行政院國家科學委員會專題研究計畫成果報告

以基因微陣列監測大鼠肝臟部分切除後,肝細胞再生相關基因之變遷

MONITORING THE VARIATION OF REGULATING GENES BY c-DNA MICROARRAY DURING LIVER REGENERATION AFTER PARTIAL HEPATECTOMY IN RATS

計畫編號：NSC 91-2314-B-002-247

執行期限：91 年 8 月 1 日至 92 年 7 月 31 日

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中文摘要

肝臟是人體最重要的維生器官之一，當肝細胞大量損傷或切除時，可因肝衰竭導致死亡。由於台灣地區 B 型肝炎帶原者眾多，肝硬化、慢性肝病、及肝癌發生率很高，肝癌加上肝病死亡人數，已是台灣地區十大死因之首，肝病對國人健康的威脅相當大。有關肝細胞在肝損傷後可以再生，雖已是公認的事實，許多研究也證實多種營養素、賀爾蒙、生長因子、藥劑等，可直接或間接影響肝細胞再生，但肝細胞再生之詳細機轉及基因控制過程，則仍不明瞭。

任何細胞之分裂與再生，必然與細胞核內 proto-oncogene 之表現有相當大的關聯，肝細胞再生也不例外。在多項以 mRNA 定量的研究報告中指出，肝臟經部分切除後，剩餘肝臟內某些基因如（c-fos、c-myc、p53 及 ras 家族基因等）的表現的確有增加現象。Arora 等人則發現抗 c-myc 物質可經由 p-450 3A 活性之調控可抑制肝細胞再生；Ozeki 及 Tsukamoto 發現 retinoic acid 可抑制 c-fos 及 c-jun 之表現，促成肝細胞凋亡而使再生程度減少；而其他基因如 p21 及 gas-6 等之表現也被提出與肝細胞再生有關，但以基因微陣列（microarray）來大量監控肝細胞再生過程中相關基因之變遷，則仍未有報告。

本計劃以重約 200 克之 Wistar 雄性大鼠做實驗，測定肝細胞再生過程中，多種基因表現在各時段之變遷。所有大鼠均接受約百分之七十之肝臟部分切除手術，各於術前及術後 2、4、6、8、10、12、24、48、72 小時及 5、7、10 天後犧牲取樣，測定(1)剩餘肝臟之重量比值；(2)剩餘肝臟之有絲分裂指標；(3)以基因微陣列尼龍膜、肝細胞 mRNA 標號、hybridization 及影像分析等方法，測定超過 300 種基因表現之變遷。結果發現：(1)剩餘肝臟之重量於切肝術後 72 小時即恢復 90％以上；(2)有絲分裂於術後 48 小時大量出現，術後 72 小時逐漸減少；(3)肝細胞再生過程中，基因表現之變化共可分為 72 種型態，包括單一尖峰、雙尖峰、遞增型、遞減型、突出型、凹陷型或混和型等，每種型態包括 40 至 218 種基因。以基因微陣列大量監測分析肝細胞再生相關基因的變化，在研究肝細胞再生基因機轉方面，的確扮演相當重要的角色。

關鍵詞：肝細胞再生、部分肝臟切除術、proto-oncogene、基因微陣列、雜交

ABSTRACT

Liver is a core organ in our body. Although it contains good regeneration ability, hepatic failure can still occur after massive hepatic injury or hepatectomy. Because of the high incidence of hepatitis B carrier and so as to many hepatoma patients, the mortality rate of hepatic disease (including hepatoma cases) is one of the tope ten causes of death in Taiwan. Although there are much controversy continues on the initiation, regulation, metabolic changes, and termination of liver regeneration after partial hepatectomy that well initiate proliferation of the remaining hepatocytes, several factors, such as hormones, growth factors, nutritional components, and pharmacological agents, have been demonstrated to directly or indirectly affect liver regeneration.

