Liver Regeneration After Hepatectomy In Rats
(I) Expression Of Aquaporins In Liver Regeneration
(II) The Effect Of Thalidomide On Liver Regeneration

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Liver Regeneration After Hepatectomy In Rats

(I) Expression Of Aquaporins In Liver Regeneration
(II) The Effect Of Thalidomide On Liver Regeneration

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中文摘要

“肝臟再生”在肝臟手術與肝移植上是一個相當重要的觀念；但是，肝臟再生的
機轉還尚未被清楚釐清。本論文分兩方面探討，第一部分探討水通道蛋白
（Aquaporin）在肝臟再生的表現；第二部分探討臨床用藥 Thalidomide 對肝臟再
生的影響。

水通道蛋白（Aquaporin）是一種位於細胞膜上的小蛋白質，它的功能是水分
子在細胞膜間的通透管道；最近研究顯示，水通道蛋白除了水的通透功能外，還
有其他和肝再生相關的生理功能，例如:血管新生、細胞移動和細胞增殖，所以本
研究的主題是探討肝臟再生中水通道蛋白家族表現的情形。我們利用三分之二切
肝的大鼠肝再生模式，以反轉錄聚合酶鏈反應(RT-PCR)、西方墨點法(western
blotting)與免疫組織化學(immunohistochemistry)來檢測與定位水通道蛋白在再生肝
臟細胞的表現。我們發現 AQP 0、8、9,和 11 主要於再生肝細胞表現；AQP8 也在
庫氏細胞於一特定時間表現；AQP9 的表現於肝切除後持續上升，尤其在肝切除的
早期表現更明顯；AQP11 短暫的在肝細胞核表現。這個研究提供了水通道蛋白
參與肝臟再生，而這些水通道蛋白與血管新生、肝癌形成，究竟扮演何種角色，
值得未來繼續研究。

目前肝癌(HCC)的治療方式仍有高復發率及預後不佳的情形，肝癌是高血管
密度分佈的惡性腫瘤。抑制血管新生的治療是目前肝癌治療非常有潛力的輔助治
療方式。據文獻指出，Thalidomide 具有抑制血管新生的作用。臨床上，Thalidomide
多用於肝癌末期無法接受手術根除性治療的病人，當成術後輔助性(adjuvant)治療
尚無文獻報導。也由於 Thalidomide 有抑制血管新生作用，肝再生也隨著血管新生
過程，Thalidomide 是否也抑制肝再生血管新生，至今尚無文獻探討。本研究以
70%切肝的大鼠肝再生模式，術前給予 Thalidomide，探討 Thalidomide 對肝再生
的影響。實驗動物將分成二組：實驗組給予飼食 Thalidomide (100mg/kg)；控制組
則無。使用 Laser Doppler flowmetry 來測量肝的表面微循環(microcirculation)。利用
西方黑點法與免疫組織化學來檢測肝內皮生長因子(VEGF)於再生肝贊細胞的表現。結果我們發現70%肝切除後，即刻進入肝再生過程，肝再生於術後96小時達到高峰。Thalidomide有延遲細胞的增生(cell proliferation)，但卻不影響再生肝贊重量的變化。兩組別中，切肝術後肝贊微循環並無明顯差異。IL-6與VEGF在切肝手術後明顯上升，在兩組別中無明顯差異。TNF-α在切肝後的再生肝中有上升趨勢，但在兩組比較下，無明顯差異。免疫組織化學的研究發現，在控制組中VEGF明顯地從pericentral至periportal由濃至淡的表現出來；但在Thalidomide這組就表現得沒有這麼強烈。由文獻記載，Thalidomide具有血管新生抑制功能，但在本實驗中Thalidomide對肝切除後的肝再生並無明顯抑制作用；利用這樣的結果，可以將Thalidomide當成肝癌手術切除後的早期輔助性治療，以抑制腫瘤的血管新生減少腫瘤復發機會而不影響術後的肝再生過程。

關鍵字：肝贊再生、水通道蛋白、大鼠肝再生模式、肝贊手術、血管新生作用、肝癌、Thalidomide、血管內皮生長因子(VEGF)腫瘤壞死因子-α(TNF-α)、細胞激素-6(IL-6)
The remarkable ability of liver to regenerate after insults has been harnessed by surgeons when designing techniques for liver resection or transplantation. However, the underlying mechanisms of liver regeneration are not fully clarified. There are two parts in this study of liver regeneration. Part I is the investigation for the expression of aquaporins (AQPs) in liver regeneration. The investigation for the influence of thalidomide on the liver regeneration is in Part II.

Aquaporins (AQPs) are small transmembrane proteins with unexpected physiological roles in addition to water transport. For example, they play pivotal roles in cell migration, angiogenesis, and cell proliferation, events that are also occurred during liver regeneration. We thus examined the possible involvement of AQPs in this regenerative process. 70% partial hepatectomy (PH) rat model was employed. The temporal expression of various AQPs in the liver following PH was determined by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and western blotting. The localization of AQPs was evaluated by immunohistochemistry. Results. As anticipated, AQP0, 8, 9, and 11 were detected mainly in hepatocytes; unexpectedly, Kupffer cells were observed to express AQP8 during a specific period of time in the regenerative process. AQP9 protein was shown to be expressed in a progressively enhanced pattern at early time points after PH. A transient expression of AQP11 in the
nucleus of hepatocytes was observed. These findings suggest the possibility that AQP might be involved in the PH-induced liver regeneration.

The treatment for hepatocellular carcinoma (HCC) still presents a major challenge in high recurrent rate. HCC is a hypervascular malignancy. Antiangiogenic therapy may be a potential adjuvant treatment for HCC. Thalidomide, a antiangiogenic agent, may inhibit VEGF induced angiogenesis and it was used for patients with advanced HCC. The present study was undertaken to investigate the effect of thalidomide on liver regeneration after hepatectomy. In this study, all rats subjected to 70% PH were divided to two groups, one treated with oral thalidomide (100mg/kg), the other not. Serial changes in hepatic microcirculation were evaluated by Laser Doppler flowmetry. The VEGF expression in liver tissue was assessed by immunohistochemical study and western blot analysis. Following PH, the liver regeneration rate increased markedly and reached a peak at 96 h in the two groups. Thalidomide did not affect the overall restoration of liver mass, although a delay in cell proliferation was observed. Prior to PH, the liver microcirculation in rats treated with thalidomide for 2 days was comparatively less than that in their corresponding controls; however, no significant difference between the two groups was detected at any time-point following PH. Western blotting showed that the expression of IL-6 and VEGF was upregulated by PH and the expression levels in the two groups were equal at all studied time-points. TNF-α
increased in the regenerative liver of thalidomide treated rats; however, there was no significant difference of TNF-α between the two groups. The immunohistochemical staining revealed a waved pattern of VEGF expression which advanced from the periportal to pericentral area in both groups, but a slower advancement was detected in thalidomide-treated rats. In conclusion, thalidomide exerted no significant effects on the expression of TNF-α, IL-6 and VEGF and did not impair the overall restoration of liver mass in a rat model of 70% PH-induced liver regeneration, providing supportive evidence for its use as a adjuvant treatment modality for liver cancers after tumors resection.

Keywords: Liver Regeneration, Aquaporin, Animal model of liver regeneration, Hepatectomy, Angiogenesis, Hepatocellular carcinoma (HCC); Thalidomide; Vascular endothelial growth factor (VEGF), Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6)
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Abbreviation

AQP: aquaporin

cDNA: complementary deoxyribonucleic acid

DNA: deoxyribonucleic acid

ENL: erythema nodosum leprosum

HRP: horseradish peroxidase

IL-6: interleukin-6

IHC: immunohistochemistry

mRNA: messenger ribonucleic acid

PBS: phosphate-buffered saline

PCNA: proliferating cell nuclear antigen

PCR: polymerase chain reactions

PEPCK: phosphoenolpyruvate carboxykinase

PH: partial hepatectomy

RT-PCR: reverse transcription polymerase chain reaction

SDS-polyacrylamide gel electrophoresis: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

TNFα: tumor necrosis factor α
5-BrdU: 5-Bromo deoxyuridine
Chapter 1  Introduction

Liver is a unique organ with capability of regeneration after various injuries, such as ischemia, viral infection, and partial resection [1]. The remarkable ability of liver to regenerate following two-thirds partial hepatectomy (PH) was first described in rats by Higgins and Anderson in 1931 [2]. This feature has been harnessed by surgeons when designing techniques such as liver resection, split-organ, and living-related donor transplantation [3]. Although tremendous efforts have been spent on the exploration of the mechanisms involved in the regenerative process, several key questions still remain unanswered [1]. To date, available information regarding liver regeneration mostly came from rat experiments, despite the physiological differences between humans and rodents. It is now well accepted that there are three main phases of liver regeneration: an initiation phase, rendering hepatocytes competent for subsequent replication; a proliferation phase, where the hepatocyte population is expanded; and a termination phase, where growth response is terminated at a defined set point [1]. In this study, there are two parts to investigate liver regeneration. Part I is the investigation for the expression of aquaporins (AQP)s in liver regeneration. The investigation for the influence of thalidomide on the liver regeneration is in Part II.

Aquaporins (AQPs) are a family of small integral proteins consisting of six transmembrane domains connected by five connecting loops, with molecular weights
ranging between 25 and 34 kDa [4]. So far, since the initial discovery of AQP1 in 1988, 13 distinct aquaporins (AQP0-12) have been reported and they are functionally subdivided into aquaporins (AQP0-2, AQP4, 5, and 8, which are primarily water selective), and aquaglyceroporins (AQP3, 7, 9, and 10, which are permeable to neutral solutes such as glycerol and other small solutes in addition to water) [4-7]. In addition to these two main groups, AQP11 and 12 are poorly characterized and their functional roles are unknown [8]. Specific physiological roles for many of the AQPs have been recognized from phenotype analysis of AQP knockout mice. In addition to water transport, various unexpected roles of AQPs were found in the past few years. For instance, the involvement of AQPs in cell migration was documented following the observation of slower migration of cultured aortic endothelial cells from AQP1-nullmice than cells from wild-type mice in response to a chemotactic stimulus [9]. Subsequent studies showed that the AQPs-facilitated cell migration was AQP- and cell-type-independent [10, 11]. On the other hand, aquaglyceroporins, despite their unidentified physiological importance as glycerol channels, might play important roles in cell proliferation and the homeostasis of metabolism. Impaired wound healing and cell regeneration was observed in AQP3-deficient mice [11, 12]. AQP7 and AQP9 are highly expressed in adipocytes and the liver, respectively [13, 14]. In the liver, AQP9 is expressed in hepatocytes within the sinusoidal surfaces of hepatocyte plates and is proposed to function in glycerol uptake
from the bloodstream for gluconeogenesis during starvation. AQP7-expressed adipocytes are crucial in the maintenance of whole-body energy balance by regulating lipogenesis and lipolysis. The release of glycerol by adipocytes and its uptake by the liver are optimally balanced by the coordinated regulation of AQP7 and AQP9. Additionally, the pathophysiological relevance of these channels has been demonstrated in studies of knockout or knockdown mice [15].

Several AQPs (0, 1, 3, 4, 5, 8, 9 and 11; AQP3 is absent in rats) have been reported to be expressed in liver [16-19]. Among them, the functional role of AQP0 and AQP11 remains unclear. AQP1, found in hepatic blood vessels and cholangiocytes, is believed to mediate water transport from plasma to bile or across the cell membrane of cholangiocytes [6, 16]. AQP3 is postulated to be involved in lipid metabolism [20]. AQP4 is implicated in the intrahepatic bile duct absorption of water [21]. AQP8 is suggested to be involved in water permeability across the canalicular membrane [22-24]. AQP9 is important for glycerol transport during hepatic gluconeogenesis and, along with AQP8, contributes to bile secretion [17, 25]. However, further studies are required to fully assess the physiological relevance of these AQPs.

Part I of this study was aimed to explore the possible involvement of AQPs in liver regeneration. The motivation comes from the accumulating observations in the literature that AQPs are involved in the angiogenesis, cell migration and proliferation that are
A partial hepatectomy-induced rat liver regeneration model was employed in this study. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blot analyses were performed to detect the expression level of AQP mRNA and protein, respectively. To confirm the presence of the AQP proteins in liver tissues, immunohistochemistry was conducted. Although only four AQPs (AQP0, 8, 9, and 11) are known to be expressed in hepatocytes, the major cell type responsible for liver regeneration, we examined all the seven AQPs (AQP0, 1, 4, 5, 8, 9, and 11) that have been identified in the hepatobiliary system considering the possibility that some AQPs might express only transiently in the regenerative process.

Thalidomide, α-N-phthalimido-glutarimide, was initially marketed as a sedative and antinausea medicine in the 1950s, but was withdrawn due to teratogenicity [26]. Unexpectedly, it has become the subject of intensive investigation in oncology since its antiangiogenic properties were first demonstrated in 1994 [27]. In that study, the bFGF-induced neovascularization in rabbit corneas was significantly reduced by thalidomide. This drug has also been shown to inhibit VEGF-induced angiogenesis [28, 29]. In addition to its antiangiogenic effect, an immunomodulatory function is also a potential mechanism of the anticancer activity of thalidomide. To date, the effectiveness of thalidomide for treating neoplastic disorders has been confirmed in diseases such as
multiple myeloma [30] and Kaposi’s sarcoma [31]. In addition, thalidomide has been tentatively used for the treatment of advanced hepatocellular carcinoma (HCC) [32-35].

