value Measurements

The mean gray value was determined for hepatocytes in each of the three regions of the hepatic lobule (Table 1). The mean gray value serves as an indicator for the intensity of labeling by CYP1A2 antibody. In neonatal liver, there was no significant difference ($P>0.05$) among hepatocytes throughout the lobule. This indicates a homogenous distribution of CYP1A2 enzyme within rat liver during the neonatal period. However, the mean gray value was significantly ($P<0.05$) reduced at the age of six weeks in the following pattern: Perivenous < midzonal < periportal. This implies a greater expression of CYP1A2 by perivenous hepatocytes around puberty.

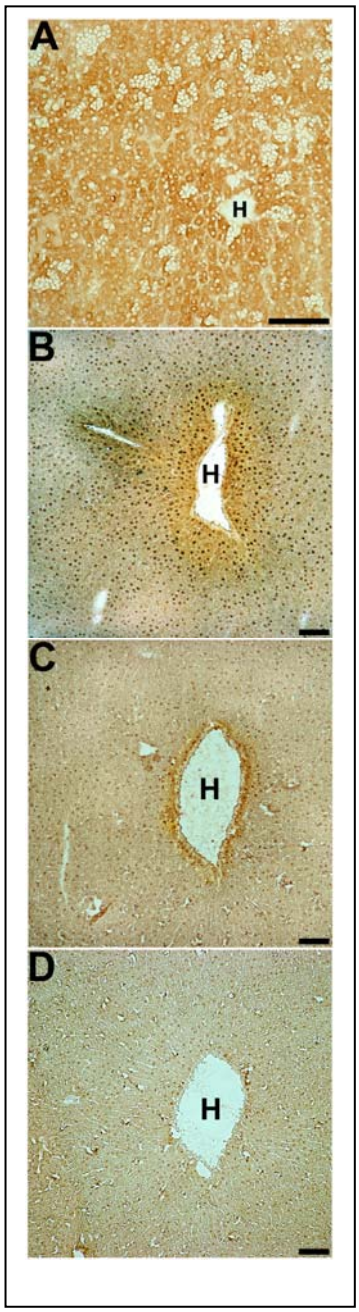

Figure (1): Immunohistochemical labeling for CYP1A2 in rat liver at three different ages of postnatal growth; (A) 1 week, (B) 6 weeks, and (C) 16 weeks. (A) Homogenous expression is observed in early neonatal livers. (B) and (C) The expression starts to repress with age to become more focally distributed around the central hepatic vein (H). (D) Control image for age 16 weeks, no secondary antibodies are applied. Scale bar = 100 μm.

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Mean gray value measurements for CYP1A2 in periportal, midzonal, and perivenous hepatocytes of rat liver at three different ages; 1, 6, and 16 weeks postnatal. Thirty contiguous hepatocytes were measured from each region. The mean gray value represents the intensity of staining by 1A2 antibody and obtains optical density values from 0 to 255 that respectively signify gradation from black to white. The numbers present are the average value ± standard deviation. Values with different letters are significantly different (P<0.05) from values of other regions within the same age group.*

Table (1):Mean gray value measurements for CYP1A2.

	1 week		6 weeks		16 weeks	
	CYP1A2	**Negative control**	**CYP1A2**	**Negative control**	**CYP1A2**	**Negative control**
Periportal	**73.64[a] ± 11.47**	**129.28± 4.24**	**131.66[a] ± 6.97**	**149.37± 6.74**	**159.99[a] ± 7.39**	**166.54 ± 4.76**
Midzonal	**71.65[a] ± 12.91**	**131.29± 3.21**	**116.76[b] ± 11.87**	**147.42± 6.43**	**159.34[a] ± 5.49**	**165.77 ± 5.60**
Perivenous	**67.92[a] ± 8.57**	**132.87± 3.69**	**89.87[c] ± 5.69**	**147.65± 7.63**	**90.35[b] ± 10.53**	**167.32 ± 7.28**

In adult rats (age 16 weeks), the mean gray value was significantly (P<0.05) lower in perivenous than in midzonal or periportal regions. Furthermore, there was no significant difference (P>0.05) in the measurements of mean gray value between 1A2 stained and negative control slides in both midzonal and periportal regions. These findings confirm our observation that CYP1A2 expression is restricted to the perivenous zone in adult rat liver.