The regenerating liver is a system in which the relationships between proto-oncogene expression and cell replication should be examined during a physiologic growth response. Proto- oncogene expression after partial hepatectomy is specific, sequential, and highly regulated. As measured by levels of mRNAs, the changes have been detected in the expression of c-fos, c-myc, p53, and the ras gene family (c-Ha-ras, c-Ki-ras, and N-ras). In contrast, expression of c-src and c-abl does not change after partial hepatectomy while c-mos transcripts cannot be detected in normal or regenerating liver. In recent study, the critical roles of some proto-oncogenes were noted in the control of cell proliferation, differentiation, and apoptosis by the new technique of complementary DNA microarray. However, mass survey and more detailed analysis by microarry method, that will be very important, are still nor reported.

The main purpose of this project is to find out the variation of more than 300 regulating genes during liver regeneration after partial hepatectomy. Male Wistar rats around 200g were used as subject. Partial hepatectomy (around 70%) were performed and they will be sacrificed at 2, 4, 6, 8, 10, 12, 24, 48, 72 hours and 5, 7, 10 days after hepatectomy. We measured: (1) weight of remnant liver; (2) mitotic index; and (3) genomic survey of the expression for more than 300 proto-oncogenes by cDNA microarray on nylon membrane, labeling of liver mRNA hybridization and image analysis. We found that: (1) the remnant liver weight increased to 90% in 72h after partial hepatectomy; (2) the mitosis of hepatocytes increased markedly at 48h then decreased at 72h after partial hepatectomy; (3) analyzing the gene expression of microarray chips, the variations could be classified into 72 different patterns including the pattern with a single peak at 2, 4, 6, 12, 48, 72h and 5, 7 days time sequence after partial hepatectomy. The variation patterns also included double peaks, enhancing trend, diminished trend, protruding curve, excavated curve and other mixed types. Each category of gene expression pattern contained 40 to 218 proto-oncogenes. The quantitative gene expression profiles should have important implications for the investigation on the mechanism of regenerating process.

Key words: liver regeneration, partial hepatectomy, proto-oncogene, microarray, hybridization

INTRODUCTION

Hepatocytes have a quiescent and highly differentiated phenotype. They rarely divide in adult humans or animals while remaining in the G_0-phase of the cell cycle. However, their capacity to replicate is not lost and is readily activated after liver resection or after injury induced by chemicals or drugs. Partial hepatectomy triggers hepatocyte proliferation whereas excessive liver mass is regulated by apoptosis. However, the knowledge on the mechanism of gene regulation on the liver regeneration is still limited.

It is reported that proto-oncogene expression after partial hepatectomy is

specific, sequential, and highly regulated.[1,2] Changes have been detected in the expression of c-fos, c-myc, p53 and the ras gene family (c-Ha-ras, c-Ki-ras, and N-ras).[3-5] In contrast, expression of c-src and c-abl does not change after partial hepatectomy while c-mos transcripts cannot be detected in normal or regenerating liver.[6,7] Recently, p21 cyclin-dependent kinase (CDK) inhibitor, Fas, interleukin (IL)-18, and several caspases which inceased apoptosis, and Bcl-2, hest shock proteins, glutathione-S-transferase genes which down regulated cell proliferation were noted to be involved in liver regeneration.[8] The mass survey about the variation of all the regulating proto-oncogenes expression according time sequence, which is not reported yet, will be very important to investigate the genetic mechanism of liver regeneration.

This study was conducted to find out the variation patterns of more than 6,000 regulating genes expressions by cDNA microarray during liver regeneration after partial hepatectomy in rats.

MATERIALS AND METHODS

Experimental Protocol

Sixty male Wistar rats (purchased from Charles River, Osaka, Japan) weighing approximately 200g were used as subjects. All of them received partial hepatectomy and they were sacrificed before and 2, 4, 6, 8, 12, 24, 48, 72 hours and 5, 7 days after hepatectomy. Six were sacrificed each time and the remnant livers were removed immediately for further tests.

Surgical Procedures

All rats are anesthetized by intraperitoneal pentobarbital (10mg/kg) injection. A midline laparotomy was performed. Partial hepatectomy was then carried out by means of aseptic extirpation of the median and left lateral lobes (around 70%) according to the procedure of Higgins and Anderson.[9] The removed liver sample was immediately weighed. Laparotomies with manipulation of liver were done in the sham operated rats. All of the surgeries were performed between 8 am and 11 am to reduce the influence of diurnal variation.