Antiangiogenic factors have been demonstrated to reduce the formation of new blood vessels [36], resulting in slower tumor growth or even tumor regression. Therefore, the combination of antiangiogenic strategies with liver resection is a promising approach to treat primary and metastatic liver cancers, such as HCC and colorectal cancer. Post-hepatectomy liver failure develops if liver regeneration is impaired, especially in antiangiogenic condition. However, the effect of the antiangiogenic agent on liver regeneration has not been fully clarified. In the Part II of the present study, we investigated the effect of thalidomide on TNF-α, IL-6 and VEGF expression and liver regeneration in rats following 70% PH.
Chapter 2  Literature Review

2.1 Liver function

Liver is the largest abdominal organ and is now thought of as anatomically consisting of eight independent segments [37]. Liver is an important organ with complex functions and high regenerative capacity [38, 39]. Liver performs many essential functions for the body, including regulation of plasma glucose levels (essential for bodily function); incorporation of toxic ammonia generated by transaminase reactions into the non-toxic urea; synthesis of many if not most of the proteins circulating in the plasma, from albumin to coagulation proteins; mobilization and export of lipids; synthesis and export to the intestine of bile, important for absorption of fat in the diet; degradation of pigments; storage of several vitamins, etc [40].

2.2 Liver regeneration

Regeneration of liver can be defined more correctly as compensatory growth, during which the remaining liver tissue expands to meet the metabolic needs of the organism. Unlike anatomic true regeneration, the expanding liver does not regain its original gross anatomic structure [41].
2.2.1 Models for liver regeneration

A number of models have been proposed for the study of liver regeneration. The most completely studied model is that of liver regeneration after partial hepatectomy. A rodent model of two-thirds hepatectomy was first proposed by Higgins and Anderson in 1931 [2]. The rodent liver is multilobar, allowing for the removal of 3 of 5 liver lobes (two-thirds of the liver mass). Within 5-7 days of the surgery, the residual liver has regenerated to a size equivalent to the original mass. This model has remained a popular model of study because there is no injury to the residual liver. The resultant sequence of events can be delineated clearly without histologic evidence of damage to the residual liver tissue.

Chemical-mediated hepatotoxic injury, including carbon tetrachloride (CCl₄), has also served as a common model of liver injury. The challenge of CC₄-mediated injury is that it triggers necrosis of lobular zones of liver, leading to an acute inflammatory response. The inflammatory response is dominated by infiltration of polymorphonuclear leukocytes and macrophages to remove necrotic hepatocytes. The intense inflammatory response is thought to affect both the onset and duration of the liver regenerative [1].

D-galactosamine is known to cause acute liver damage in animal models. The mechanism of D-galactosamine hepatotoxicity is not understood completely, but D-galactosamine is believed to induce an intracellular deficiency of uridine metabolites, leading to acute liver failure [42].
An important distinction must be made regarding the origin of the cells that replace missing hepatocytes after PH and in the growth processes that follow parenchymal cell necrosis [43, 44]. After CCl₄-induced injury or PH, liver mass is replenished by replication of existing hepatocytes, without activation of the progenitor cell compartment [41]. In the regeneration of liver that follows loss of parenchymal cells induced by other toxins, such as D-galactosamine, replication and differentiation of intrahepatic progenitor cells occurs [45, 46]

### 2.2.2 Molecular basis of liver regeneration

A resected rodent liver undergoes compensatory growth in which the initial liver mass (and its functions) is restored within approximately 1 week after surgery. This compensatory growth after PH is due to the synchronized entry of approximately 95% of hepatocytes into the S phase of the cell cycle, followed by mitosis. In rat and mouse hepatocytes, a first peak of DNA synthesis occurs at around 24 h and 40 h post-PH, respectively. However, DNA replication in non-parenchymal cells is delayed. Subsequent levels of DNA synthesis in hepatocytes are lower, as complete restoration of the initial liver mass requires an average of ~1.6 cycles of replication per cell for all cells [47].

Within minutes of liver resection, hepatocytes undergo a coordinated cellular activation termed the ‘acute phase response after PH’. This highly regulated process is
mediated by multiple growth factors and cytokines that coordinate to transduce the response signal into kinase and transcription factor activation. As a result of the acute response, a surviving hepatocyte initiates the transcription of more than 100 early genes and accumulates triacylglycerol and cholesterol esters in intracellular lipid droplets. These droplets supply the energy and building materials needed to support rapid cell division and tissue regrowth and so are essential for normal liver regeneration [48, 49].

2.2.3 Cytokines

Cytokines are also important for the initiation of liver regeneration. These intercellular messengers, particularly the inflammatory cytokines TNFα and IL-6 produced by activated Kupffer cells, prime hepatocytes to enter the cell cycle and respond to the mitogenic effects of growth factors ( Appendix 1). Indeed, experiments with knockout mice suggest that TNFR1 and IL-6 may be essential for complete liver regeneration. After PH, levels of TNFα and IL-6 increase very rapidly. Both these cytokines are components of the TNFα→ TNFR1→ NF-κB→ IL-6→ STAT3 pathway that drives hepatocyte gene expression. Each component of this pathway appears sequentially in the regenerating rodent liver during the first 12 h after PH [50]. However, it is not known how PH triggers the initial increase in TNFα levels.
2.2.4 Growth factors

While the cytokine network acts at the priming phase of liver regeneration, growth factors drive cell cycle progression from the G1 phase through to the S phase. Hepatocyte growth factor (HGF), transforming growth factor-α (TGFα) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) are growth factors of major importance for liver regeneration [51, 52]. HGF is produced by mesenchymal cells in the liver and other tissues and may act on hepatocytes by a paracrine or endocrine mechanism. HGF binding to the c-met receptor leads to the activation of the MAP kinases ERK1 and 2 [53]. TGFα and HB-EGF are members of the epidermal growth factor (EGF) family of ligands, and both bind to EGF receptors that are known to activate a phosphorylation cascade leading to DNA replication [54].

2.2.5 Termination of liver regeneration

Though much less studied and even less understood, pathways leading to termination of liver regeneration should be equally as important as those initiating the process. All evidence from work with animals points out that liver mass adjustment is precisely determined and that some degree of apoptosis at the end of the regenerative process may play a role [55]. Livers from large animals (dogs) transplanted to smaller animals decrease in size, while the opposite is also true (as shown with livers from small to large dogs and from baboons to humans) [56]. These findings have raised the issue of an
existence of a “hepatostat” control system, which ensures that liver weight is as it should be (and not more) for the performance of its homeostatic functions [1]. TGFβ1 does not have mito-inhibitory effects on hepatocytes during regeneration but may do so at later stages. Deposition of new matrix (in part stimulated by members of the TGFβ family) may also stop hepatocyte proliferation. Studies with hepatocyte cultures have demonstrated that extracellular matrix is always inhibitory to proliferation of hepatocyte monolayers [57].

It is not clear whether the full development of new sinusoid capillaries, (leading to higher oxygen levels, suppression of HIF1α, suppression of VEGF, suppression of HGF production by endothelial cells, decreased hepatocyte proliferation, etc.) might also play a role. The mechanisms controlling the proportionality between liver weight and body weight are also poorly understood [40]. If a balance between mitogens (HGF, EGF) and mito-inhibitors (TGFβ1, activin) controls hepatocyte proliferation, then it is possible that a signaling pathway adjusting liver weight could operate if the mitogens bound to the hepatic biomatrix were derived from sources outside the liver (such as lung (for HGF) and duodenum (for EGF)). More mitogens per hepatocyte would be deposited to the matrix when a liver from a small animal were to be implanted into a large one, under that scenario, leading to imbalance between mitogens and mitoinhibitors and hepatocyte proliferation, new matrix synthesis, etc, until the balance was restored [40]. The above,
however, is purely speculative at this time and experimental work needs to be done to sort out several alternatives.

2.3 Biological relevance of membrane water transport

Water is the most abundant component of living organisms, and its movement into and out of the living cell is fundamental to life. Maintenance of fluid homeostasis, a housekeeping function of pivotal importance, critically depends on the efficient regulation of water supply and distribution. Fluid homeostasis is a highly intricate function that requires a coordinated and precise regulation of solute and water transport systems [58]. Living cells have developed complex physiological systems for sensing and responding to changes in fluid composition and volume [59].

2.4 Aquaporins

Water can cross the biological membranes by simple diffusion, through the lipid bilayer, or by facilitated diffusion, through specialized proteinaceous water channels [58, 60, 61]. Following the early definition of water channels as aquaporins (AQPs), it became evident that the water permeability of membranes containing AQPs is 5- to 100-times higher than that of membranes lacking such channels [62, 63]. Driven by osmotic forces, each single AQP pore can conduct billions of water molecules per second.
AQP1, the first water channel, was discovered in 1989-1991 in human red cells by Peter Agre, who was awarded the 2003 Nobel Prize in Chemistry in recognition of this seminal discovery. To date, 13 AQPs (i.e., AQP 0-12) have been found in mammals, with a large number (i.e., AQP0, AQP1, AQP4, AQP5, AQP8, AQP9, AQP11) being localized to the hepatobiliary system [16-19, 64-68].

### 2.4.1 Structure of AQPs

AQPs are small (25-34 kd), hydrophobic proteins consisting of 6 transmembrane domains with 5 connecting loops (A-E) and with cytoplasmically oriented amino and carboxy termini [69]. Connecting loops B (cytoplasmic) and E (extracellular) contain NPA boxes; each of them represents the signature asparagine-proline-alanine motif conserved among family members (Appendix 2. A-B). NPA boxes form a single aqueous pathway within a symmetrical structure that resembles an hourglass [69, 70]. The water channel itself consists of extracellular and cytoplasmic vestibules connected by an extended narrow pore or selectivity filter with a diameter of 2.8 Å, a width sufficient to allow passage of water molecules in single file in either direction [71, 72]. In the plasma membrane, AQPs are assembled as homotetramers that contain 4 single water channels (Appendix 2. C).

### 2.4.2 AQPs in bile formation
Given that one of the key functions of the hepatobiliary system is bile formation, and that bile consists of more than 98% water, the involvement of AQPs in bile formation seems likely [16-19, 64-68]. Whereas cholangiocytes account for only 3% to 5% of the liver cell population, they nevertheless play a significant role in bile formation (Appendix 3 & 4), producing as much as 40% of total bile volume. Cholangiocytes, analogous to hepatocytes, establish osmotic gradients by the secretion of ions, primarily Cl\(^-\) and HCO\(_3^-\), and by the absorption of solutes, primarily bile salts and glucose, processes that in turn may drive passive transcellular AQP-mediated movement of water[16, 64, 65]. AQPs are also involved in transport of small solutes (e.g., glycerol) across the hepatocyte basolateral plasma membrane [17, 18] and may transport water across the membranes of intracellular organelles [68].

### 2.4.3 AQPs in hepatocytes

Rat hepatocytes express at least 3 AQPs (AQP0, AQP8, and AQP9) in variable amounts (AQP8 >> AQP9 > AQP0) [14, 17, 23, 25, 73-76]. AQP0 and AQP8 are mainly localized intracellularly and to a lesser degree on the canalicular plasma membrane [23, 25, 75], whereas AQP9 is principally localized on the hepatocyte basolateral plasma membrane [23]. AQP0 and AQP8 are expressed to a larger degree in hepatocytes surrounding the central vein, whereas AQP9 is uniformly distributed within the hepatic lobe [23]. A
message for AQP11 was also found in liver [19]. Overall, accumulating data support important and diverse functions for AQPs in hepatocytes.

2.4.3.1 AQP0

AQP0 was originally localized to the lens fibers of the eye[6]. Rat liver is the only other known organ that expresses AQP0. In the lens, AQP0 constitutes 50% of the total membrane protein in the fiber cells, where it has a structural role as a cell-cell adhesion molecule in addition to functioning as a low-capacity water channel[6]. The physiological role of AQP0 in hepatocytes is unknown.

2.4.3.2 AQP8

AQP8 is expressed in many organs and tissues; in the rat and mouse, the liver is a major site of AQP8 expression (Appendix 3). The subcellular distribution of AQP8 in hepatocytes varies with species. In rat hepatocytes, AQP8 is primarily localized intracellularly, and to a lesser degree on the canalicular plasma membrane [23]. In mouse hepatocytes, different groups report different AQP8 localization, that is, widespread expression in intracellular membranes including smooth endoplasmic reticulum, subapical vesicles, and mitochondria [22] or, in contrast, strong localization on the plasma membrane with weak intracellular localization [77]. AQP8 is involved in water
permeability across the canalicular plasma membrane, supporting its potential importance in canalicular bile formation. Under basal (i.e., non-stimulated) conditions, AQP8 is largely localized in intracellular vesicles; on stimulation by choleretic agonists such as dibutyryl cyclic adenosine monophosphate (cAMP) or glucagon, AQP8 redistributes to the canalicular plasma membrane, thereby increasing the apical cell surface permeability and facilitating osmotic water transport [23, 25, 76, 78, 79]. At least 2 spliced isoforms of AQP8 are present in hepatocytes and that 1 of them might be involved in canalicular water permeability, whereas the other, which is expressed in mitochondria, might be involved in the homeostatic control of mitochondrial volume [64, 68]. It has been suggested that AQP8-mediated water transport into and out of mitochondria may be particularly important for control of mitochondrial volume that occurs during active oxidative phosphorylation and apoptotic signaling [68]. However, no experimental evidence demonstrates a specific role for AQP8 in mitochondria or in other intracellular organelles.

