EVIDENCE OF NITRIC OXIDE BEING THE INITIAL TRIGGER OF THE LIVER REGENERATION CASCADE

BY

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TO MY FAMILY, MY LOVELY DAUGHTER,

AND MY DEAR PARENTS

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ABSTRACT

Liver regeneration is a cascade of events (elevation of growth factors, cytokines and hormones, and early gene expression) leading to rapid hepatocyte proliferation to restore its lost cell mass in response to partial hepatectomy (PHX) or cell injury. The initial trigger for the cascade is still unidentified despite intensive research started over a century ago.

The hypotheses of the project comprise: 1. the hemodynamic change (↑ blood flow/liver mass ratio) occurring immediately upon PHX serves as the initial trigger; 2. this ratio change alters the concentration of flow-dependent factor(s) leading to production of proliferative factors (PF, which represents a combination of growth factors, cytokines and hormones) that represent the onset of the regeneration cascade.

Experimentally, a 2/3 PHX rat model, an *in vitro* hepatocyte bioassay measuring the PF production in the plasma and an *in vivo* method directly measuring liver regeneration by liver weight were utilized. This approach was combined with the pharmacological manipulation of flow-dependent factors (adenosine, nitric oxide and prostaglandins) to assess their effects on the PF production and liver weight

regeneration, in order to test their roles in the initiation of the regenerative response. Selective portal vein branch ligation (PVL), an analogous hemodynamic model to PHX, was also used to test the hypotheses. PVL results in shunting of portal flow to 1/3 of the liver thus causing a similar flow/mass ratio change as produced by 2/3 PHX.

The optimized bioassay results showed an early and transient increase of PF in the PHX rat plasma (1-4 hr, peaking at 4 hr after PHX), which is closely related to regeneration and can indicate the initiation of the cascade. The developed bioassay can quantitatively distinguish the PF levels in 1/3 and 2/3 PHX rat plasma. A flow-dependent factor, nitric oxide, was demonstrated to be a key triggering factor for the regeneration cascade and prostaglandins are also involved in the initiation, possibly as a mediator mechanism for the nitric oxide effects. PF is also detected in the PVL rat plasma, indicating a similar initiation to the PHX model.

In conclusion, data support the hypotheses of increased blood flow/liver mass upon PHX resulting in increased shear-stress and release of flow-dependent factors, which then lead to the PF production and onset of the liver regeneration.

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LIST OF ABBREVIATIONS

Ach: Acetylcholine

Ado: Adenosine

aFGF: Acidic fibroblast growth factor

ALR: Augmentor of liver regeneration

AP-1: Activator protein-1

bNOS: Brain nitric oxide synthase

cGMP: Guanosine 3',5'-cyclic monophosphate

COX: Cyclo-oxygenase

EDRF: Endothelium-derived relaxing factor

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

eNOS: Endothelial nitric oxide synthase

FA: Femoral artery

FV: Femoral vein

GdCl: Gadolinium chloride

GEA: Mesoionic 3-aryl substituted oxatriazole-5-imine derivatives

GF: Growth factors

GTN: Glyceryl trinitrate

HB-EGF: Heparin-binding epidermal growth factor-like growth factor

HGF: Hepatocyte growth factor

HGFA: Hepatocyte growth factor activator

HPTA: Hepatopoietin A

HSS: Hepatic stimulatory substance

ICAM-1: Intercellular adhesion molecule-1

IFNγ: Interferon gamma

IL-1: Interluekin 1

IL-4: Interluekin 4

IL-6: Interluekin 6

IL-10: Interluekin 10

INDO: Indomethacin

iNOS: Inducible nitric oxide synthase

KGF: Keratinocyte growth factor

L-Arg: L-Arginine

L-NA: N^G-nitro-L-arginine

L-NAA: N^G-amino-L-arginine

L-NAME: N^G-nitro-L-arginine methyl ester

L-NIO: N-iminoethyl-L-ornithine

L-NMMA: N^G-monomethyl-L-arginine

LPS: Lipopolysaccharide

NE: Norepinephrine

NF-1: Nuclear factor-1

NF-κB: Nuclear factor κB

NO: Nitric oxide

NOS: Nitric oxide synthase

PCNA: Proliferating cell nuclear antigen

PF: Proliferative factors

PGs: Prostaglandins

PHX: Partial hepatectomy

PVL: Portal vein ligation

SIN-1: 3-Morpholino-sydnonimine

SIN-10: Molsidomine

SNAP: S-nitroso-N-acetyl-DL-penicillamine

SNP: Sodium nitroprusside

TGFα: Transforming growth factor α

TGFβ: Transforming growth factor β

TNFα: Tumor necrosis factor α

tPA: Tissue type plasminogen activator

VCAM-1: Vascular cell adhesion molecule-1

I. INTRODUCTION

IA. OVERVIEW OF LIVER REGENERATION STUDY

What is liver regeneration? Liver regeneration is a rapid regrowth of remnant liver tissue (by an early hypertrophy and a later hyperplasia) to restore its original size after loss of cells by surgical resection or by chemical and viral injuries (Michalopoulos, 1990; Fausto and Webber, 1994). This phenomenon is the basis of a Greek legend in which the ancient Greek Gods punished Prometheus with a daily partial hepatectomy by an eagle after he stole the secret of fire from the Gods and passed that to the human being. Liver regeneration has been observed in all of the examined vertebrate species (Michalopoulos, 1990; Sigel,1969). It has been used as a model to study controlled cell growth for over a century (Milne, 1909). Despite this, some basic fundamental questions still remain unsolved.