Measurements

(1)Evaluation of the remnant liver

Observation of the liver surface and color. Then weighing the liver immediately after sacrifice, and the ratio of remnant liver weight/body weight will be calculated.

(2)Mitotic index of remnant liver

The small pieces of liver tissue for hisopathological examination at certain postoperative period will be fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin for microscpic observation. The mitotic activity will be determined by counting the number of parenchymal cells undergoing mitosis in 50 randomly-selected fields under magnification ×400. The results will be expressed as the mitotic index (the total number of mitoses per 50 different fields examined).

(3)Genomic survey of remnant liver by cDNA microarray

a. Non-isotopic labeling of liver mRNA

Total RNA was extracted from remnant livers of sacrificed rats in each postoperative time sequence. The tissue was homogenized in 3 ml of solution A containing 4M guanidine thiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, and 0.1M betamercaptoethanol at pH 7.0, followed by phenol extraction, isopropanol precipitation, and ethanol precipitation. Quality of RNA was examined by agarose gel electrophoresis. Messenger RNA was purified using Qiagen Oligotex extraction Kit.

Five micrograms of mRNA was annealed with 6 uM random hexamer in a total volume of 50 ul. The cDNA synthesis

was performed in a 100 ul mixture containing 0.5 mM each dATP, dCTP, dGTP: 40 uM dTTP, 40 uM biotin-16-dUTP (Boehringer Mannheim), 10 mM DTT; 0.5 units/ul Human Placental Ribonuclease Inhibitor (HT Biotechnology Ltd., UK), and 200 units of Superscript RT II (GIBCO-BRL, Gaithersburg, MD). The mixture was incubated for 90 min at 42ºC and terminated by heating at 90ºC for 5 min. The RNA was degraded by addition of 5.5 ul of 3N NaOH and incubated at 50ºC for 30min. The mixture was neutralized by addition of 5.5 ul of 3M acetic acid and precipitated by addition of 50 ul of 7.5M ammonium acetate, 20 ug of linear polyacrylamide, and 1125 ul ethanol. The pellet was suspended in 36 ul deionized water.

b. Hybridization and image analysis of microarray

The membrane containing 384 spots cDNA was pre-hybridized in 5 ml 1×hybridization buffer containing 5×SSC, 0.1% SDS, 1% BM blocking buffer (Boehringer Mannheim), and 10 ug/ml denatured salmon sperm DNA, at 60ºC for 1 h. The probe was mixed with 2 ul of 10 ug/ul poly d(A) 10 and 2 ul of 10 ug/ul human Cot-1 DNA (Gibco BRL) and 40 ul of 2×hybridization buffer to a final volume of 80 ul, followed by denaturation of the probe mixture at 95ºC for 5 min and then cooling on ice. The membrane was annealed with the probe mixture in a hybridization chamber, incubated at 95ºC for 5 min, and then at 58ºC for 12-16h. The membrane was washed twice with 5 ml of 2×SSC, 0.1%SDS for 5 min at room temperature, followed by three washes for 15 min each with 5 ml of 0.1×SSC, 0.1% SDS at 58ºC. The membrane was blocked with 5 ml of 1% BM blocking reagent containing 2% dextran sulfate at room temperature for 1 h, followed by incubation with 5 ml mixture containing 700×diluted Streptavidin-β-galactosidase (1.38 U/ml, enzyme activity) (Gibco BRL), 4% polyethylene glycol 8000 (Sigma), and 0.3% BSA in TBS buffer (10 mM Tris, pH 7.4, 150 mM NaCl) at room temperature for 1 hour. The membrane was then washed with TBS buffer three times for 5 min each. The membrane was then treated with 5 ml X-gal substrate containing 1.2 mM X-gal, 1 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ in TBS buffer for 45 min at 37ºC with gentle shaking, followed by mini-Q water wash and air dry. Color image was generated using UMAX PowerLook 3000 flatbed scanner at a resolution 3048 dpi and processed by ScanAlyze.

Data analysis

The images captured by a scanner could be digitized by a commercial analysis software such as GenePix 3.0 (Axon instruments) or the program written in-house. The pre-treated data will be then clustered by hierarchical method or self-organizing maps. These softwares are free and available from Stanford university and Massachusetts Institute of Technology once got the license permission for non-profit use.