2.4.3.3 AQP9

AQP9, an aquaglyceroporin permeable to glycerol, urea, and certain small, uncharged solutes with minor water transport capacity, is a liver-specific glycerol channel expressed on the hepatocyte sinusoidal (basolateral) plasma membrane (Appendix 3) [14, 17, 23,
The exact role of AQP9 in hepatocytes is obscure. Potentially, AQP9 may mediate water transport between the sinusoidal blood and the hepatocyte interior, and thus, together with AQP8, contribute to canalicular bile secretion [23]. However, because AQP9 is also highly permeable to glycerol and urea, it may provide an entry route for glycerol and an exit route for the urea and a number of other solutes produced within hepatocytes [17, 18].

2.4.4 AQPs in cholangiocytes

Rat cholangiocytes express more AQPs than any cell type reported to date [seven AQPs from the known thirteen (AQP0, AQP1, AQP4, AQP5, AQP8, AQP9, and AQP11)] [16, 65]. Among these, two AQPs (AQP1 and AQP4) have been well characterized by molecular, biochemical, and functional studies, whereas 5 others (AQP0, AQP5, AQP8, AQP9, and AQP11) have been detected only at a transcript level.

AQP1 is present in both cholangiocyte apical and basolateral plasma membrane domains and in an intracellular vesicle compartment (Appendix 4); however, the subcellular location of AQP1 varies depending on physiological conditions [80, 81].

AQP4, in contrast, is constitutively expressed exclusively on the cholangiocyte basolateral plasmamembrane domain [82]. These AQPs account for the water permeability of both the apical and basolateral cholangiocyte plasma membrane domains,
AQP1 facilitating mainly the apical transport of water, and AQP4 modulating its basolateral movement [80-82].

### 2.4.5 AQPs in the hepatic blood vessels

AQP1 is highly expressed in the peribiliary vascular plexus, a mesh-like arrangement of blood vessels that surround the intrahepatic bile ducts [6, 16], suggesting a potential functional role of this AQP in facilitating water transport from plasma to bile.

### 2.5 Thalidomide

In the 1950s, thalidomide came out as a sedative and anti-nausea medicine, but eventually it was withdrawn from the market in 1961 as it was found to be a potent teratogen to a human fetus. Prenatal use of thalidomide caused severe developmental defects such as amelia (lack of limb) and phocomelia (seal limbs) [26]. Nonetheless, studies on thalidomide continued, and in 1965, Jacob Sheshkin, a dermatologist from Israel, found that administration of this drug in a patient with erythema nodosum leprosum (ENL) improved the skin lesions [83]. When reports from different regions confirmed these results, thalidomide reemerged as an effective treatment for ENL. In 1998, thalidomide was approved by the U.S. Food and Drug Administration (FDA) for the acute management of and as maintenance therapy for cutaneous manifestations of ENL [84].
Thalidomide has now been used in the investigational treatment of a myriad of dermatologic conditions including aphthous stomatitis, Behcet's syndrome, lupus erythematosus, prurigo nodularis, as well as novel uses in the treatment of Kaposi’s sarcoma, pyoderma gangrenosum, and lichen planus [85]. In addition to dermatologic diseases, multiple malignant tumors including multiple myeloma, hepatocellular carcinoma [35], melanoma [86], and prostate cancer [87] have been treated with thalidomide. A possible mechanism of thalidomide to induce antitumor activity was suggested through antiangiogenic action by inhibition of cytokine-induced nuclear factor-κB (NF-κB), vascular endothelial growth factor (VEGF), prostagrandin synthesis and tumor necrosis factor-α (TNF-α) [88-91] Richardson et al. [92] described the pathways of thalidomide activity against multiple myeloma (MM) (Appendix 5). However, the precise anti-tumor mechanism of thalidomide remains to be clarified.
Part (I)

Expression Of Aquaporins In Liver Regeneration
Chapter 3  Material And Methods

3.1 Animals

The protocols in this study were submitted and approved by the E-Da Hospital (Kaohsiung, Taiwan, ROC) Institutional Animal Care and Use Committee. All animal procedures were in compliance with our institutional guidelines. Male Sprague-Dawley rats initially weighing 250-300 g were acclimated to a 12-h day-night cycle and given free access to standard rodent chow and water. Rats were deprived of food for 24 h before hepatic surgery but were permitted free access to water.

3.2 Experimental design

Rats (n = 5 for each time point) were then sacrificed at 1, 6, 24, 48, 72, 96, 144, and 192 h after 70% partial hepatectomy and the regenerated livers were harvested. The resected quiescent liver was used as 0 h control. For Western blotting and RT-PCR assays, liver tissue harvested was rinsed thoroughly in cold PBS and snap-frozen in liquid nitrogen, and the samples were stored at –80C until use. For tissue staining, the liver tissue was fixed in 4% neutral buffered formalin, embedded in paraffin, and sectioned at 5 mm/slide.

3.3 Surgical procedure: 70% partial hepatectomy

The animals were anesthetized with Zoletil 50 (Virbac, Brazil; 50 mg/kg, IP) and
subjected to 70% partial hepatectomy according to the method of Higgins and Anderson [2]. Briefly, a midline incision was made in the rat’s upper abdomen and the median and the left lateral lobes were ligated and removed without injuring the remaining liver tissue, resulting in about 70% PH, as showed in Figure 1.

3.4 Hepatic regeneration rate

The rate of liver regeneration was evaluated using the formula of Kwon et al. [93]:

Hepatic regeneration rate (%) = D/E x 100, where D is the weight of the liver per 100 g of body weight at death and E = R/0.7. E is the estimated liver weight per 100 g body weight before hepatectomy, which was calculated from the weight of resected liver (R).

3.5 Reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. Total RNA (5 μg) was reverse-transcribed with random primers. The cDNA was then amplified by using the polymerase chain reaction with primers specific against various AQPs (Table 1). The PCR products were electrophoresed in 1% agarose gels and the bands were visualized by ethidium bromide staining. Densitometric analysis was performed and corrected for loading using β-actin gene.
3.6 Western blotting

Liver tissue was homogenized in ice-cold lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride] containing protease inhibitor cocktail. The homogenate was centrifuged and the supernatant collected. Protein concentrations of supernatants were determined. Samples of supernatants containing 100 μg protein were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred to Hybond-Polyvinylidene difluoride membranes by electroelution. After 3 h of blocking with Trisbuffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membranes were incubated overnight with antibodies to proliferating cell nuclear antigen (PCNA, dilution 1:1000; Epitomics, CA, USA), AQP0, 1, 4, 5, 8, 9, 11 (2 mg/ml for each AQP; Alpha Diagnostic, TX, USA), or β-actin (dilution 1:100; Sigma-Aldrich, MO, USA) diluted in the blocking solution. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, the signals were detected by enhanced chemiluminescence detection kit (Amersham, NJ, USA). The chemiluminescent signal was captured by a UVP BioSpectrum500 imaging system (UVP, CA, USA).

3.7 Immunohistochemistry

Liver sections were de-paraffinized, rehydrated, and placed in citrate buffer (10 mM,
pH 6.0) and microwaved twice for 7 minutes to improve staining by antigen unmasking. After washing and quenching of endogenous peroxidase with 3% hydrogen peroxide for 15 minutes, sections were blocked and incubated overnight at 4°C with antibodies to AQP0, 1, 4, 5, 8, 9, 11 (10 μg/ml for each AQP; Alpha Diagnostic), PCNA (dilution 1:500; Epitomics), or cluster of differentiation 68 (CD68, dilution 1:100; Abbiotec, CA, USA), washed in PBS and incubated with secondary antibody (biotinylated anti-rabbit IgG, 30 minutes at 25°C) followed by HRP-conjugated streptavidin. Negative controls were performed by omitting the primary antibody or pre-incubating the antibody with peptide used as the immunogen. The reactions were visualized by incubation with a DakoCytomation 3,3'-diaminobenzidine chromogen solution (Dako, CA, USA). The sections were then mounted with glass coverslips.

3.8 Statistical analysis

All data are expressed as means ± SEM. Statistical evaluations were made by Student’s t-test by Sigma-Stat Software (Jandel Scientific, CA, USA). P < 0.05 was considered significant.
Chapter 4  Results

All the experimental rats survived the two-thirds partial hepatectomy. Detectable liver mass regeneration was initially observed at 24 h after PH and increased progressively to the maximum at 96 h (Figure 2A). Correspondingly, significant increase in DNA synthesis, as indicated by strong PCNA protein expression, was noted immediately after liver resection and reached the plateau during 48-72 h followed by an abrupt decrease after 72 h (Figure 2B, Figure 3).

4.1 AQP0

There was no significant variation, compared with the basal condition (0 h), in the expression of AQP0 in both mRNA and protein levels throughout the entire study period (Figure 4A). Results of immunohistochemistry using anti-AQP0 antibody showed intense intracellular staining, predominantly in pericentral hepatocytes (Figure 4A).

4.2 AQP1

No significant difference in the expression level of AQP1 mRNA or protein among different time points was observed (Figure 4B). Pronounced anti-AQP1 immunolabeling was detected on the surface of central vein and sinusoid, presumably reacting with the endothelial cells lining the central vein and sinusoid. Hepatocytes were negative for
4.3 AQP4, 5

The expression level of AQP5 mRNA or protein at each studied time point following PH was statistically equal to that at the basal state (Figure 4C). Similar observations were also made for the expression of AQP4. By immunohistochemistry, both AQP4 (Figure 6) and AQP5 (Figure 7) were expressed similarly before and after PH, with immunoreactivity mainly presented in bile ductules of the portal triad. Neither AQP4 nor AQP5 reactivity on the hepatocytes was detected.

4.4 AQP8

After PH, the expression level of AQP8 mRNA increased progressively from 24 h and reached the peak at 144 h (Figure 8A); while a significant increase in protein level was detected at 48–96 h and followed by a decrease (Figure 8B). In addition to 28 kDa, AQP8 also showed a band approximately at 35 kDa on the immunoblot which supposedly is the glycosylated form [25]. During the quiescent state and the early hours following PH, staining for AQP8 was mainly intracellular and was stronger in hepatocytes surrounding the central vein (Figure 8C, Figure 9). This is consistent with previous reports [23, 25]. Pre-absorption of the antibody with the antigen peptides abolished all staining (Figure 8C,
pronounced AQP8 labeling was detected in cells within the sinusoid and reached the peak at 48–72 h and decreased thereafter. These spindleshaped cells were further identified to be Kupffer cells by positive immunoreactivity for CD68, a macrophage marker (Figure 8C, 96 h, inset).

**4.5 AQP9**

The expression level of AQP9 mRNA during the first 6 h following PH was not different from the basal level (Figure 10A). At 24 h and the subsequent time points, significantly increased expression levels were observed. As to the protein expression profile, a time-dependent increase was detected and reached the maximum at 48-72 h (Figure 10B). Similar to AQP8, a protein band of 35 kDa was also positively stained with anti-AQP9 antibody (data not shown), in addition to 28 kDa. By immunohistochemistry, AQP9 was localized exclusively in the basolateral (sinusoidal) membrane of hepatocytes (Figure 10C, Figure 11).

**4.6 AQP11**

RT-PCR and Western blot analysis revealed that significant changes in the expression levels of AQP11 occurred only at early time points after PH (Figure 12A and 12B).
Nevertheless, results of immunohistochemical staining exhibited altered localizations of AQP11 protein at different time points. During the rest state and early hours of liver regeneration, AQP11 protein was mostly confined to the cytosol (Figure 12C). At 24 h and 48 h, intense stain was observed mainly in the nucleus of hepatocytes and then subsided. Pre-absorption of the antibody with the antigen peptides abolished all staining (Figure 12C, Neg).
Chapter 5  Discussion

This study demonstrated the temporal mRNA and protein expression profiles of AQPs (AQP0, 1, 4, 5, 8, 9, and 11) in rat liver during the process of partial hepatectomy-induced liver regeneration. As anticipated, AQP0, 8, 9, and 11 were detected mainly in hepatocytes; unexpectedly, Kupffer cells were observed to express AQP8 during a specific period of time in the regenerative process. To our knowledge, this is the first report documenting the expression of AQP8 in Kupffer cells.

The maximal expression of PCNA, a marker for DNA synthesis, was observed to occur at 48 h post-hepatectomy in the present study. In contrast, the peak for hepatocyte proliferation during liver regeneration in the rat as determined by Ki-67 or 5-Bromo deoxyuridine (5-BrdU) labeling is at 24 h [1]. The discrepancy might lie in the methodologies employed in different studies. Ki-67 staining is used to determine the growth of a specific cell population such as hepatocytes in the liver. In contrast, the approach we used is detection of the overall PCNA expression in liver homogenate which is derived from various types of cells with different proliferation rates.

AQP0 had previously been thought to be expressed exclusively in the lens fiber cells of the eye [6]. In addition to water transport function, recent studies strongly suggest its structural role as a cell-to-cell adhesion protein [94, 95]. Although murine liver is the only known organ besides the lens that expresses AQP0, the physiological role of AQP0 in
hepatocytes remains to be identified. In this current study, no significant temporal variations in cellular localization and expression of AQP0 were observed, implying its minor role in liver regeneration.

AQP8 is the most abundant aquaporin in rat hepatocytes [22] and no previous reports have demonstrated its expression in Kupffer cells. Between 48 h and 72 h after PH, pronounced expression of AQP8 was observed in Kupffer cells, coinciding with the peak for Kupffer cell proliferation after PH which has been proposed to be at 48 h or 72 h according to different studies [38, 96, 97]. Although further studies are required, this finding raises many possibilities as to the functional roles of AQP8 in Kupffer cells. For example, it is reasonable to speculate that AQP8 might be important for the repopulation of Kupffer cells following PH. If the hypothesis is proven to be true, the importance of AQP8 in liver regeneration cannot be understated, since it has been well-acknowledged that Kupffer cells play pivotal roles in liver regeneration mainly by releasing proliferative cytokines, interleukin-6 (IL-6), and tumor necrosis factor α (TNF α) [98].