IA-i. THEORIES OF LIVER REGENERATION CASCADE

To study this fascinating phenomenon, liver regeneration is often experimentally induced by performing surgical

partial hepatectomy (PHX) or exposure of rats to a chemical toxin such as carbon tetrachloride. 2/3 PHX in rats is the most common way of inducing liver regeneration.

Following PHX, a series of molecular and cellular responses occur both inside and outside the liver, leading to a very rapid liver tissue regeneration. There are various theoretical models in the literature describing the overall picture of the events occurring after PHX. In the model presented by Leffert and Koch (Leffert and Koch, 1980; Koch and Leffert 1979), adult hepatocyte proliferation was initiated by altered monovalent cation fluxes and increased hormone stimulation, which resulted in changes in RNA and protein metabolism, increased DNA synthesis and increased mitotic index. Recently, Fausto and Webber put forward a comprehensive model of liver regeneration (Fausto and Webber, 1994; Fausto et al. 1995). According to this model, a cascade of events and their interactions occur sequentially after PHX: various transcriptional factor activations, a wide variety of early gene expression, hepatic specific and nonspecific growth factor production in the plasma followed by hepatocyte priming and transition from G_0 to G_1 phase to enter the cell cycle and proliferate. This model is illustrated in Figure 1.

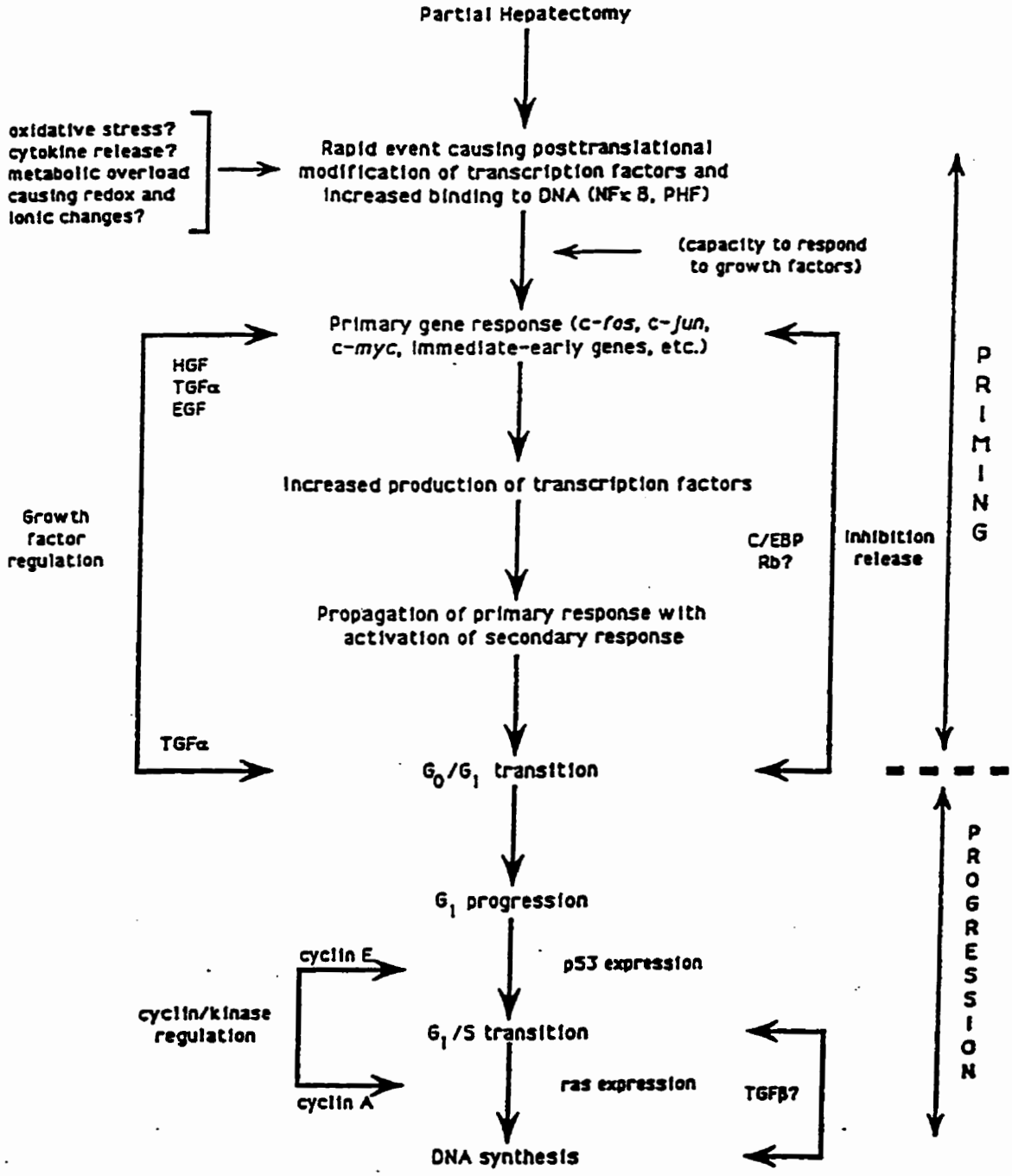


Figure 1. Sequential events after PHX.

(Adopted from Fausto and Webber, 1994)

IA-ii. BASIC QUESTIONS AND CONTROVERSIES IN LIVER REGENERATION STUDY

No matter which model is used to describe the cascade of events in liver regeneration, some basic questions have always been asked and yet not answered.

a. What Triggers Liver Regeneration?