RESULTS

The rats were living well during experimental stage. After sacrifice, the remnant liver weight increased to 90% in 72h after partial hepatectomy. The mitosis index showed that the mitosis of hepatocytes in the remnant liver increased to 104±12 at 48h, then decreased to 24±6 at 72h after partial hepatectomy.

The mRNA extracted from the remnant liver was collected at the indicated intervals (before and 2, 4, 6, 8, 12, 24, 48, 72 hours and 5, 7 days after partial hepatectomy) and analyzed by Northern blot hybridization as shown in Fig 1. The mRNA examined by agarose gel electropgoresis were well qualified in the 28s/18s ratio of 2, and the 260nm/280nm OD ratio of 2 by spectrophotometry.

The colorimetric image of cDNA microarray hybridization with 6,144 putative

genes on the nylon membrane of the remnant liver before and 6, 24, 72 hours after partial hepatectomy were shown in Fig 2. The scattering of the scanned spots were not eventually even and the deep-colored spots were not much in the cDNA microarray chip from the normal liver tissue before hepatectomy. The spots increased in number and in the density on the chips at 6, 24, 72, hours after partial hepatectomy. The variation was uneven and not in a regular pattern.

When the microarray chip was analyzed by flatted scanner and the GenePix in each time sequence, the variations of all the 6,144 proto-oncogenes expression could be classified into 72 different patterns (Fig 3.). The changing curves of the genes expression could have a single peak which occurred at 2h (c29, 86 genes), 4h (c7, 80 genes), 6h (c0, 178 genes), 12h (c24, 117 genes), 48h (c64, 71 genes), 72h (c40, 73 genes), 5days (c69, 141 genes), and 7days (c44, 93 genes). The curves could have a double-peaks pattern which occurred at different time sequence (c16, 58 genes; c68, 70 genes). An increasing (c61, 65 genes) or a decreasing (c21, 67 genes) tendency curve could be seen in some proto-oncogenes expressions. The changing curves could also be a protruding pattern (c25, 48 genes; c33, 56 genes) or an excavated form (c38, 98 genes; c47, 101 genes; c55, 65 genes). Mixed type changing curves in other unclassified variation patterns of genes expressions were also detected and figured out. Each category of gene expression pattern contained 40 to 218 identified proto-oncogenes.

DISCUSSION

There are 4 transcription factors, NFKB, STAT3 (both are strongly induced by TNF), AP-1, and C/EBP β are activated after partial hepatectomy and they might play important roles in the initiation of liver regeneration.[10-13] Harber et al proved that 70 genes were induced with relation to liver regeneration during 9 days after partial hepatectomy.[14] Because the initiation and termination of liver regeneration is a self-regulating growth process, the progression phase must be dependent on proto-oncogene regulatory mechanism.

Analysis of genes expression by cDNA microarray technology led to the identification of many regeneration-related genes expressions during liver regeneration stage after partial hepatectomy. We analyzed 6,144 genes according the time sequency after partial hepatectomy by PCR-amplified cDNA fragments and arraying machine, and found that a lot of genes changed there expressions in totally 72 patterns of changing curves. The extraction of mRNA in the regenerated liver were well qualified by both Northern blot hybridization and colorimetric image of microarray chips as shown in Fig 1. and Fig 2.. The computerized analysis of changing patterns of the genes expression showed that it might have a single peak at early, intermediate, late phase, or have two separated peaks, a protruding curve, an excavated curve, ascending or descending trends with a different number of genes in each. It's hard to conclude how many or which genes were involved in the regenerating mechanism. However, many of these genes should play very important roles at some specific timing during the liver regeneration. The effects may be direct or indirect, enhanced of diminished.