Discussion

This study represents an improvement in the analysis technology for immunohistochemical evaluation of CYP1A2 expression in hepatocytes by using a computer software program that measures the intensity of staining within individual cells. This methodology provides a better, more accurate representation of intrahepatic CYP1A2 expression during postnatal development. The study shows that the lobular distribution of CYP1A2 within rat liver varies as a function of age. While the expression tends to be more homogenous in the neonatal period, it is repressed to be more focally distributed around the central vein in adult rats. This temporal change in expression has an important toxicological significance that is related to compounds undergoing CYP1A2 metabolism or bioactivation. It indicates an age-related difference in the capability of hepatocytes within different regions of the liver lobule to resist the hepatotoxicity that may occur upon exposure to these compounds.

There is substantial evidence in the literature that CYP1A2 is expressed postnatally, while CYP1A1 is present prenatally. [13, 14] It is suggested that CYP1A2 is the last major CYP enzyme to develop during the neonatal period. [15] As detected by western analysis, induction of CYP1A2 expression occurs usually 5 to 6 days after birth. [10, 16] Rat livers of age 1 week used in this study were satisfactory to detect the expression of CYP1A2 during the neonatal period.

Several approaches have been used to study the expression and regulation of CYP1A enzymes including RNA blot, [14] RT-PCR, [17] immunoblot [15] and immunohistochemistry. [11, 13, 16] Immunohistochemistry is most preferable among these procedures since mRNA levels do not always reflect the protein expression accurately and because of the ability to detect the expression in individual cells. This study uses immunohistochemical technique that includes a CYP1A2 antibody which does not cross-react with other CYP enzymes, especially CYP1A1. Furthermore, the immunohistochemical procedure applied in this study depends on inducing immunochemical reactions in fresh frozen tissue sections. This approach is considered more sensitive than using paraffin-embedded sections. It is well established that antigen loss form paraffin-embedded tissue may result during the preparation process. [18] Indeed, a reduced intensity of labeling for CYP enzymes within rat liver was reported when using the paraffin-embedded method. [19]

Previous Immunohistochemical studies have spotted the expression of CYP1A2 within the livers of different species. Rich et al. (1993) [16] have found that CYP1A2 expression is restricted to centrilobular region within hepatic lobules of neonatal rabbits, and spreads with age to become evenly distributed throughout the lobule after weaning. In adult human liver, the expression of CYP1A2 is confined to hepatocytes in the perivenous region. [13] This localized expression pattern resembles what we found in adult rat liver. In fact, it has been reported that rat and human 1A2 enzymes do mediate similar reactions and share analogous regularity mechanisms. [20] This finding may help in identifying the rat as an appropriate model for reasonable extrapolation of metabolism and toxicology data involving CYP1A2 substrates.

Age-related changes in intralobular distribution of 1A2 demonstrated in this study are presumably due to nutritional and hormonal factors. It has been established that the type of diet and hormone levels are varying significantly at different stages during postnatal growth. [21] Pineau et al. (1991) [22] have reported a significant increase in the expression level of CYP 1A enzymes that corresponds accordingly with

weaning in rabbits. This change in the level of expression may probably combine a change in the distribution pattern of 1A enzymes. However, some researchers argue that the concentration of diet gradients such as glucose, amino acids and fatty acids are too shallow in the hepatic sinusoids to have a major regulatory effect on the distributional pattern of liver enzymes. [2]