The most frequently asked and least understood question is what triggers the liver to start regeneration. Substantial research has been conducted focusing on looking for the factor(s), appearing at the earliest time point after PHX, that is capable of triggering the whole cascade. This is also the most controversial part in the various theoretical models of liver regeneration. Searching for the earliest trigger of liver regeneration has been considered as important as the studies of the big-bang theory of the universe by Michalopoulos in a recent review article (Michalopoulos and DeFrances, 1997).

According to the model proposed by Leffert and Koch (1980), a promotion stage following PHX (0-6 hr), when RNA and protein metabolic changes and DNA synthesis started to occur, was initiated by Na^+ influx. These changes were

potentiated (3-18 hr after PHX) by combined hormonal effects of insulin and glucagon. The Na^+ influx inhibitor, amiloride, blocked the hepatocyte growth transition *in vitro* and *in vivo* after 70% PHX. Michalopoulos (1990) proposed two hypotheses on the initiation: 1. Extrahepatic stimulating signals of HPTA/HGF (hepatopoietin A/hepatocyte growth factor, a complete hepatocyte mitogen) and norepinephrine (an hepatocyte comitogen, via α_1 receptor), generated after 2/3 PHX, provide a complete mitogenic stimulation for hepatocytes to enter the cell cycle to proliferate. 2. Following PHX, metabolic signals prime hepatocytes to enter the G_1 phase and produce autocrine TGFα (transforming growth factor α) and aFGF (acidic fibroblast growth factor) to stimulate the DNA synthesis in hepatocytes themselves. In the comprehensive model proposed by Fausto and Webber (1994), the initiating signals following PHX are totally uncertain (oxidative stress? cytokine release? metabolic overload?). In recent years, more attention has been paid to certain cytokines released early after PHX.

The main drawback in different initiation hypotheses is that the proposed signals do not occur instantaneously after PHX. Therefore, the real trigger has not been considered.

In his recent review article, Michalopoulos proposed

that "Any hypotheses made to explain the mechanism of initiation of liver regeneration have to account for the mitogenic stimuli for hepatocytes appearing in circulation during regeneration and for the rapid changes occurring in hepatocytes within 30 min after PHX" (Michalopoulos and DeFrances, 1997). Regarding what is the real initiating signal, the article only repeated some existing hypotheses. HGF was still proposed to be the initial trigger since its plasma concentration started to rise within 1 hr after PHX (Lindroos, 1991).

Cytokines TNFα (tumor necrosis factor α) and IL-6 produced by Kupffer cells in the liver have also been suggested to play important roles in the early signals for liver regeneration. Evidence is as follows. TNFα could cause activation of transcription factors (NF-κB and AP1, Grilli et al., 1993; Liou and Baltimore, 1993). Antibodies to TNFα administered before PHX caused a decrease in DNA synthesis and inhibited some early gene expressions following PHX (Akerman, et al., 1992; Diehl et al., 1994). IL-6 concentration was increased after PHX and its secretion could be regulated by TNFα (Michalopoulos, 1997; Akerman et al. 1992). Type I TNFα receptor knockout mice showed severely impaired DNA synthesis after PHX which was corrected by IL-6 injection before PHX (Yamada et al., 1997). Hepatocyte complete mitogens, EGF (Epidermal growth factor) and TGFα,

were again proposed to be important in the early stage of liver regeneration.

Overall, there have been many different factors considered to be a possible initiator for the liver regeneration cascade. Nevertheless, none of the factors could provide a satisfactory explanation of the trigger, and more importantly, a most essential factor that will change instantaneously after PHX, a blood flow dependent factor produced under varied blood flow conditions by endothelial cells, has been missed out in all of these hypotheses (detailed in Section IC).

b. What Stops Liver Regeneration?

The next frequently asked question is what terminates the liver regeneration process at the point when the exact mass of the original liver is replaced. Very little is known about this aspect. There is much less research done in this area than in the area of initiation of liver regeneration. The candidate for the terminating signal selected by *in vitro* hepatocyte culture is TGFβ (TGFβ1, transforming growth factor β), which could inhibit the increase of DNA synthesis in cultured hepatocytes stimulated by other complete hepatocyte mitogens (Fausto et al., 1991; Russell et al., 1988;

Michalopoulos, 1990). However, the supporting evidence is paradoxical: 1. TGFβ1 selectively blocked the hepatocyte proliferative response stimulated only by TGFα *in vivo* but not the responses stimulated by EGF, HGF or HSS (hepatic stimulatory substance) in a dog Eck fistula (portacaval shunt) model (Francavilla et al., 1992); 2. TGFβ mRNAs increase between 3 to 4 hr after PHX, reaching a peak at 48-72 hr when hepatocyte DNA is still actively synthesizing (Michalopoulos and DeFrances, 1997; Braun et al., 1988), although some reports showed that these mRNAs can only be translated into inactive precursors of TGFβ molecules (Pistoi and Morello, 1996). 3. Dual effects of TGFβ1 on both the inhibition of hepatocyte proliferation and stimulation of hepatocyte motility response to EGF have been reported (Stolz and Michalopoulos, 1997).