Su et al used the microarray analysis and reported that gene expression between 0 and 4h after partial hepatectomy is corresponding to the priming phase of liver regeneration.[15] The genomic program involves transcription-factor regeneration, stress and inflammatory responses, cytoskeletal and extracellular matrix modification, and regulation of cell-cycle entry. The changes of genes expressions provide a detailed and comprehensive map of the priming stage of liver regeneration. To differentiate the roles of Paeoniae Radix (PRE) in the apoptosis of hepatoma and hepatectomy, Lee et al used cDNA microarray technology and found that

gene expression of BNIP3 was up-regulated while ZK1, RAD23B, and HSPD1 were down-regulated during early apoptosis.[16] Detailed analysis of the genes expression by the method of cDNA microarray, and comparison with the liver regeneration phenotype might give us the answer that how many and which specific genes could have regulated the initiation, differentiation and the self-termination during the regeneration process.

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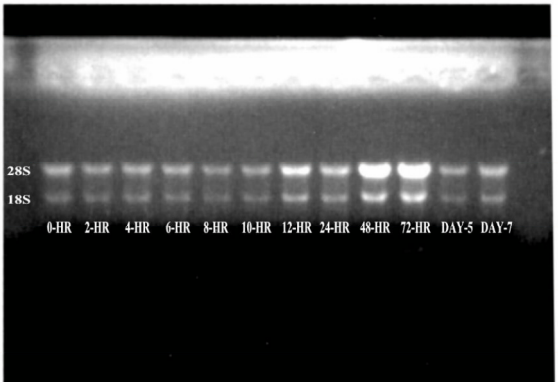

Fig 1. The mRNA extracted from the remnant liver showed a qualified picture by agarose gel electrophoresis.

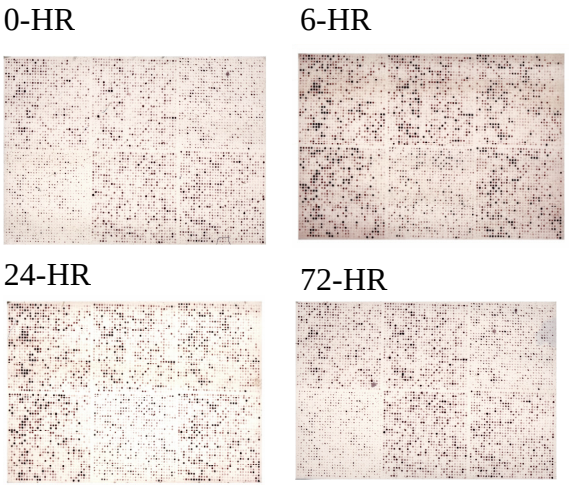

Fig 2. The colorimetric image of cDNA microarray hybridization chips with 6,144 genes showed uneven changed patterns before (0-HR), and 6, 24, 72 hours after partial hepatectomy.

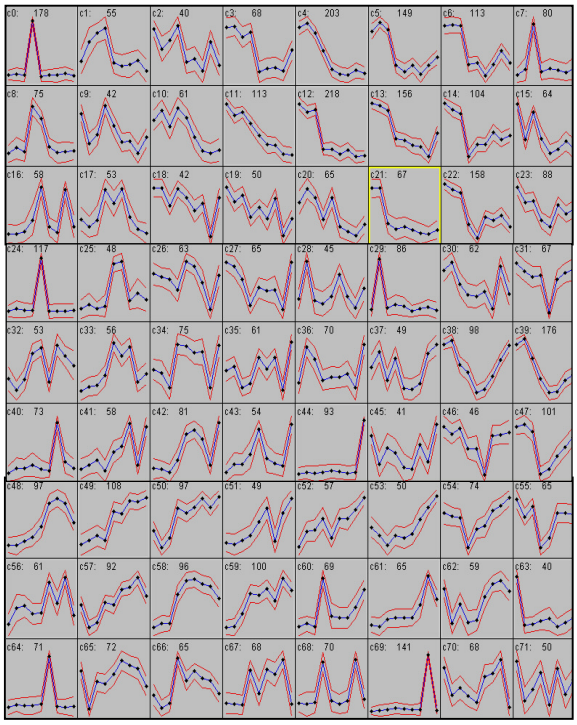

Fig 3. Seventy-two different patterns of genes expressions in the remnant liver during liver regeneration according the time sequence of 0, 2, 4, 6, 12, 48, 72 hours and 5, 7 days after partial hepatectomy (C= sequence number of categories; the following number= numbers of genes included in this category; the longitudinal line is the intensity of gene expression; the longitudinal line is the intensity of gene expression).