The regulatory effect of hormones on several CYP members has been reported. Treatment with growth hormone has been found to suppress the expression of CYP 2B and 3A subfamily enzymes and, on the other hand, stimulates 2C members expression. [2] Growth hormone does not only affect the expression of these enzymes, but also influence their hepatic intralobular distribution. [2] Oinonen et al. (1996) [23] have reported that GH treatment stimulates the periportal transcription of 1A2 mRNA and reduces its perivenous level. However, this observation is limited to mRNA level only and no data yet available regarding the effect of GH on 1A2 distribution at the protein level. Also, other circulating hormones are speculated to influence 1A2 distribution in a different manner to GH. Insulin, for example, has been found to suppress the accumulation of 1A2 mRNA in cultured hepatocytes. [24] A higher ratio of insulin is expected in the periportal region of the lobule. [25] This suggests more periportal reduction of 1A2 mRNA by the action of insulin.

It is anticipated that the distribution of CYP1A2 enzyme is a consequence of a summation effect based on a complex interaction among several regulatory factors. This study shows that the zonated perivenous pattern of CYP1A2 expression becomes more prominent after puberty. Hormonal factors are expected to be the main attributors to this zonated pattern, since no observable change in the type of the diet was made around this age period. However, further investigations are required to reveal the exact hormonal regulators and the mechanism by which they are affecting the distribution of CYP1A2.

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انتشار أنزيم CYP1A2 داخل فصيص الكبد أثناء النمو

محمد علّوه[1]، زهير علّوه[2]

1- قسم التشريح، كلية الطب، جامعة العلوم والتكنولوجيا الأردنية، اربد، الأردن؛ 2- قسم العلوم التطبيقية، كلية الحصن، جامعة البلقاء، الحصن، الأردن.

الملخص

يهدف هذا البحث إلى دراسة انتشار أنزيم CYP1A2 داخل فصيص الكبد عبر مراحل عمرية مختلفة وباستخدام التحليل المناعي النسيجي الكيميائي. لقد تم جمع أنسجة كبدية من جرذان ذات نوع Sprague-Dawley و على أعمار أسبوع، ستة أسابيع، وستة عشر أسبوعاً بعد الولادة. بعد ذلك تم استعمال أجسام مضادة خاصة قابلة للارتباط بهذا الأنزيم فقط، ومن ثم إحداث تفاعل كيميائي مع هذه الأجسام المرتبطة على نسيج الكبد لإنتاج صبغة بنية اللون. تم تحديد شدة هذه الصبغة باستخدام برنامج حاسوبي متخصص في ثلاث مناطق داخل الفصيص. هذه المناطق الثلاث هي (1) المنطقة الطرفية: وتقع على حواف الفصيص حيث الأقنية والجيوب البابية. (2) المنطقة المركزية: وتقع في مركز الفصيص حيث الوريد المركزي. (3) المنطقة الوسطى: وتقع ما بين المنطقتين الطرفية و المركزية. بيّنت نتائج هذا البحث أن انتشار أنزيم CYP1A2 يكون متجانساً داخل الفصيص خلال مراحل العمر المبكّرة، ومن ثم يبدأ بالانحسار نحو مركز الفصيص عند سن البلوغ، إلى أن يصبح إفراز هذا الأنزيم مقتصراً على الخلايا الكبدية الموجودة في مركز الفصيص فقط عند سن الرشد. وتدل هذه النتائج على أن هناك عوامل عمرية تلعب دورا مؤثراً في انتشار هذا الأنزيم داخل الكبد كالعوامل الهرمونية والغذائية مثلاً. بالإضافة إلى ذلك فإن هذا التباين في إفراز CYP1A2 ضمن مناطق الفصيص المختلفة سيؤدي إلى تباين في حساسية هذه المناطق لسمّية المواد التي يقوم هذا الأنزيم بأكسدتها.

الكلمات الدالة: CYP1A2، التحليل المناعي النسيجي الكيميائي، الكبد.