Here again, a blood flow dependent factor could be a good candidate for the termination of the regeneration, although it has not yet been proposed in the literature.

c. Clonogenicity of Hepatocytes

Can adult hepatocytes undergo proliferation? The common understanding is that mature hepatocytes rarely divide under normal conditions both *in vivo* and *in vitro* (Fausto et al.,

1995; Block et al., 1996). It was reported only recently that hepatocytes have almost unlimited clonogenicity both *in vivo* and *in vitro* (Michalopoulos and DeFrances, 1997). Using transgenic mouse models, Rhim et al. showed that transplanted adult hepatocytes could undergo more than 12 divisions *in vivo* and were still able to proliferate in response to PHX (Rhim et al., 1994). The liver of a mutant-hepatic-dysfunctional transgenic mouse could be repopulated by as few as 1000 transplanted wild-type hepatocytes, demonstrating a tremendous replicative potential of mature hepatocytes (Overturf et al., 1996). Block et al. have also shown the capacity of cultured hepatocytes *in vitro* to proliferate clonogenically under the defined medium condition and stimulation of hepatocyte mitogens (HGF, EGF and TGFα) (Block et al., 1996).

d. Do Hepatocytes Have To Be Dedifferentiated In Order To Proliferate?

This is another controversial area. Some people believe that hepatocytes have to dedifferentiate first before they can proliferate. However, certain experimental evidence does not support this view (Fausto and Webber, 1994). Specific hepatic functions, such as glucose metabolism, plasma protein

synthesis, bile secretion and detoxification of toxic compounds, were well maintained during the liver regeneration process (Michalopoulos and DeFrances, 1997). Reports on whether or not fetal markers appear in the regenerating liver have been controversial (Bonney et al., 1973; Petropoulos et al., 1983; Lemire and Fausto, 1991). Solid evidence showed that a large number of immediate-early and delayed-early genes encoding various transcription factors and oncogene products were activated after PHX (Taub, 1996). These early genes could be induced superimposed on the constitutive-hepatic gene expression, making it possible for the regenerating hepatocytes to proliferate and maintain differentiated specific functions simultaneously (Taub, 1996). These reports represent only one type of openion. The area still remains to be controversial at the present.

e. Structural Changes In The Regenerating Liver

As with the major responses in hepatocytes following PHX, the hepatic extracellular matrix also undergoes major structural changes from an early degradation to a later reconstruction after PHX. The extracellular matrix, composed of collagens, elastin, structural glycoproteins and proteoglycans, has been shown to play significant roles in

the interplay with hepatocytes to restore the normal hepatic architecture (Martinez-Hernandez and Amenta, 1995).

During liver regeneration, hepatocytes proliferate before the vascular proliferation, resulting in an increased cell/matrix ratio with clusters of newly regenerated hepatocytes without sinusoids. Gradually, the vascular proliferation starts within the hepatocyte clusters, restoring the normal cell/matrix relationship and normal liver architecture (Martinez-Hernandez and Amenta, 1993: 1995). Gap junctions between hepatocytes have been shown to play an important role in cell proliferation and serve as the signal propagation pathway for hepatic sympathetic nerve effects on carbohydrate metabolism (Iwai et al., 1991; Seseke et al., 1992). An impaired effect of sympathetic nerves on glycogenolysis was observed at an early stage after PHX (48-72 hr) due to a transient reduction of a gap junction protein, connexin 32 (Iwai et al., 1991; Neveu et al., 1995). Both connexin 32 level and glucose output response returned to normal by day 11 after PHX (Iwai et al., 1991).

In addition to the parenchymal cell and vascular proliferation, other nonparenchymal cells also undergo proliferation, with a DNA synthesis peak occuring 24 hr later than that of the parenchymal cells, which occurs at 24 hr posthepatectomy (Taub, 1996).

IA-iii. STUDY MODELS USED IN THE LITERATURE

There are a few animal models used to study the liver regeneration process.

a. Partial Hepatectomy (PHX)

The most commonly used model is performing 2/3 PHX in rats. The methodology was well described by Higgins and Anderson in 1931. Removal of the left lateral and median lobes results in 2/3 (68%) PHX; removal of the left lateral lobe results in 1/3 PHX (Higgins and Anderson, 1931). The major advantage of using the PHX model is that it is a pure cell replication model where cell injury, fibrosis and inflammatory responses are not present (Fausto and Webber, 1994; Michalopoulos and DeFrances, 1997). Therefore, the initiation and control mechanisms of the hepatocyte proliferation can be studied without the interference of other situations.

b. CCl_4 Model

Another model often used is administration of the chemical hepatotoxin, carbon tetrachloride (CCl_4), to the

rats. This method induces liver regeneration by causing cell loss by necrosis (Michalopoulos, 1990). In this model, cell proliferation is present together with cell injury and death, fibrosis and inflammatory responses. Therefore it presents a more complex picture for studying the control mechanism in liver regeneration.

Comparing the PHX and CCl_4 models, there is evidence indicating that a similar series of events, but different timing, is taking place in their regeneration processes. Proto-oncogenes c-fos and c-myc expression patterns were found to be identical in the two models. DNA synthesis peaked at 24 hr after PHX, but between 48-60 hr after CCl_4 treatment (Schmiedeberg, et al., 1993; Lindroos et al., 1991). HGFA (HGF activator), one of the four identified activators of the most potent hepatocyte mitogen HGF (HGF activator, factor XIIa, urokinase, and tissue-type plasminogen activator, tPA), were activated in CCl_4 induced liver injury (Miyazawa et al., 1996). The plasma HGF levels were found greatly increased between 2-6 hr after 2/3 PHX and between 2-24 hr after CCl_4 treatment (Lindroos et al., 1991).