AQP9 is an aquaglyceroporin and has been proposed to be the entry pathway for plasma glycerol deriving from adipose lipolysis, a major substrate for hepatic gluconeogenesis [17, 18, 24]. Following two-thirds PH, the increased gluconeogenesis is one of the adaptive responses in the remnant liver to provide sufficient glucose for the maintenance of metabolic homeostasis in the whole organism [99-102]. Several
immediate early genes encoding enzymes and proteins that are required for regulation of gluconeogenic response following hepatectomy have been documented. For example, the mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK), the key gluconeogenic enzyme, increased rapidly and peaked at 6 and 72 h after liver resection and remained high throughout the entire study period (168 h) [100]. This expression profile generally coincided with that of AQP9 observed in this study. We thus proposed that the enhanced expression of AQP9 might be relevant for PH-induced increase in gluconeogenesis.

The expression of AQP11 mRNA was reported to be highest in the testis, and moderate in the kidney and liver [19, 103, 104]. Immunohistochemical studies revealed that AQP11 was expressed intracellularly in renal proximal tubules [19] and in neurons [104]. Similarly, intracellular localization of AQP11 was also observed in this study. To the best of our knowledge, this is the first report demonstrating the expression of AQP11 in hepatocytes. The transient expression of AQP11 in the cell nucleus, compared to other AQPs, is not a common finding; however, some other AQPs have also been detected in the nucleus. For example, AQP9 protein was present and localized in the nucleus, cytoplasm, and cell membrane of the human granulosa cells [105]. In addition, AQP5 was immunolocalized in the nucleus of ovarian cancer SKOV3 cells [106]. The roles of AQPs in the cell nucleus are currently uncharacterized although a regulatory function of cell growth has been speculated [106].
AQP11, despite its function as a water channel being uncertain, has been proven to be important for the development of the proximal tubule as its absence led to fatal polycystic kidneys in neonatal mice [19]. In our study, the physiological significance for the transient redistribution of AQP11 from the cytoplasm to nucleus of hepatocytes needs further investigation.
Chapter 6 Conclusion

This first step study in the understanding of the role of AQPs in liver regeneration demonstrates that two-thirds partial hepatectomy induces differential mRNA and protein expression profiles of various AQPs in the liver. Although data presented are phenomenal observations, they implicate the possibility that aquaporins are involved in liver regeneration.
Part (II)
The Effect Of Thalidomide On Liver Regeneration
Chapter 7  Materials and Methods

7.1 Animals

Male Sprague-Dawley rats initially weighing 250-300 g were used. All animals were housed in a temperature and humidity controlled environment, and they received humane care with free access to standard chow and water throughout the study period. The protocols in this study were submitted to and approved by the E-Da Hospital Institutional Animal Care and Use Committee (IACUC-97007). All animal procedures were in compliance with our institutional guidelines.

7.2 Experimental design

A total of 50 rats were subjected to 70% PH and equally divided into two groups: the control and thalidomide groups. Two days prior to PH, rats in the thalidomide group were daily administered thalidomide (100 mg/kg, TTY BioPharm, Taipei, Taiwan) in olive oil by intragastric administration. Control rats received olive oil only. Animals in the two groups were equally divided into 5 subgroups according to observation intervals, which were 0, 48, 96, 144 and 192 h after PH as showed in the time schedule (Figure 13).

7.3 Surgical procedure – 70% partial hepatectomy

Liver regeneration was induced by 70% PH (Figure 1) as described by Higgins and
Animals were anesthetized with ketamine (100 mg/kg, intraperitoneal injection). After a midline laparotomy, the liver was exposed and the left and medial lobes were ligated (4-0 silk) and resected. Glucose solution (5 ml; 5%; 37°C) was injected into the abdominal cavity and the abdominal wound was closed in two layers with 4-0 silk. The resected liver was termed ‘quiescent liver’ in this study. The resected quiescent liver was used as 0 h control. For Western blotting assays, liver tissue harvested was rinsed thoroughly in cold PBS and snap-frozen in liquid nitrogen, and the samples were stored at –80°C until use. For tissue staining, the liver tissue was fixed in 4% neutral buffered formalin, embedded in paraffin, and sectioned at 5 mm/slide.

7.4 Liver regeneration rate

The rate of liver regeneration was evaluated using the formula of Kwon et al. [93]: Liver regeneration rate (%) = D/E x 100, where D is the weight of the liver per 100 g of body weight at death and E is the estimated liver weight per 100 g body weight prior to hepatectomy, which was calculated from the weight of resected liver (R); E = R/0.7.

7.5 Laser Doppler flowmetry analysis of microcirculation

Laser-Doppler flowmetry analysis has been widely used and is considered as a suitable
The technique for the analysis of hepatic microcirculation [107-110]. The principle of laser Doppler flowmetry combines laser technology with the Doppler effect caused by the movement of red blood cells in the microcirculation to estimate red blood cell flux [107]. The strength of this technique is in observing changes in flow, either over time or over an area of the exposed tissue. Before 70% PH and animal sacrifice, the surface of the liver and kidney was scanned by Moor LDI 2 imager (Moor Instruments Ltd., Devon, UK) to assess the perfusion hemodynamics. The Doppler shift is proportional to a blood flow-related variable and is expressed in arbitrary perfusion units (PU). Microcirculation density was quantified using software provided by the manufacturer (Moor LDI system software V5).

7.6 Western blot analysis for VEGF, TNF-α and IL-6

Livers were homogenized by Ultrasonic cell disruptor (Microson™ XL-2000; Misonix, Farmingdale, NY, USA) in tissue protein extraction buffer (T-PER®, Pierce, Rockford, IL, USA) containing protease inhibitors (Protease Inhibitor Cocktail 100X, Pierce) and the homogenate was centrifuged to obtain the supernatant. Protein concentrations were determined and the samples were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (ECL, Amersham, Buckinghamshire, UK). After blocking and washing, blots were incubated overnight at
4°C with rabbit affinity-purified antibodies against proliferating cell nuclear antigen (PCNA; dilution, 1:1,000; Epitomics, Burlingame, CA, USA), VEGF (dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), IL-6 (dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), TNF-α (dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or β-actin (dilution, 1:100; Sigma, St. Louis, MO, USA). The blots were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1h. Finally, the signals were detected using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA). The chemiluminescent signal was captured by a UVP BioSpectrum500 imaging system (UVP, Upland, CA, USA).

7.7 Immunohistochemistry for VEGF

Fresh liver samples were immediately immersed into 10% neutral formalin. Paraffin-embedded liver samples were cut into 5-μm sections. Liver sections were de-paraffinized, rehydrated and placed in citrate buffer (10 mM, pH 6.0) and microwaved twice for 7 min to improve staining by antigen unmasking. After dewaxing and rehydration, liver sections were placed in citrate buffer (10 mM, pH 6.0) and microwaved twice for 7 min to improve staining by antigen unmasking. The activity of endogenous peroxidase was removed by incubation with 3% H2O2 for 15 min at room temperature.
VEGF was identified by rabbit anti-rat VEGF polyclonal antibody (dilution, 1:500; SC-152, Santa Cruz Biotechnology, Inc.) followed by HRP-conjugated goat anti-rabbit secondary antibody (Dako™ REAL™ EnVision Detection System, K5007; Carpinteria, CA, USA). Positive signals were shown by 3,3’-diaminobenzidine (DAB) response. Sections were then counterstained with hematoxylin.

7.8 Statistical analysis

Student's t-test was used to compare sample means with paired or unpaired controls, as appropriate. Results are expressed as means ± SEM. $p < 0.05$ was considered to indicate a statistically significant result.
Chapter 8  Results

8.1 Liver regeneration rate

All the experimental rats survived the 70% liver resection and thalidomide treatment. Following hepatectomy, the restituted liver mass in the two groups markedly increased with a peak at 96h and then declined (Figure 14A). Unexpectedly, the calculated regeneration rate in the thalidomide group at the first two post-hepatectomy time-points (48 and 96h) was significantly higher than that in the control rats, while no difference
8.2 Expression of PCNA

PCNA is a protein marker for DNA synthesis and is commonly used as an indicator for cell proliferation. At resting state, weak expression of PCNA was detected in both groups. Following hepatectomy, the expression level of PCNA increased significantly and reached a peak at 48 h in the control group, 96 h in the thalidomide group and declined abruptly thereafter (Figure 14B).

8.3 Hepatic microcirculation

To quantify the circulatory effect of thalidomide on liver regeneration, hepatic blood flow was assessed by Laser Doppler flowmetry (Figure 15) before PH (0 h, quiescent liver) and sacrifice (regenerating liver). Prior to PH, the hepatic microcirculation in rats treated with thalidomide for 2 days was comparatively less than that in their corresponding controls (Figure 16). However, no significant difference in blood flow in the regenerating liver between the control and thalidomide groups was detected at any studied time-point. In the thalidomide group, however, liver regeneration induced by PH increased the microcirculation (Figure 16).

8.4 Western blot analysis

8.4.1 Western blot analysis of VEGF
Prior to liver resection (0 h), a low expression level of VEGF was detected in control rats and rats treated with thalidomide for 48 h (Figure 17). Hepatectomy induced marked expression of VEGF, which peaked at 48-96 h and declined rapidly in the two groups. No significant difference in the expression level between the two groups at any studied time-point was found.

8.4.2 Western blot analysis of IL-6

In the quiescent or regenerative livers, there was no significant difference between the thalidomide group and control group ($p = 0.3$ and $0.12$; Figure 18). However, liver regeneration markedly increased IL-6 expression between quiescent liver and regenerative liver in the control and thalidomide groups ($p = 0.001$ and $0.001$) (Figure 18).

8.4.3 Western blot analysis of TNF-α

In the quiescent or regenerative livers, there was no significant difference of TNF-α expression between the thalidomide group and control group ($p = 0.49$, and $0.07$; Figure 19). In the control group, there was no significant difference between the quiescent and regenerative livers ($p = 0.40$). However, in the thalidomide treated rats, the level of TNF-α markedly increased in the regenerated liver at 96 h after PH ($p = 0.02$).
8.5 Immunohistochemical staining of VEGF

Positive VEGF immunoreactivity was mainly localized in the cytoplasm of hepatocytes. Prior to PH, faint staining was observed in the two groups. At 48 h after PH, VEGF was mainly expressed in the periportal area in both groups (data not shown). At 96 h, the positive immunoreactivity was limited to the pericentral area in the control group (Figure 20A-C), while observed in pericentral and periportal hepatocytes in the thalidomide group (Figure 20D-F). At the subsequent time-points, markedly weaker expression of VEGF was observed and mostly located in the pericentral area in the two groups (data not shown).
Chapter 9  Discussion

The present study demonstrated that thalidomide delayed the PH-induced hepatic cell proliferation but did not impair the overall liver regeneration. In addition, the PH-induced upregulation of VEGF was not inhibited by thalidomide. The maximal expression of PCNA, a marker for DNA synthesis, was observed to occur at 48 h post-hepatectomy in the control group of this study. However, the peak for hepatocyte proliferation during liver regeneration in the rat as determined by Ki-67 or 5-bromodeoxyuridine (5-BrdU) labeling is at 24 h [38]. There are at least two possible explanations for this discrepancy. One is that 24 h was not one of the selected time-points in our study. The other is the methodologies used in different studies. The approach we employed in this study was detection of the overall PCNA expression in liver homogenate, which is derived from various types of cells with different proliferation rates. By contrast, Ki-67 staining is used to determine the growth of a specific cell population, such as hepatocytes in the liver. In the present study, the maximal PCNA expression detected in the thalidomide group was 96 h after liver resection, a significant delay as compared with control rats; nevertheless, the overall restoration of liver mass was not affected.

The significance for the observed transient greater liver regeneration rate in the thalidomide group requires further investigation. However, we speculated that it may be due to the non-specific effect of thalidomide, such as increasing the water content in liver...
tissue based on the transient watery appearance of thalidomide-treated liver (our unpublished observation). If so, the liver regeneration rate may thus be overestimated.

Laser Doppler flowmetry is a technique for the non-invasive blood flow monitoring and is considered to be a suitable technique for the analysis of hepatic microcirculation [107, 108, 111]. In the present study, thalidomide impaired hepatic microcirculation in the quiescent liver, but not regenerating liver. The reduced blood flow in the thalidomide-treated quiescent liver may be associated with the inhibitory effect of thalidomide on the release of tumor necrosis factor (TNF)-α and nitric oxide, two potent vasodilators, as suggested by a previous study in which thalidomide ameliorated the portal pressure and hyperdynamic circulation in partially portal vein-ligated rats by reducing TNF-α and nitric oxide production [112]. After PH, this inhibitory effect of thalidomide was eliminated by rapid release of TNF-α, resulting in similar hepatic microcirculation in the two groups.

VEGF is an important factor in the early phase of liver regeneration [113]. In this study, treatment with thalidomide for 48 h before PH did not affect the expression of VEGF, as evidenced by the insignificant difference in the expression level between control and thalidomide groups at 0 h. Following PH, VEGF was markedly upregulated and the expression profile during the regenerative process was similar in the two groups.

These observations suggest that thalidomide exerts no significant effect on the expression
of VEGF either in quiescent liver or in PH-induced regenerating liver. The immunohistochemical result showing that VEGF was predominantly expressed in the periportal hepatocytes at 48 h after PH is consistent with a previous study [114]. At 96 h, the positive staining was observed only in the pericentral area in the control group, while observed in the periportal and pericentral areas in the thalidomide group. This time-dependent alteration in the main expression site in the liver suggests a waved pattern for the expression of VEGF, which advances from the periportal to pericentral area. Although the significance for the observed difference in the expression areas at 96 h between groups requires further studies for clarification, we hypothesize that it may reflect the slower angiogenesis in thalidomide-treated rats.