In terms of the initial trigger of the regeneration, it may or may not be different in the two models, although there is not yet a definite answer at the present. In the PHX model, there is an acute, one step trigger, while in the CCl_4 model, the occurrence of the trigger is not so clear cut.

c. Portal Vein Ligation (PVL)

Another model used for studying cell proliferation in liver hypertrophy and cell death in liver atrophy is the rat selective portal vein branch ligation model (Mizuno et al., 1996; Ikeda et al., 1995). It was originally developed for various purposes: morphological studies, presurgical treatment of hepatic malignant tumors, liver regeneration, atrophy and studies on cirrhosis (Winternitz, 1911; Rous and Larimore, 1920; DeWeese et al., 1951; Steiner and Martinez, 1961; Kozaka, 1963; Honjo et al., 1975; Mizuno et al., 1996). Ligation of the left portal vein branch occludes the portal blood supply to the median and left lateral lobes, creating an analogous model to 2/3 PHX wherein all portal blood perfuses 1/3 of the liver. The unligated lobes (right lateral and caudate lobes) undergo hypertrophy while the ligated lobes undergo atrophy shortly after PVL (Steiner and Martinez, 1961). In the ligated lobes, most of the hepatocytes will undergo apoptosis and be cleared by macrophages (Kupffer cells) (Ikeda et al., 1995). Various other combinations of ligation of portal vein, hepatic artery and bile duct branches have also been reported to be used in different studies (Steiner and Martinez, 1961).

d. Mouse Model

A genetically engineered transgenic mouse model has also been used in liver regeneration study. It is a powerful tool to study the *in vivo* effect of a particular growth factor or inhibitor for regeneration and to assess the regenerative capacity of mature hepatocytes in diseased liver (hepatic gene therapy). HGF/SF and TGFα expressing transgenic mice are available now. EGFR (EGF receptor) -/- and $TGF\beta_1$ knockout mice are also available in the literature (Pistoi and Morello, 1996). The advanced genetic engineering technology has opened a new avenue for liver regeneration study.

IA-iv. EARLY STUDIES ON LIVER REGENERATION

a. Earliest Studies

The Prometheus' myth could be traced back to the ancient time of the Greek Gods. The first ideas of the possibility of liver tissue to regenerate were introduced by Cruveilhier (1833) and Andral (1834) in the 1830s (Milne, 1909; Fishback, 1929). The first valuable experimental work to produce liver cell regeneration was performed by Von Podwyssozki in 1886 (Von Podwyssozki, 1886). After he removed small wedge-shaped

areas from rat and rabbit livers, he found the adjacent liver cells showed proliferative activities in 15-20 hr (Milne, 1909). These preliminary original ideas and experiments were widely accepted and confirmed by other researchers later on.

b. Blood Flow Theories In 1930s-1950s

Higgins and Anderson developed the rat PHX model in 1931, which has become the best experimental model to study liver regeneration in modern times (Higgins and Anderson, 1931). Using PHX as a standard and an effective tool, many laboratories attempted to look for the factors that might be controlling the liver regeneration process in the early years. The general agreement reached was that "the most important limiting factor in liver regeneration is the portal blood supply" (Weinbren, 1955; Stephenson, 1932; Mann, 1940). The so-called "Vascular Theory", which emphasized the importance of the filling of the liver with venous blood in determining liver regeneration, was widely accepted in the 1950s (Sigel, 1969).

Weinbren demonstrated that there was a liver weight increase in the right posterior lobe after PHX and a weight decrease in the same lobe after deprivation of portal blood supply to the lobe (Weinbren, 1955). He also demonstrated

that diversion of the portal vein from the right posterior lobe did not impair its ability to regenerate after the PHX at a later time when considerable liver atrophy had already been induced in the lobe. This observation started to challenge the "Vascular Theory" seriously. Fisher et al. showed the dependence of liver regeneration on blood in dogs. He observed an 80% regeneration in PHX dogs (43% PHX with the left and left central lobes removed), 0% regeneration in PHX plus portacaval shunt and 103% regeneration in PHX plus portacaval shunt plus arterialization of the liver blood supply (Fisher et al., 1954). The major limitations of the early studies were the lack of proper controls in many of the experimental designs and lack of advanced technology. Therefore, data generated from different laboratories showed substantial variations, controversies and confusion (Weinbren, 1955; Stephenson, 1932; Mann, 1940; Grindlay and Bollman, 1952; Child, 1953; Lieberman and Short, 1965).

The possibility of the existence of humoral factors involved in liver regeneration started to draw attention from scientists in the 1950s (Glinos and Gey, 1952; Glinos, 1956). It was observed that sera from 2/3 PHX rats could support longer growth period of hepatic explants *in vitro* than normal sera, with a lower serum concentration being more stimulatory than higher concentration. The hypothesis was made that PHX

reduced the concentration of inhibitory constituents in the serum, resulting in the liver regeneration response (Glinos and Gey, 1952). Some studies using parabiotic rats also supported the same idea (Christensen and Jacobsen, 1949-1950; Bucher et al., 1951; Wenneker and Sussman, 1951).

c. Blood Flow Theories In 1960s-1970s

By the 1960s, more workers had reported results which were against the Vascular Theory of the filling of the liver with blood to cause cell proliferation; the amount of blood flow was no longer considered to play a determinant role, but only play a permissive role for other mechanisms (Thomson and Clarke, 1965; Clarke et al., 1968; Sigel, 1969). The "Humoral Theory" emerged during that time, which indicated that humoral factors induced the regenerative response in all remaining liver tissue with a blood supply, and that the humoral stimulus would disappear when liver cell number returned to normal and thus turn off the regeneration (Sigel, 1969). The liver blood flow was regarded as important only in maintaining normal liver tissue and delivering the humoral stimuli to hepatocytes (Sigal, 1969). The primary role of extracellular control of liver regeneration was assigned to humoral factors. The supporting evidence for the Humoral

Theory, however, was still inadequate at that time.

By early 1970s, Starzl et al., using modifications of portal venous inflow in a two-segmented splanchnic flow division model in the dog liver, first reported that pancreatic hormones, especially insulin and glucagon, were the hepatotrophic factors present in the splanchnic venous blood (Starzl et al., 1973). In subsequent years, the hepatotrophic role of insulin was reconfirmed by the same group of people (Starzl, 1975).

Using *in vitro* primary monolayer culture of the differentiated fetal rat hepatocytes, Leffert has further confirmed the stimulatory and comitogenic effects of insulin on hepatocyte DNA synthesis and demonstrated the complex hormonal interactions in the control of hepatocyte DNA synthesis *in vitro* (Leffert, 1974). Growth hormone and hydrocortisone could enhance the DNA synthesis in fetal hepatocytes only when insulin was added together to the culture and had no effects by themselves. Glucagon inhibited the insulin-stimulated DNA synthesis in fetal hepatocytes. More interestingly, L-arginine could enhance insulin-stimulated hepatocyte DNA synthesis. (3',5') cGMP, with no effect by itself, was able to increase the hepatocyte DNA synthesis in the presence of insulin. The interpretation of these findings was that "one of the ealiest prereplicative

events would appear to be an interaction between arginine, insulin and the cell membrane" (Leffert, 1974). Leffert's work has provided the *in vitro* evidence supporting the "Humoral Theory" *in vivo*. His findings have also indicated a potential role of nitric oxide in the hepatocyte proliferation through facilitating the effects of insulin. The results of metabolic studies in our lab also demonstrated that the blockade of nitric oxide production by a nitric oxide synthase inhibitor L-NAME caused decreased sensitivity of glucose uptake by the skeletal muscle under the stimulation of insulin, i.e. L-NAME caused insulin resistence.

It can be seen from above, in the attempt to study hepatotrophic factors in the blood circulation, the "Humoral Theory" has dominated the literature since 1970s.

IA-v. NEW AREAS IN LIVER REGENERATION STUDY

In the review series on liver regeneration published in the FASEB Journal in 1995-1996, a variety of topic areas have been covered: hepatic extracellular matrix, growth factors and cytokines, signal transduction, transcriptional controls, mRNA stability, liver developmental analysis, transgenic mice and stem cells (Martinez-Hernandez and Amenta, 1995; Fausto

et al., 1995; Diehl and Rai, 1996; Taub, 1996; Kren and Steer, 1996; Ponder, 1996; Pistoi and Morollo, 1996; Columbano and Shinozuka, 1996; Thorgeirsson, 1996). These are the areas that have been intensively studied.

A newly developed field is the role of Kupffer cells in liver regeneration. Kupffer cells are tissue macrophages located in the lumen of sinusoids of the liver. In the current literature, their role in liver regeneration is linked through the TNF production by Kupffer cells under liver injury (e.g. endotoxin stimulation). TNFα has been shown to increase DNA synthesis through an IL-6 pathway (Michalopoulos, 1997; Yamada et al., 1997). One method presently used to study Kupffer cells is to administer gadolinium chloride (GdCl), which depletes Kupffer cells *in vivo*. The exact role of Kupffer cells in liver regeneration, whether stimulatory or inhibitory, is unclear at present. There are reports in the literature giving the exact opposite results. Shiratori et al. demonstated that serum TNFα and IL-6 levels were increased shortly after PHX in an LPS-responsive mouse strain. GdCl pretreatment in the mouse reduced the increase of both serum TNFα and IL-6 concentrations, reduced PCNA labeling index and reduced liver regeneration, indicating a stimulatory role of Kupffer cells in liver regeneration in LPS-responsive mice (Shiratori et

al., 1996). Another report by Rai et al. in the same year showed that GdCl treated rats had 6-8 fold higher TNFα mRNA in the liver and 12-16 fold higher serum IL-6 level following PHX compared to control rats. GdCl also increased the transcription factor AP-1 binding to DNA, increased [^{3}H]thymidine incorporation, PCNA expression and mitotic index in hepatocytes, thus indicating an inhibitory role of Kupffer cells in liver regeneration (Rai et al., 1996). There is also one report describing that the production of IL-1, IL-6 and prostaglandin E_2 (PGE2) by Kupffer cells is increased after PHX, and PGE2 controlled the IL-1 and IL-6 production by Kupffer cells in an autoregulatory fashion (Goss et al., 1993). From our study, another extremely important link of Kupffer cells to liver regeneration is the nitric oxide production by Kupffer cells (see later).

IB. GROWTH FACTORS (GF) FOR HEPATOCYTE PROLIFERATION

Shortly after PHX, a variety of hepatic specific and nonspecific growth factors, hormones and cytokines increase in concentration in the blood circulation and liver. This early increase in GF levels are followed by a wide range of change in immediate- and delayed-early gene expression encoding transcriptional factors, glucose and cellular

metabolic enzymes and proteins in the liver (Taub, 1996), which then prime the quiescent hepatocytes (G_0 phase) to enter the G_1 phase and proliferate along the cell cycle. This cascade of events, which occurs after liver regeneration is triggered following PHX, has been intensively studied in the past decade. The members in the GF family are increasing continuously and their production profiles have been described in the literature.