The role of thalidomide on TNF-α levels in vivo has been investigated in numerous animal models and patients. A number of studies showed the effect of thalidomide on decreasing serum TNF-α levels [115-120]. ENL patients treated with thalidomide not only observed improvement in the clinical manifestations of ENL[115] but also decreased serum TNF-α levels from pretreatment levels [116]. In contrast, some other clinical studies have contradicted the effects of thalidomide on TNF-α in vivo. Despite achieving positive therapeutic results, plasma TNF-α levels were increased with thalidomide in sarcoidosis [121], ENL[122], and HIV oral aphthous ulcers[123]. In this study, the expression of TNF-α in regenerative liver after PH increased by thalidomide (Figure 19).
Therefore, the in vivo effect of thalidomide on TNF-α levels is still controversial.

The role of thalidomide on IL-6, a macrophage-derived cytokine, has been examined in mice and humans. Thalidomide-treated mice infected with tuberculosis had reduced plasma level of IL-6 [124]. In vitro studies showed that thalidomide decreased IL-6 levels from human peripheral blood mononuclear cells (phytohaemagglutinin-stimulated) [125] and human lung fibroblasts (spontaneous production) [126], but had no effect on IL-6 release from human alveolar macrophages obtained from patients with interstitial lung disease (lipopolysaccharide-stimulated) [127]. In this study, PH-induced hepatic regeneration caused the increased IL-6 expression in the liver, consistent with previous study [128]. Thalidomide, however, has no inhibitive effect on IL-6 level both in the quiescent and regenerative livers (Figure 18).
Chapter 10 Conclusion

Thalidomide has the potential inhibitive effect on cytokines including TNF-α and IL-6. Thalidomide is also an antiangiogenic agent with anti-VEGF effect. Liver regeneration is a complex signal interaction; nevertheless, there is no effect of thalidomide on the expressions of TNF-α, IL-6 and VEGF in this study. Clinically, surgeons are concerned for the postoperative medication which might impair the post-hepatectomy liver regeneration. In conclusion, our results demonstrate that thalidomide exerts no significant inhibitive effect on the expression of TNF-α, IL-6 and VEGF and does not impair the overall PH-induced restoration of liver mass, providing supportive evidence that thalidomide may be used as a postoperative adjuvant treatment modality for liver cancers.
Figure 1. Surgical procedure of 70% hepatectomy. A. The rat was anesthetized with ketamine (100 mg / kg, intraperitoneal injection ). Four limbs were fixed on the table. Abdominal skin was shaved and disinfected with Tincture. B. Mid-line laparotomy was done to expose liver. C. Liver was mobilized. D. Medial and left lobes were ligated with 4-0 silk and then divided. E. The right and omental lobes showed hyperemia without ischemic change. F. Abdominal bleeding was checked. Wound was
closed with 4-0 silk.  

G. Resected medial and left lobes (70% of the total liver)
Table 1. Primers for AQP expression.

<table>
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<th>mRNA</th>
<th>Primer sequence (5’-3’)</th>
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<td>AQP0</td>
<td>F: ACCGGCTCAAGAGTTTTCTGGA</td>
<td>189</td>
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<tr>
<td></td>
<td>R: TCCCGAGATTCCTTTTCATT</td>
<td></td>
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<tr>
<td>AQP1</td>
<td>F: CTTGCTGCTGCTGCTGCTGCTG</td>
<td>344</td>
</tr>
<tr>
<td></td>
<td>R: AATTTGGCCCAAGTTCTG</td>
<td></td>
</tr>
<tr>
<td>AQP4</td>
<td>F: TTTGACATCATATAGAGGCGC</td>
<td>212</td>
</tr>
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<td>R: GTCAATGTCATCAATGA</td>
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<tr>
<td>AQP5</td>
<td>F: GCCAGATCTAATCCAGCGT</td>
<td>373</td>
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<td></td>
<td>R: AAGGATGCGGCTGCTGAT</td>
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<tr>
<td>AQP8</td>
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<td>495</td>
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<tr>
<td></td>
<td>R: GGAGGCGCTCATGCGCGAT</td>
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Abbreviations: AQP = aquaporin; F = forward; R = reverse
Figure 2. Liver regeneration following partial hepatectomy. (A) Hepatic regeneration rate (%) = D/E x 100, where E = R/0.7 and D is the weight of the liver per 100 g of body weight when sacrificed. E represents the estimated liver weight per 100 g body weight before hepatectomy, which was calculated from resectional liver weight (R). Results shown are the means ± SEM of data from five different rats/group per time point. *p < 0.05 versus 0 h control. (B) Densitometric analysis of hepatic PCNA protein expression. The expression of PCNA was normalized against that of β-actin. The expression at 0h is arbitrarily normalized to 1.
Figure 3. Immunohistochemistry for hepatic PCNA after 70% hepatectomy (0-192h). Significant increase of PCNA protein expression was noted immediately after liver resection and reached the plateau during 48-72 h followed by an abrupt decrease after 72 h.
Figure 4. Expression of AQP0, 1, and 5 in rat liver at different time points following partial hepatectomy. Total RNA was isolated from liver tissues and the RT-PCR products were separated by 1% agarose gel electrophoresis. The β-actin transcript was used as a reference template. Proteins extracted from liver tissues were subjected to Western blot analysis and the expression of AQP was normalized against that of β-actin. (A) AQP0. Top, Representative gel for RT-PCR products. Middle, Representative immunoblot for AQP0 protein. Bottom, Immunohistochemistry for AQP0 in 5 μM paraffin liver section. Arrows indicate the pericentral hepatocytes. (B) AQP1. Top, Representative gel for RT-PCR products. Middle, Representative immunoblot for AQP1 protein. Bottom, Immunohistochemistry for AQP1. Arrow indicates the positive immunostaining on the surface of sinusoid. (C) AQP5. Top, Representative gel for RT-PCR products. Middle, Representative immunoblot for AQP5 protein. Bottom, Immunohistochemistry for AQP5. Arrow indicates the bile ductule. Scale bars: 50 μm.
Figure 5. Immunohistochemistry for hepatic AQP1 (0-192h) after hepatectomy, 200 x. Pronounced anti-AQP1 immunolabeling was detected on the surface of central vein and sinusoid, presumably reacting with the endothelial cells lining the central vein and sinusoid. Hepatocytes were negative for anti-AQP1 reactivity.
Figure 6. Immunohistochemistry for hepatic AQP4 (0-192h) after hepatectomy, 200 x. AQP4 was expressed after PH, with immunoreactivity mainly presented in bile ductules of the portal triad. AQP4 reactivity on the hepatocytes was not detected.
<table>
<thead>
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<td>0</td>
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Expression
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**Figure 7.** Immunohistochemistry for hepatic AQP5 (0-192h) after hepatectomy, 200 x. AQP5 was expressed after PH, with immunoreactivity mainly presented in bile ductules of the portal triad. AQP5 reactivity on the hepatocytes was not detected.
Figure 8. Expression of AQP8 in rat liver at different time points following partial hepatectomy. (A) RT-PCR analysis. The expression of AQP8 was normalized against that of the housekeeper gene, β-actin. **Top**, Representative gel. **Bottom**, Densitometric analysis of AQP8 mRNA expression. The expression at 0 h is arbitrarily normalized to 1. Results shown are the means ± SEM of data from five different rats/group per time point. *p < 0.05 versus 0 h control. (B) Western blot analysis. The expression of AQP8 (non-glycosylated form) was normalized against that of β-actin. **Top**, Representative immunoblot. **Bottom**, Densitometric analysis of AQP8 protein expression. The expression at 0 h is arbitrarily normalized to 1. (C) Immunolocalization of AQP8 protein in liver section. Inset in 0 h shows the result after the antibody to AQP8 was pre-incubated with the antigen peptide. Arrows in the inset of 24 h indicate positive CD68 (a macrophage marker) staining in the sinusoid. Scale bars: 50 μm.
Figure 9. Immunohistochemistry for hepatic AQP8 (0-192h) after hepatectomy, 200 x. During the quiescent state and the early hours following PH, staining for AQP8 was mainly intracellular and was stronger in hepatocytes surrounding the central vein. Starting at 24 h, in addition to faint signals in pericentral hepatocytes, pronounced AQP8 labeling was detected in cells within the sinusoid and reached the peak at 48–72 h and decreased thereafter.
Figure 10. Expression of AQP9 in rat liver at different time points following partial hepatectomy. (A) RT-PCR analysis. The expression of AQP9 is normalized against that of the housekeeper gene, β-actin. **Top.** Representative gel. **Bottom.** Densitometric analysis of AQP9 mRNA expression. The expression at 0 h is arbitrarily normalized to 1. Results shown are the means ± SEM of data from five different rats/group per time point. *p < 0.05 versus 0 h control. (B) Western blot analysis. The expression of AQP9 was normalized against that of β-actin. **Top.** Representative immunoblot. **Bottom.** Densitometric analysis of hepatic AQP9 protein expression. The expression at 0 h is arbitrarily normalized to 1. (C) Immunolocalization of AQP9 protein in liver section. Scale bars: 50 μm.
Figure 11. Immunohistochemistry for hepatic AQP9 (0-192h) after hepatectomy, 200 x. AQP9 was localized exclusively in the basolateral (sinusoidal) membrane of hepatocytes.
Figure 12. Expression of AQP11 in rat liver at different time points following partial hepatectomy. (A) RT-PCR analysis. The expression of AQP11 is normalized against that of the housekeeper gene, b-actin. Top, Representative gel. Bottom, Densitometric analysis of AQP11 mRNA expression. The expression at 0 h is arbitrarily normalized to 1. Results shown are the means ± SEM of data from five different rats/group per time point. *p < 0.05 versus 0 h control. (B) Western blot analysis. The expression of AQP11 was normalized against that of b-actin. Top, Representative immunoblot. Bottom, Densitometric analysis of hepatic AQP11 protein expression. The expression at 0 h is arbitrarily normalized to 1. (C) Immunolocalization of AQP11. Pre-absorption of the antibody with the antigen peptides abolished all staining (Neg). Scale bars: 50 μm.
Figure 13. Time schedule for administration of thalidomide. Thalidomide was given intragastrically. The first dose was administrated 48h before heptectomy, and then daily with 100mg/kg.
Figure 14. Liver regeneration following partial hepatectomy. (A) Liver regeneration rate (%) = D/E x 100, where D is the weight of the liver per 100 g of body weight when sacrificed and E represents the estimated liver weight per 100 g body weight prior to hepatectomy, which was calculated from resected liver weight (R); E = R/0.7. Results shown are the means ± SEM of data from 5 different rats/group per time-point. *p < 0.05 versus control. (B) Densitometric analysis of hepatic PCNA protein expression. The expression of β-actin was used as loading control. PCNA, proliferating cell nuclear antigen.
Figure 15. The liver surface scan by Laser Doppler flowmetry