IB-i. GROWTH FACTOR FAMILIES

Using *in vitro* hepatocyte cultures in chemically defined serum-free medium, a number of hepatocyte growth stimulatory factors have been identified. They can be categorized into "complete hepatocyte mitogens" and "incomplete comitogens" (Michalopoulos, 1990; Fausto et al., 1995; Fausto and Webber, 1994). These terms were derived from the studies using *in vitro* culture. For their effects on DNA synthesis to be seen *in vivo*, the conditions may be more complex. Some complete mitogens (TGFα and HGF, see below) were shown to be able to increase DNA synthesis only in the "primed" hepatocytes (in 1/3 PHX rats) but not in the normal quiesent hepatocytes *in vivo* (Webber et al., 1994).

a. Complete Hepatocyte Mitogens

These are substances capable of independently stimulating quiescent hepatocytes into DNA synthesis and mitosis in cultures containing chemically defined media in the absence of serum. Well defined complete hepatocyte mitogens include epidermal growth factor (EGF), transforming growth factor α (TGFα), hepatocyte growth factor (HGF, hepatopoietin A), hepatopoietin B, acidic fibroblast growth factor (αFGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF) and keratinocyte growth factor (KGF). These factors have been shown to be able to stimulate hepatocyte DNA synthesis in culture or *in vivo* without requiring the presence of other agents (Fausto et al., 1995; Michalopoulos, 1990; Kiso et al., 1995). The observed potency of some of these mitogens are HGF > TGFα > EGF > αFGF (Ni and Yager, 1994).

b. Incomplete Comitogens

Incomplete comitogens are substances that can only enhance the mitogenic effects of complete mitogens but by themselves do not have direct mitogenic effects on hepatocytes in serum-free cultures. These factors include

mostly hormones and some growth factors: norepinephrine (NE), which is the strongest hepatocyte comitogen, insulin, glucagon, hepatic stimulatory substance (HSS), augmenter of liver regeneration (ALR), vasopressin, estrogens, angiotensin II and III (Michalopoulos, 1990; Fausto et al., 1995). These factors are able to both enhance the effects of complete mitogens and decrease the effects of inhibitors of hepatocyte proliferation.

c. Cytokines (CK)

The number of CK found involved in the liver regeneration cascade is also increasing. The most important CK is TNFα, which is produced by Kupffer cells after endotoxin stimulation or PHX (Decker, 1990; Shiratori et al., 1996). Endothelial cells and biliary epithelial cells also produce TNF (Rai et al., 1996). TNFα has been shown to be able to activate transcriptional factors NF-κB and AP-1 in the early signaling pathway to stimulate liver regeneration (Fausto et al., 1995; Michalopoulos and DeFrances, 1997). Administration of antibodies to TNFα before PHX resulted in decreased DNA synthesis and inhibited expression of some early genes (Diehl et al., 1994). TNFα type I receptor knockout mice showed severely impaired DNA synthesis after

PHX, which was corrected by IL-6 injection before PHX (Yamada et al., 1997). The relationship of TNFα and NO synthesis has been studied. It was shown that there was a correspondant increase of TNFα and TNFα rec. transcripts in the regenerating liver (from 1-3 hr) and increased NO synthesis in hepatocytes from regenerating liver between 1-6 hr after PHX (Obolenskaya and Decker, 1995). TNFα has been shown to be a required CK to induce hepatocyte NO production (Curran et al., 1990). IL-6 and IL-1 are also involved in the early signaling of liver regeneration. IL-6 is secreted by Kupffer cells and the secretion is stimulated by TNFα (Michalopoulos and DeFrances, 1997; Yamada et al., 1997).

IB-ii. GROWTH FACTOR PROFILES

With the increasing number of members in the GF family, more and more reports describing their production after PHX have appeared in the literature. The time profiles of various GF and CK and their mRNA expressions are summarized in Table 1. The concentration changes of these GF and inhibitors occur either before or are correlated with the hepatocyte DNA synthesis (24 hr after PHX) and proliferation after PHX *in vivo*.

Table 1. Time Profile of Various GF and CK production after PHX.

Molecule measured		Time of Increase (hr after PHX)	Site of Detection	Reference
EGFrec	mRNA	<8 hr	Liver	Fausto et al. 1995; Mullhaupt et al. 1994.
rec	binding	<8 hr		
TGFα	mRNA	2-3 to 12-24 hr	Hepatocytes	Michalopoulos and DeFrances, 1997; Fausto et al., 1995.
	peptide	24-48 hr		
HGF	mRNA	3-6 to 24 hr	Ito cells	Michalopoulos and DeFrances, 1997; Lindroos et al,.1991.
	peptide	2-6 hr	Plasma	
HB-EGF	mRNA	1.5-6 hr	Liver	Kiso et al., 1995.
		6 hr	Nonhepatocytes	
αFGF	peptide	~24 hr-7 days	Hepatocytes, nonhepatocytes	Kan et al., 1989.

Table 1 (continued).

Molecule measured		Time of Increase (hr after PHX)	Site of Detection	Reference
NE		2-4 hr	Plasma	Lindroos et al., 1991.
Insulin		↓ after PHX	Plasma	Michalopoulos and DeFrances, 1997.
Glucagon		↑ after PHX	Plasma	
Estrogen		24-48 hr	Serum	Francavilla et al.,1989, 1986.
TNFα	total RNA	1-3 hr	Liver	Oblenskaya and Decker, 1995.
	rec RNA	1-3 hr	Liver	
TNFα		3-14 hr	Serum	Shiratori et al., 1996.
IL-6		24 hr	Plasma, serum	Michalopoulos and DeFrances 1997; Shiratori et al., 1996.

Table 1 (continued).

Molecule measured	Time of Increase (hr after PHX)	Site of Detection	Reference
TGFβ1 mRNA (Inhibitor) rec	4 to 18-72 hr ↓ after PHX	Plasma Hepatocytes	Brun et al., 1988;Michalopoulos and DeFrances,1997.