The liver surface scan after hepatectomy in the thalidomide group by laser Doppler flowmetry
Figure 16. Laser Doppler flowmetry of the hepatic microcirculation. Hepatic blood flow was measured before hepatectomy (0 h, quiescent liver) and at 96 h post-PH (regenerating liver). The liver microcirculation at 0 h in the thalidomide group was obtained from rats treated with thalidomide for 48 h before measurement. * $p < 0.05$ versus quiescent liver in control group; # $p < 0.05$ versus thalidomide-treated regenerating liver at 96 h. PH = partial hepatectomy.
Figure 17. Expression of VEGF in rat liver at different time-points following partial hepatectomy. The expression of VEGF was normalized against that of β-actin. (A) Representative immunoblot. (B) Densitometric analysis. Results shown are the mean ± SEM of data from 5 different rats/group per time-point. *p < 0.05 versus 0 h control. The expression at 0 h is arbitrarily normalized to 1. VEGF, vascular endothelial growth factor.
Figure 18. Expression of IL-6 in quiescent liver (0 h) and regenerative liver (96 h) as detected by Western blot analysis. In the quiescent or regenerative livers, there was no significant difference between the thalidomide group and control group ($p = 0.3$ and 0.12). However, liver regeneration markedly increased IL-6 expression between quiescent liver and regenerative liver in the control and thalidomide groups ($p = 0.001$ and 0.001).
Figure 19. Expression of TNF-α in quiescent liver (0 h) and regenerative liver (96 h) as detected by Western blot analysis. In the quiescent or regenerative livers, there was no significant difference of TNF-α expression between the thalidomide group and control group ($p = 0.49$, and $0.07$). In the control group, there was no significant difference between the quiescent and regenerative livers ($p = 0.40$). However, in the thalidomide treated rats, the level of TNF-α markedly increased in the regenerated liver at 96 h after PH ($p = 0.02$).
Figure 20. Immunohistochemistry for VEGF in regenerating liver at 96 h after partial hepatectomy. VEGF was stained with rabbit anti-rat VEGF antibody followed by HRP-conjugated goat anti-rabbit secondary antibody. Positive signals were shown by DAB response. Sections were then counterstained with hematoxylin. Control group (A) pericentral area (x40); (B) pericentral area (x400); (C) periportal area (x400). Thalidomide group (D) pericentral and periportal areas (x40); (E) pericentral area (x400); (F) periportal area (x400). Scale bars, 50 μm. VEGF, vascular endothelial growth factor; HRP, horseradish peroxidase; DAB, 3,3'-diaminobenzidine.
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Appendix 1. Signaling pathways in liver regeneration. After partial hepatectomy (PH) of normal mice, cytokines including tumor necrosis factor-α (TNFα) and interleukin (IL)-6 are released from activated Kupffer cells. These bind to their receptors on hepatocytes, leading to activation of transcription factors (STAT3, NF-κB, c-Fos and c-Jun in cytoplasm and nucleus) and to the expression of regeneration genes. (Adapted from Hata, S., et al. [129])
Appendix 2. Molecular organization of AQP monomers. (A-B) Topology of a single AQP monomer. Each AQP monomer consists of six transmembrane domains (numbered 1-6) connected by five loops (A-E). Connecting loops B (intracellular) and E (extracellular) contain NPA boxes that form a single aqueous pore by folding into membrane. The arrow shows that water can move through the channel in both directions. (C) Four AQP monomers assembled in the membrane into a tetramer are shown from above. The aqueous pore does not reside in the center of the tetramer. Instead, each tetramer contains four aqueous pores formed by connecting loops B and E of each monomer. Asterisks denote the location of the water pore in each AQP monomer. (Adapted from Masyuk, A.I., et al. [130].)
Appendix 3. The scenario of bile formation is depicted in hepatocytes, with respect to hepatobiliary transporters and solutes. For clarity reasons, transporters are reported in different parts of the panel, although they all operate within the same cell. Left hepatocyte: at the sinusoidal domain of the basolateral plasma membrane of hepatocytes, two transport systems operate: OATP (namely OATP1B1: SLCO1B1 and OATP1B3: SLCO1B3) for uptake of unconjugated BA, OA, OC and efflux of GSH and bicarbonate) and NTCP (SLC10A1) for sodium-dependent uptake of BA. NTCP function requires the Na⁺/K⁺-ATPase generating an inwardly directed Na⁺ gradient and a K⁺ channel (which generates in part the membrane potential). Right hepatocyte: at the domain of the basolateral membrane additional transporters include a Na⁺-bicarbonate cotransporter (symporter), a Na⁺-H⁺ exchanger; MRP3, MRP4, and OSTa/b all three contribute to BA, OA, and solute transport. At the canalicular membrane (apical plasma membrane) of hepatocytes, three ATP-binding cassette (ABC) transporters operate: ABCB4 (also named Multidrug-resistance-3 P-glycoprotein MDR3) for phospholipid (PL), ABCB11 (also named Bile salt export pump BSEP) for bile acid (BA), and ABCG5/G8 for cholesterol (Chol) secretion which concur to formation of micelles and vesicles in bile (see text for further details). The NPC1L1 protein is responsible for sterol import and is found in the liver in humans but is absent in rodents [131]. Drug metabolites are secreted by the MDR1. Sulphated (BA-S') or glucuronidated (BA-G') bile acids, and OA are secreted by the MRPs. Canalicular membrane-associated ATP-independent transport systems include the GSH transporter, the AE2 for secretion of bicarbonate, and a chloride channel (distinct from the cystic fibrosis transmembrane regulator protein, CFTR). Canalicular formation and secretion of water in bile are depicted in the right hepatocyte. Water flows basolaterally from the sinusoidal blood into the hepatocyte through AQP9. Under
choleretic stimuli, an increase of intracellular cAMP, subapical vesicles containing AQP8 are translocated by exocytosis to the canalicular membrane. Here, AQP8 facilitates the osmotically-driven efflux of water in the bile canaliculus. AQP8 is also present in mitochondria (inner membrane) and in the smooth endoplasmic reticulum, where it is suggested to play other functions unrelated to bile formation (not shown).

Abbreviations: AE2, chloride–bicarbonate anion exchanger isoform 2; APM, apical plasma membrane (canalicular membrane); AQP8, aquaporin-8; AQP9, aquaporin-9; BA, bile acids; BA-G, glucuronidated bile acids; BA-S, sulphated bile acids; BLPM, basolateral plasma membrane; Chol, cholesterol; GSH, glutathione; MDR1, Multidrug-resistance-1 P-glycoprotein; MRP2, Multidrug resistance-associated protein 2; MRP3, Multidrug resistance-associated protein 3; MRP4, Multidrug resistance-associated protein 4; NCTP, sodiumtaurocholate cotransporting polypeptide; NPC1L1, Niemann-Pick C1 Like 1 protein; OA, organic anion; OATP, sodium-independent organic-anion transporting polypeptide; OC, organic cation; OS, organic solutes; OSTa/b, Organic solute transporter a/b; PL, phospholipids; SAV, subapical plasma membrane; IC, intracellular location.

(Adapted from Portincasa, P., et al. [132])
Appendix 4. The scenario of bile formation is depicted in intrahepatic bile duct cholangiocytes, with respect to hepatobiliary transporters and solutes. For clarity reasons, transporters are depicted in different parts of the panel, although they all belong to the same cell. Left cholangiocyte: proposed model for the coupling of solute and water transport and somatostatin-induced absorption of ductal bile in rat cholangiocyte. Upper cholangiocyte: main transporters involved in bile duct secretion. The increase in intracellular cAMP induced by choleretic hormones such as secretin activates CFTR leading to the exocytotic insertion of AQP1 in the cholangiocyte apical membrane. The efflux of Cl\(^-\) leads to the extrusion of HCO\(_3\)\(^-\) (via the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger, AE2) and Na\(^+\) (paracellular arrow) and possibly K\(^+\) (through a paracellular pathway; not depicted in the figure). The osmotic gradient created by these solute transports drives a transcellular movement of water into the bile duct lumen. The existence of a subapical vesicles containing transporters involved in bile duct secretion including CFTR, AE2 and AQP1 has been recently suggested in rat cholangiocytes [133]. This vesicles translocate exocytotically into the apical membrane under stimulation of secretin. Water is imported mostly by AQP4 and secreted into the lumen by AQP1. Lower cholangiocyte: main transporters involved in bile duct absorption. Somatostatin decreases the ductal bile secretion by reducing the intracellular levels of cAMP and, consequently, inhibiting the exocytotic insertion of AQP1 and the activation of CFTR in the apical membrane. The net water ductal absorption caused by somatostatin is the consequence of stimulating glucose and bile salt absorption by SGLT1 and ASBT, respectively. The sodium/hydrogen
exchanger isoform NHE3 has also been suggested to be involved in bile duct secretion. Of note, in this working model, based on bidirectional property, AQP4 would move water into or out of the cholangiocyte, depending on the secretive or absorptive status of the bile duct. Right cholangiocytes: at the apical membrane, glucose and amino acids are reabsorbed by the GLUT1 and amino acid carriers. On the basolateral membrane bile acids may exit by the MRP3 and the heteromeric Organic solute transporters OSTα/β.

Abbreviations: ASBT, apical Na⁺-coupled bile salt transporter; AQP1, aquaporin-1; AE2, Cl⁻/HCO₃⁻ anion exchanger; AQP4, aquaporin-4; BA, bile acids; BA-G, glucuronidated bile acids; BA-S, sulphated bile acids; CFTR, cystic fibrosis transmembrane conductance regulator Cl⁻ channel; Chol, cholesterol; GLUT1, glucose transporter isoform 1; MRP3, Multidrug resistance-associated protein 3; NHE3, Na⁺/H⁺ exchanger isoform 3; OA, organic anion; OC, organic cation; OS, organic solutes; SGLT1, Na⁺-coupled glucose transporter. See also Appendix 3 for abbreviations.
(Adapted from Portincasa, P., et al. [132])
Appendix 5. Pathways of thalidomide activity against multiple myeloma (MM) in the host microenvironment. A, Induction of apoptosis or growth arrest of MM cells; B, antiangiogenesis in bone marrow (BM) milieu; C, inhibition of MM cell adherence to BM stromal cells (BMSCs); D, inhibition of cytokine secretion from MM cells and BMSCs; E, augmentation of host anti-MM activity. bFGF = basic fibroblast growth factor; CTL = cytotoxic T lymphocytes; ICAM-1 = intercellular adhesion molecule 1; IFN = interferon; IGF-1 = insulin-like growth factor 1; IL = interleukin; NF-κB = nuclear factor κB; NK = natural killer cells; SDF-1α = stromal cell-derived factor 1α; TGF-β1 = transforming growth factor β1; TNFα = tumor necrosis factor α; VCAM-1 = vascular cell adhesion molecule; VEGF = vascular endothelial growth factor.

(Adapted from Richardson P., et al. [92])
Expression of aquaporins in rat liver regeneration

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Abstract
Objective. The remarkable ability of liver to regenerate after insults has been harnessed by surgeons when designing techniques for liver resection or transplantation. However, the underlying mechanisms of liver regeneration are not fully clarified. On the other hand, aquaporins (AQPs) are small transmembrane proteins with unexpected physiological roles in addition to water transport. For example, they play pivotal roles in cell migration, angiogenesis, and cell proliferation, events that are also occurred during liver regeneration. We thus examined the possible involvement of AQPs in this regenerative process.

Material and methods. A two-thirds partial hepatectomy (PH) rat model was employed. The temporal expression of various AQPs in the liver following PH was determined by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. The localization of AQPs was evaluated by immunohistochemistry.

Results. As anticipated, AQP0, 8, 9, and 11 were detected mainly in hepatocytes; unexpectedly, Kupffer cells were observed to express AQP8 during a specific period of time in the regenerative process. AQP9 protein was shown to be expressed in a progressively enhanced pattern at early time points after PH. A transient expression of AQP11 in the nucleus of hepatocytes was observed.

Conclusion. These findings suggest the possibility that AQP might be involved in the PH-induced liver regeneration.

Key Words: aquaporin, liver regeneration, partial hepatectomy

Introduction
Liver is a unique organ with capability of regeneration after various injuries, such as ischemia, viral infection, and partial resection [1]. The remarkable ability of liver to regenerate following two-thirds partial hepatectomy (PH) was first described in rats by Higgins and Anderson in 1931 [2]. This feature has been harnessed by surgeons when designing techniques such as liver resection, split-organ, and living-related donor transplantation [3]. Although tremendous efforts have been spent on the exploration of the mechanisms involved in the regenerative process, several key questions still remain unanswered [1]. To date, available information regarding liver regeneration mostly came from rat experiments, despite the physiological differences between humans and rodents. It is now well accepted that there are three main phases of liver regeneration: an initiation phase, rendering hepatocytes competent for subsequent replication; a proliferation phase, where the hepatocyte population is expanded; and a termination phase, where growth response is terminated at a defined set point [1].

Aquaporins (AQPs) are a family of small integral proteins consisting of six transmembrane domains connected by five connecting loops, with molecular weights ranging between 25 and 34 kDa [4]. So far, since the initial discovery of AQP1 in 1988, 13 distinct
Aquaporins (AQP0–12) have been reported and they are functionally subdivided into aquaporins (AQP0–2, AQP4, 5, and 8, which are primarily water selective), and aquaglyceroporins (AQP3, 7, 9, and 10, which are permeable to neutral solutes such as glycerol and other small solutes in addition to water) [4–7]. In addition to these two main groups, AQP11 and 12 are poorly characterized and their functional roles are unknown [8]. Specific physiological roles for many of the AQPs have been recognized from phenotype analysis of AQP knockout mice. In addition to water transport, various unexpected roles of AQPs were found in the past few years. For instance, the involvement of AQPs in cell migration was documented following the observation of slower migration of cultured aortic endothelial cells from AQP1-null mice than cells from wild-type mice in response to a chemotactic stimulus [9]. Subsequent studies showed that the AQPs-facilitated cell migration was AQP2 and cell-type-independent [10,11]. On the other hand, aquaglyceroporins, despite their unidentified physiological importance as glycerol channels, might play important roles in cell proliferation and the homeostasis of metabolism. Impaired wound healing and cell regeneration was observed in AQP3-deficient mice [11,12]. AQP7 and AQP9 are highly expressed in adipocytes and the liver, respectively [13,14]. In the liver, AQP9 is expressed in hepatocytes within the sinusoidal surfaces of hepatocytes plates and is proposed to function in glycerol uptake from the bloodstream for gluconeogenesis during starvation. AQP7-expressed adipocytes are crucial in the maintenance of whole-body energy balance by regulating lipogenesis and lipolysis. The release of glycerol by adipocytes and its uptake by the liver are optimally balanced by the coordinated regulation of AQP7 and AQP9. Additionally, the pathophysiological relevance of these channels has been demonstrated in studies of knockout or knockdown mice [15].

Several AQPs (0, 1, 3, 4, 5, 8, 9 and 11; AQP3 is absent in rats) have been reported to be expressed in liver [16–19]. Among them, the functional role of AQP0 and AQP11 remains unclear. AQP1, found in hepatic blood vessels and cholangiocytes, is believed to mediate water transport from plasma to bile or across the cell membrane of cholangiocytes [6,16]. AQP3 is postulated to be involved in lipid metabolism [20]. AQP4 is implicated in the intrahepatic bile duct absorption of water [21]. AQP8 is suggested to be involved in water permeability across the canalicular membrane [22–24]. AQP9 is important for glycerol transport during hepatic gluconeogenesis and, along with AQP8, contributes to bile secretion [17,25]. However, further studies are required to fully assess the physiological relevance of these AQPs.

This study was aimed to explore the possible involvement of AQPs in liver regeneration. The motivation comes from the accumulating observations in the literature that AQPs are involved in the angiogenesis, cell migration and proliferation that are events also occurred during the process of liver regeneration [1]. A partial hepatectomy-induced rat liver regeneration model was employed in this study. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blot analyses were performed to detect the expression level of AQP mRNAs and proteins, respectively. To confirm the presence of the AQP proteins in liver tissues, immunohistochemistry was conducted. Although only four AQPs (AQP0, 8, 9, and 11) are known to be expressed in hepatocytes, the major cell type responsible for liver regeneration, we examined all the seven AQPs (AQP0, 1, 4, 5, 8, 9, and 11) that have been identified in the hepatobiliary system considering the possibility that some AQPs might express only transiently in the regenerative process.

Material and methods

Animals and partial hepatectomy

The protocols in this study were submitted and approved by the E-Da Hospital (Kaohsiung, Taiwan, ROC) Institutional Animal Care and Use Committee. All animal procedures were in compliance with our institutional guidelines. Male Sprague-Dawley rats initially weighing 250–300 g were acclimated to a 12-h day–night cycle and given free access to standard rodent chow and water. Rats were deprived of food for 24 h before hepatic surgery but were permitted free access to water.

The animals were anesthetized with Zoletil 50 (Virbac, Brazil; 50 mg/kg, IP) and subjected to 70% partial hepatectomy according to the method of Higgins and Anderson [2]. Briefly, a midline incision was made in the rat’s upper abdomen and the median and the left lateral lobes were ligated and removed without injuring the remaining liver tissue, resulting in about 70% PH. Rats (n = 5 for each time point) were then sacrificed at 1, 6, 24, 48, 72, 96, 144, and 192 h after PH and the regenerated livers were harvested. The resected quiescent liver was used as 0 h control. For Western blotting and RT-PCR assays, liver tissue harvested was rinsed thoroughly in cold PBS and snap-frozen in liquid nitrogen, and the samples were stored at −80°C until use. For tissue staining, the liver tissue was fixed in 4% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm/slide.
The rate of liver regeneration was evaluated using the formula of Kwon et al. [26]: Hepatic regeneration rate (\(\%\)) = \(\frac{D}{E} \times 100\), where \(D\) is the weight of the liver per 100 g of body weight at death and \(E = R/0.7\). \(E\) is the estimated liver weight per 100 g body weight before hepatectomy, which was calculated from the weight of resected liver (R).

Reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. Total RNA (5 \(\mu\)g) was reverse-transcribed with random primers. The cDNA was then amplified by using the polymerase chain reaction with primers specific against various AQPs (Table I). The PCR products were electrophoresed in 1% agarose gels and the bands were visualized by ethidium bromide staining. Densitometric analysis was performed and corrected for loading using \(\beta\)-actin gene.

Western blotting

Liver tissue was homogenized in ice-cold lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride] containing protease inhibitor cocktail. The homogenate was centrifuged and the supernatant collected. Protein concentrations of supernatants were determined. Samples of supernatants containing 100 \(\mu\)g protein were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred to Hybond-Polyvinylidene difluoride membranes by electroelution. After 3 h of blocking with Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membranes were incubated overnight with antibodies to proliferating cell nuclear antigen (PCNA, dilution 1:1000; Epitomics, CA, USA), AQPO, 1, 4, 5, 8, 9, 11 (2 \(\mu\)g/ml for each AQP; Alpha Diagnostic, TX, USA), or \(\beta\)-actin (dilution 1:100; Sigma-Aldrich, MO, USA) diluted in the blocking solution. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. The signals were detected by enhanced chemiluminescence detection kit (Amersham, NJ, USA). The chemiluminescent signal was captured by a UVP BioSpectrum500 imaging system (UVP, CA, USA).

Immunohistochemistry

Liver sections were de-paraffinized, rehydrated, and placed in citrate buffer (10 mM, pH6.0) and microwaved twice for 7 minutes to improve staining by antigen unmasking. After washing and quenching of endogenous peroxidase with 3% hydrogen peroxide for 15 minutes, sections were blocked and incubated overnight at 4°C with antibodies to AQPO, 1, 4, 5, 8, 9, 11 (10 \(\mu\)g/ml for each AQP; Alpha Diagnostic), PCNA (dilution 1:500; Epitomics), or cluster of differentiation 68 (CD68, dilution 1:100; Abbiotec, CA, USA) diluted in blocking solution. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, the signals were detected by enhanced chemiluminescence detection kit (Amersham, NJ, USA). The chemiluminescent signal was captured by a UVP BioSpectrum500 imaging system (UVP, CA, USA).

### Table I. Primers for AQP expression.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence (5’–3’)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQPO</td>
<td>F ACGGCTCAAGAGTGTTTCTGA</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>R TCCCCACAOCTCTTTCTTCTAT</td>
<td></td>
</tr>
<tr>
<td>AQP1</td>
<td>F CTGGTGCTGATGTCTTCTGA</td>
<td>344</td>
</tr>
<tr>
<td></td>
<td>R ATTTCCGGAATCGATGTCTTCTC</td>
<td></td>
</tr>
<tr>
<td>AQP4</td>
<td>F TTAGGCAACTGACAAGCAGCC</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>R GTAATGTCGATGATACCGATGC</td>
<td></td>
</tr>
<tr>
<td>AQP5</td>
<td>F GCCAGCACATCGGACCACATT</td>
<td>373</td>
</tr>
<tr>
<td></td>
<td>R AAAGATCGGCTGAGGTTGACAT</td>
<td></td>
</tr>
<tr>
<td>AQP8</td>
<td>F GCCACAGCTCACTACACCTGC</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>R CCGAGCAGTTCGATCCAGGG</td>
<td></td>
</tr>
<tr>
<td>AQP9</td>
<td>F GATGGACTCATGACCCATTGTC</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>R CAAATCAGGACCCAGACAGG</td>
<td></td>
</tr>
<tr>
<td>AQP11</td>
<td>F ACCCTCTAGGGTGAGGGAAC</td>
<td>867</td>
</tr>
<tr>
<td></td>
<td>R CACCGACTTGTGGACTTTGGC</td>
<td></td>
</tr>
<tr>
<td>(\beta)-actin</td>
<td>F ACAATGGCTGAGCTGGGAGG</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>R GGACGCCGCTCATTGCCGATAG</td>
<td></td>
</tr>
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</table>

Abbreviations: AQP = aquaporin; F = forward; R = reverse.
initially observed at 24 h after PH and increased progressively to the maximum at 96 h, 144 h (Figure 1A). Correspondingly, significant increase in DNA synthesis, as indicated by strong PCNA protein expression, was noted immediately after liver resection and peaked at 48 h followed by an abrupt decrease after 72 h (Figure 1B). This expression profile was also confirmed by immunohistochemistry (data not shown).

*AQP0*

There was no significant variation, compared with the basal condition (0 h), in the expression of AQP0 in both mRNA and protein levels throughout the entire study period (Figure 2A). Results of immunohistochemistry using anti-AQP0 antibody showed intense intracellular staining, predominantly in pericentral hepatocytes.

*AQP1*

No significant difference in the expression level of AQP1 mRNA or protein among different time points was observed (Figure 2B). Pronounced anti-AQP1 immunolabeling was detected on the surface of central vein and sinusoid, presumably reacting with the endothelial cells lining the central vein and sinusoid. Hepatocytes were negative for anti-AQP1 reactivity.

*AQP4, 5*

The expression level of AQP5 mRNA or protein at each studied time point following PH was statistically equal to that at the basal state (Figure 2C). Similar observations were also made for the expression of AQP4. By immunohistochemistry, both AQP4 and AQP5 were expressed similarly before and after PH, with immunoreactivity mainly presented in bile ductules of the portal triad. Neither AQP4 nor AQP5 reactivity on the hepatocytes was detected.

*AQP8*

After PH, the expression level of AQP8 mRNA increased progressively from 24 h and reached the peak at 144 h (Figure 3A); while a significant increase in protein level was detected at 48–96 h and followed by a decrease (Figure 3B). In addition to 28 kDa, AQP8 also showed a band approximately at 35 kDa on the immunoblot which supposedly is the glycosylated form [25]. During the quiescent state and the early hours following PH, staining for AQP8 was mainly intracellular and was stronger in hepatocytes surrounding the central vein (Figure 3C). This is consistent with previous reports [23,25].

**Results**

All the experimental rats survived the two-thirds partial hepatectomy. Detectable liver mass regeneration was

Statistical analysis

All data are expressed as means ± SEM. Statistical evaluations were made by Student’s t-test by SigmaStat Software (Jandel Scientific, CA, USA). A p < 0.05 was considered significant.
absorption of the antibody with the antigen peptides abolished all staining (Figure 3C, 0 h, inset). Starting at 24 h, in addition to faint signals in pericentral hepatocytes, pronounced AQP8 labeling was detected in cells within the sinusoid and reached the peak at 48–72 h and decreased thereafter. These spindle-shaped cells were further identified to be Kupffer cells by positive immunoreactivity for CD68, a macrophage marker (Figure 3C, 96 h, inset).

**AQP9**

The expression level of AQP9 mRNA during the first 6 h following PH was not different from the basal level (Figure 4A). At 24 h and the subsequent time points, significantly increased expression levels were observed. As to the protein expression profile, a time-dependent increase was detected and reached the maximum at 48–72 h (Figure 4B). Similar to AQP8, a protein band of 35 kDa was also positively stained with anti-AQP9 antibody (data not shown), in addition to 28 kDa. By immunohistochemistry, AQP9 was localized exclusively in the basolateral (sinusoidal) membrane of hepatocytes (Figure 4C).

**AQP11**

RT-PCR and Western blot analysis revealed that significant changes in the expression levels of AQP11 occurred only at early time points after PH (Figure 5A and B). Nevertheless, results of immunohistochemical staining exhibited altered localizations of AQP11 protein at different time points. During the rest state and early hours of liver regeneration, AQP11 protein was mostly confined to the cytosol (Figure 5C). At 24 h and 48 h, intense stain was observed mainly in the nucleus of hepatocytes.
and then subsided. Pre-absorption of the antibody with the antigen peptides abolished all staining (Figure 5C, Neg).

**Discussion**

This study demonstrated the temporal mRNA and protein expression profiles of AQPs (AQP0, 1, 4, 5, 8, 9, and 11) in rat liver during the process of partial hepatectomy-induced liver regeneration. As anticipated, AQP0, 8, 9, and 11 were detected mainly in hepatocytes; unexpectedly, Kupffer cells were observed to express AQP8 during a specific period of time in the regenerative process. To our knowledge, this is the first report documenting the expression of AQP8 in Kupffer cells. The maximal expression of PCNA, a marker for DNA synthesis, was observed to occur at 48 h post-
hepatectomy in the present study. In contrast, the peak for hepatocyte proliferation during liver regeneration in the rat as determined by Ki67 or 5-Bromo deoxyuridine (5-BrdU) labeling is at 24 h [1]. The discrepancy might lie in the methodologies employed in different studies. Ki-67 staining is used to determine the growth of a specific cell population such as hepatocytes in the liver. In contrast, the approach we used is detection of the overall PCNA expression in liver homogenate which is derived from various types of cells with different proliferation rates. AQP0 had previously been thought to be expressed exclusively in the lens fiber cells of the eye [6]. In addition to water transport function, recent studies strongly suggest its structural role as a cell-to-cell

Figure 4. Expression of AQP9 in rat liver at different time points following partial hepatectomy. (A) RT-PCR analysis. The expression of AQP9 is normalized against that of the housekeeper gene, β-actin. Top, Representative gel. Bottom, Densitometric analysis of AQP9 mRNA expression. The expression at 0 h is arbitrarily normalized to 1. Results shown are the means ± SEM of data from five different rats/group per time point. *p < 0.05 versus 0 h control. (B) Western blot analysis. The expression of AQP9 was normalized against that of β-actin. Top, Representative immunoblot. Bottom, Densitometric analysis of hepatic AQP9 protein expression. The expression at 0 h is arbitrarily normalized to 1. (C) Immunolocalization of AQP9 protein in liver section. Scale bars: 50 μm.
adhesion protein [27,28]. Although murine liver is the only known organ besides the lens that expresses AQP0, the physiological role of AQP0 in hepatocytes remains to be identified. In this current study, no significant temporal variations in cellular localization and expression of AQP0 were observed, implying its minor role in liver regeneration.

AQP8 is the most abundant aquaporin in rat hepatocytes [22] and no previous reports have demonstrated its expression in Kupffer cells. Between 48 h and 72 h after PH, pronounced expression of AQP8 was observed in Kupffer cells, coinciding with the peak for Kupffer cell proliferation after PH which has been proposed to be at 48 h or 72 h according to different studies [29–31]. Although further studies are required, this finding raises many possibilities as to the functional roles of AQP8 in Kupffer cells. For example, it is reasonable to speculate that AQP8 might be important for the repopulation of Kupffer cells following PH. If the hypothesis
is proven to be true, the importance of AQP8 in liver regeneration cannot be understated, since it has been well-acknowledged that Kupffer cells play pivotal roles in liver regeneration mainly by releasing proliferative cytokines, interleukin-6 (IL-6), and tumor necrosis factor α (TNFα) [32].

AQP9 is an aquaglyceroporin and has been proposed to be the entry pathway for plasma glycerol deriving from adipose lipolysis, a major substrate for hepatic gluconeogenesis [17,18,24]. Following two-thirds PH, the increased gluconeogenesis is one of the adaptive responses in the remnant liver to provide sufficient glucose for the maintenance of metabolic homeostasis in the whole organism [33–36]. Several immediate-early genes encoding enzymes and proteins that are required for regulation of gluconeogenic response following hepatectomy have been documented. For example, the mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK), the key gluconeogenic enzyme, increased rapidly and peaked at 6 and 72 h after liver resection and remained high throughout the entire study period (168 h) [34]. This expression profile generally coincided with that of AQP9 observed in this study. We thus proposed that the enhanced expression of AQP9 might be relevant for PH-induced increase in gluconeogenesis.

The expression of AQP11 mRNA was reported to be highest in the testis, and moderate in the kidney and liver [19,37,38]. Immunohistochemical studies revealed that AQP11 was expressed intracellularly in renal proximal tubules [19] and in neurons [38]. Similarly, intracellular localization of AQP11 was also observed in this study. To the best of our knowledge, this is the first report demonstrating the expression of AQP11 in hepatocytes. The transient expression of AQP11 in the cell nucleus, compared to other AQPs, is not a common finding; however, some other AQPs have also been detected in the nucleus. For example, AQP9 protein was present and localized in the nucleus, cytoplasm, and cell membrane of the human granulosa cells [39]. In addition, AQP5 was immunolocalized in the nucleus of ovarian cancer SKOV3 cells [40]. The roles of AQPs in the cell nucleus are currently uncharacterized although a regulatory function of cell growth has been speculated [40].

AQP11, despite its function as a water channel is uncertain, has been proven to be important for the development of the proximal tubule as its absence led to fatal polycystic kidneys in neonatal mice [19]. In our study, the physiological significance for the transient redistribution of AQP11 from the cytoplasm to nucleus of hepatocytes needs further investigation.

In summary, this first step study in the understanding of the role of AQPs in liver regeneration demonstrates that two-thirds partial hepatectomy induces differential mRNA and protein expression profiles of various AQPs in the liver. Although data presented are phenomenal observations, they implicate the possibility that AQPs are involved in liver regeneration.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Effect of thalidomide on the expression of vascular endothelial growth factor in a rat model of liver regeneration

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Abstract. Liver regeneration is an angiogenesis-associated phenomenon. The present study investigated the influence of thalidomide, an antiangiogenic agent, on vascular endothelial growth factor (VEGF) expression and liver regeneration after 70% partial hepatectomy (PH) in rats. PH was performed on 50 rats dosed with either thalidomide (100 mg/kg) or a vehicle (controls) by intragastric administration. Serial changes in hepatic microcirculation were evaluated by laser Doppler flowmetry. The VEGF expression in liver tissue was assessed by immunohistochemical study and western blot analysis. Following hepatectomy, the liver regeneration rate increased markedly and reached a peak at 96 h in the two groups. Thalidomide did not affect the overall restoration of liver mass, although a delay in cell proliferation was observed. Prior to PH, the liver microcirculation in rats treated with thalidomide for 2 days was comparatively less than that in their corresponding controls; however, no significant difference between the groups was detected at any time-point following PH. Western blotting showed that the expression of VEGF was upregulated by hepatectomy and the expression levels in the two groups were equal at all studied time-points. The immunohistochemical staining revealed a waved pattern of VEGF expression which advanced from the periportal to pericentral area in both groups, but a slower advancement was detected in thalidomide-treated rats. In conclusion, thalidomide exerted no significant effects on the expression of VEGF and did not impair the overall restoration of liver mass in a rat model of PH-induced liver regeneration, providing supportive evidence for its use as an adjunct treatment modality for liver cancers.

Introduction

Liver regeneration is a tissue repair response of the liver following damage due to various causes, including viral infection, chemical intoxication and partial hepatectomy (PH). Although the exact underlying mechanisms have not been fully characterized, the process is acknowledged to be tightly regulated through controlled delivery of ‘start and stop’ signals, including numerous cytokines and growth factors, to maintain a constant liver-to-body mass ratio (1-3). A number of the growth factors involved in a regenerating liver are known for their angiogenic properties (4). Among the various angiogenic factors that have been identified, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), VEGF has been demonstrated to be a major angiogenic factor following PH (5,6).

Thalidomide, N-phthalimido-glutarimide, was initially marketed as a sedative and antinausea medicine in the 1950s, but was withdrawn due to teratogenicity (7). Unexpectedly, it has become the subject of intensive investigation in oncology since its antiangiogenic properties were first demonstrated in 1994 (8). In that study, the bFGF-induced neovascularization in rabbit corneas was significantly reduced by thalidomide. This drug has also been shown to inhibit VEGF-induced angiogenesis (9,10). In addition to its antiangiogenic effect, an immunomodulatory function is also a potential mechanism of the anticancer activity of thalidomide. To date, the effectiveness of thalidomide for treating neoplastic disorders has been confirmed in diseases such as multiple myeloma (11) and Kaposi's sarcoma (12). In addition, thalidomide has been...
Animals. Male Sprague-Dawley rats initially weighing 250-300 g were used. All animals were housed in a temperature and humidity controlled environment, and they received humane care with free access to standard chow and water throughout the study period. The protocols in this study were submitted to and approved by the E-Da Hospital (Taiwan) Institutional Animal Care and Use Committee (IACUC-97007). All animal procedures were in compliance with our institutional guidelines.

Materials and methods

Animals. Male Sprague-Dawley rats initially weighing 250-300 g were used. All animals were housed in a temperature and humidity controlled environment, and they received humane care with free access to standard chow and water throughout the study period. The protocols in this study were submitted to and approved by the E-Da Hospital (Taiwan) Institutional Animal Care and Use Committee (IACUC-97007). All animal procedures were in compliance with our institutional guidelines.

Experimental design. A total of 50 rats were subjected to 70% PH and equally divided into two groups: the control and thalidomide groups. Two days prior to PH, rats in the thalidomide group were daily administered thalidomide (100 mg/kg, TTY BioPharm, Taipei, Taiwan) in olive oil by intragastric administration. Control rats received olive oil only. Animals in the two groups were equally divided into 5 subgroups according to observation intervals, which were 0, 48, 96, 144 and 192 h after PH.

PH. Liver regeneration was induced by 70% PH as described by Higgins and Anderson (18). Animals were anesthetized with ketamine (100 mg/kg, intraperitoneal injection). After a midline laparotomy, the liver was exposed and the left and medial lobes were ligated (4-0 silk) and resected. Glucose solution (5 mL; 5%; 37°C) was injected into the abdominal cavity and the abdominal wound was closed in two layers with 4-0 silk. The resected liver was termed ‘quiescent liver’ in this study.

Hepatic regeneration rate. The rate of liver regeneration was evaluated using the formula of Kwon et al (19): Hepatic regeneration rate (%) = D/E x 100, where D is the weight of the liver per 100 g of body weight at death and E is the estimated liver weight per 100 g body weight prior to hepatectomy, which was calculated from the weight of resected liver (R); E = R/0.7.

Laser Doppler flowmetry analysis of microcirculation. The principle of laser Doppler flowmetry combines laser technology with the Doppler effect caused by the movement of red blood cells in the microcirculation to estimate red blood cell flux (20). The strength of this technique is in observing changes in flow, either over time or over an area of the exposed tissue. Before 70% PH and animal sacrifice, the surface of the liver was scanned by a Moor LDI 2 imager (Moor Instruments Ltd., Devon, UK) to assess the perfusion hemodynamics. The Doppler shift is proportional to a blood flow-related variable and is expressed in arbitrary perfusion units (PU). Microcirculation density was quantified using software provided by the manufacturer (Moor LDI system software V5).

Western blot analysis. Livers were homogenized by Ultrasonic cell disruptor (Microson™ XL-2000; Misonix, Farmingdale, NY, USA) in tissue protein extraction buffer (T-PER®, Pierce, Rockford, IL, USA) containing protease inhibitors (Protease Inhibitor Cocktail 100X, Pierce) and the homogenate was centrifuged to obtain the supernatant. Protein concentrations were determined and the samples were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (ECL, Amersham, Buckinghamshire, UK). After blocking and washing, blots were incubated overnight at 4°C with rabbit affinity-purified antibodies against proliferating cell nuclear antigen (PCNA; dilution, 1:1,000; Epitomics, Burlingame, CA, USA), VEGF (dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or β-actin (dilution, 1:1,000, Sigma, St. Louis, MO, USA). The blots were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Finally, the signals were detected using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA). The chemiluminescent signal was captured by a UVP BioSpectrum500 imaging system (UVP, Upland, CA, USA).

Immunohistochemistry. Fresh liver samples were immediately immersed into 10% neutral formalin. Paraffin-embedded liver samples were cut into 5-μm sections. Liver sections were de-paraffinized, rehydrated and placed in citrate buffer (10 mM, pH 6.0) and microwaved twice for 7 min to improve staining by antigen unmasking. After dewaxing and rehydration, liver sections were placed in citrate buffer (10 mM, pH 6.0) and microwaved twice for 7 min to improve staining by antigen unmasking. The activity of endogenous peroxidase was removed by incubation with 3% H2O2 for 15 min at room temperature. VEGF was identified by rabbit anti-rat VEGF polyclonal antibody (dilution, 1:500; SC-152, Santa Cruz Biotechnology, Inc.) followed by HRP-conjugated goat anti-rabbit secondary antibody (Dako™ REAL™ EnVision Detection System, K5007; Carpinteria, CA, USA). Positive signals were shown by 3,3′-diaminobenzidine (DAB) response. Sections were then counterstained with hematoxylin.

Statistical analysis. Student’s t-test was used to compare sample means with paired or unpaired controls, as appropriate. Results are expressed as means ± SEM. P<0.05 was considered to indicate a statistically significant result.

Results

Liver regeneration rate. All the experimental rats survived the 70% liver resection and thalidomide treatment. Following hepatectomy, the restituted liver mass in the two groups markedly increased with a peak at 96 h and then declined (Fig. 1A).
Expression of PCNA.

**Western blot analysis of VEGF.** Prior to liver resection (0 h), a low expression level of VEGF was detected in control rats and rats treated with thalidomide for 48 h (Fig. 3). Hepatectomy induced marked expression of VEGF, which peaked at 48-96 h and declined rapidly in the two groups. No significant difference in expression level between the groups at any studied time-point was found.

Immunohistochemical staining of VEGF. Positive VEGF immunoreactivity was mainly localized in the cytoplasm of hepatocytes. Prior to PH, faint staining was observed in the two groups. At 48 h after PH, VEGF was mainly expressed in the perportal area in both groups (data not shown). At 96 h, the positive immunoreactivity was limited to the pericentral area in the control group (Fig. 4A-C), while observed in pericentral and periportal hepatocytes in the thalidomide group (Fig. 4D-F). At the subsequent time-points, markedly weaker expression of VEGF was observed and mostly located in the pericentral area in the two groups (data not shown).
Discussion

The present study demonstrated that thalidomide delayed the PH-induced hepatic cell proliferation but did not impair the overall liver regeneration. In addition, the PH-induced upregulation of VEGF was not inhibited by thalidomide.

The maximal expression of PCNA, a marker for DNA synthesis, was observed to occur at 48 h post-hepatectomy in the control group of this study. However, the peak for hepatocyte proliferation during liver regeneration in the rat as determined by Ki-67 or 5-bromodeoxyuridine (5-BrdU) labeling is at 24 h (1). There are at least two possible explanations for this discrepancy. One is that 24 h was not one of the selected time-points in our study. The other is the methodologies used in different studies. The approach we employed in this study was detection of the overall PCNA expression in liver homogenate, which is derived from various types of cells with different proliferation rates. By contrast, Ki-67 staining is used to determine the growth of a specific cell population, such as hepatocytes in the liver. In the present study, the maximal PCNA expression detected in the thalidomide group was 96 h after liver resection, a significant delay as compared with control rats; nevertheless, the overall restoration of liver mass was not affected.

The significance for the observed transient greater liver regeneration rate in the thalidomide group requires further investigation. However, we speculated that it may be due to the non-specific effect of thalidomide, such as increasing the water content in liver tissue based on the transient watery appearance of thalidomide-treated liver (our unpublished observation). If so, the liver regeneration rate may thus be overestimated.

Laser Doppler flowmetry is a technique for the non-invasive blood flow monitoring and is considered to be a suitable technique for the analysis of hepatic microcirculation (20–22). In the present study, thalidomide impaired hepatic microcirculation in the quiescent, but not regenerating, liver. The reduced blood flow in the thalidomide-treated quiescent liver may be associated with the inhibitory effect of thalidomide on the release of tumor necrosis factor (TNF)-α and nitric oxide, two potent vasodilators, as suggested by a previous study in which thalidomide ameliorated the portal pressure and hyperdynamic circulation in partially portal vein-ligated rats by
reducing TNF-α and nitric oxide production (23). After PH, this inhibitory effect of thalidomide was eliminated by rapid release of TNF-α (1), resulting in similar hepatic microcirculation in the two groups.

VEGF is an important factor in the early phase of liver regeneration (24). In this study, treatment with thalidomide for 48 h before PH did not affect the expression of VEGF, as evidenced by the insignificant difference in the expression level between control and thalidomide groups at 0 h. Following PH, VEGF was markedly upregulated and the expression profile during the regenerative process was similar in the two groups. These observations suggest that thalidomide exerts no significant effect on the expression of VEGF either in quiescent liver or in PH-induced regenerating liver. The immunohistochemical result showing that VEGF was predominantly expressed in the periportal hepatocytes at 48 h post-PH is consistent with a previous study (5). At 96 h, the positive staining was observed only in the pericentral area in the control group, while observed in the periportal and pericentral areas in the thalidomide group. This time-dependent alteration in the main expression site in the liver suggests a wave pattern for the expression of VEGF, which advances from the periportal to pericentral area. Although the significance for the observed difference in the expression areas at 96 h between groups requires further studies for clarification, we hypothesize that it may reflect the slower angiogenesis in thalidomide-treated rats.

In conclusion, our results demonstrate that thalidomide exerts no significant effect on the expression of VEGF and does not impair the overall PH-induced restoration of liver mass, providing supportive evidence that thalidomide may be used as an adjunct treatment modality for liver cancers.

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