IC. FLOW-DEPENDENT FACTORS

Flow-dependent factors are substances produced by vascular endothelium or from cellular metabolism under varied blood flow conditions. In the hepatic circulation, some flow-dependent factors, such as adenosine, nitric oxide (NO) and prostaglandins (PGs) have been reported to play important roles in regulating local hepatic blood flow.

IC-i. ADENOSINE

Adenosine, a metabolic vasodilator, was shown to be a key intrinsic regulator of the hepatic arterial blood flow by Lautt in 1977 and was recently reviewed (Lautt, 1996; Lautt and Greenway, 1996). He demonstrated that adenosine was produced locally into the Space of Mall (a small fluid space between the terminal hepatic artery, portal vein and the limiting plate of hepatocytes) where the terminal branches of hepatic artery and portal vein intertwine to enter the sinusoids of the liver. Adenosine was produced at a constant rate, independent of the hepatic oxygen supply. The local concentration of adenosine was adjusted by both the portal and hepatic arterial blood flow. Increased blood flow in either vessel results in an increased washout of adenosine into the blood stream in the sinusoids, reducing local adenosine concentration and causing vasoconstriction of the terminal hepatic arterioles. In contrast, decreased blood flow would reduce the adenosine washout, increasing local adenosine concentration and causing vasodilation of the hepatic arterioles (Lautt, 1985; Ezzat and Lautt, 1987).

IC-ii. NITRIC OXIDE (NO)

Niric oxide, which was originally discovered as endothelium-derived relaxing factor (EDRF) by Furchgott and

Zawadzki in 1980 (Furchgott and Zawadzki, 1980), is another extremely important flow-dependent factor not only in the hepatic blood circulation, but also in the vascular system throughout the body. NO has been observed to mediate the endothelium-dependent relaxation under a variety of stimuli: acetylcholine (Ach), adenine nucleotides, thrombin, bradykinin, hypoxia, increased blood flow and elecrical stimulation (Moncada et al, 1991). NO is synthesized from the amino acid L-arginine (L-arg) by NO synthase (NOS). It has been demonstrated that the endothelial NOS gene expression and protein level could be increased by increased shear stress, which is a frictional force on the blood vessel wall produced by lateral blood flow (Griendling and Alexander, 1996; Forstermann et al., 1994).

The vessel wall shear stress (τ) is calculated by: $\tau = \eta\gamma$, where η is the blood viscosity, γ is the wall shear rate. Wall shear rate $\gamma = (m + 2)Q/(\pi r^3)$. Q is the total blood flow, r is the internal radius of the vessel. m value depends on the flow condition: $m = 2$ in the laminar flow, $m > 2$ in turbulent flow situations (Kamika and Togawa, 1980; Macedo and Lautt, 1996). According to this relationship, reduced vessel radius by vasoconstriction with maintained constant blood flow will cause increased shear stress. From the previous work in the lab, it was demonstrated that NO was able to suppress the vasoconstriction induced by both

sympathetic nerve stimulation and exogenous norepinephrine infusion under situations where shear stress was allowed to increase (vasoconstriction with constant blood flow). This shear stress-triggered suppressive effect of NO was demonstrated in the hepatic circulation (hepatic artery and portal vein) and superior mesenteric artery, with the site of suppression being both post junctional on the smooth muscle cells (hepatic artery and portal vein) and prejunctional on the sympathetic nerve endings (superior mesenteric artery) (Macedo and Lautt, 1996; Macedo and Lautt, in press).

IC-iii. PROSTAGLANDINS (PGs)

Another important group of endothelial derived endogenous flow-dependent factors is prostaglandins, which play some functions similar to those of NO: vasodilation, inhibition of platelet aggregation and mediation of inflammatory responses (Xu et al., 1995; Sautebin et al., 1995). Prostaglandins are generated from arachidonic acid metabolism by a rate-limiting enzyme cyclo-oxygenase (COX). Two isoforms of COX have been identified: COX-1, the constitutive form, present in various organs, such as stomach, gut and kidney; COX-2, the inducible form, expressed in endothelial cells and macrophages after stimulation with

bacterial lipopolysaccharide (LPS) or cytokines (Sautebin et al., 1995; Maier et al., 1990; Masferrer et al., 1990). Substantial evidence has been obtained from *in vitro* and *in vivo* studies in the literature, demonstrating that there is an important interaction between the effects of NO and PGs. In terms of what type of interaction there is between them, it is highly controversial. An *in vivo* study on the interaction of PGs and NO in the microcirculation showed blockade of either of the two molecules significantly reduced the basal diameter of cremaster muscle arterioles. When given in sequence, only the inhibitor given first (Indo or L-NNA, N^G-nitro-L-arginine) caused increased basal tone, suggesting PGs and NO act via their own second messenger (PGs → ↑cAMP; NO → ↑cGMP) to a final common pathway to cause the smooth muscle cell relaxation (Kaley and Koller, 1995). Using isolated pressurized arterioles of rat gracilis muscle, their study also indicated that NO and PGs are co-released when flow and shear stress are increased. The NO donor, sin-1, has been shown to potentiate arachidonic acid-induced paw edema in the rat (Santebin et al., 1995). The NO production was shown to be decreased during hypoxic culture of vascular endothelium, but PGI_2 and PGE_2 were significantly stimulated, and Indo was able to restore the decreased NO production to normal level, suggesting an inhibition of NO production by

PGs (Xu et al., 1995). A variety of other studies have been reported (Salvemini et al., 1995a, 1995b; Klein-Nulend et al., 1995; Salvemini et al., 1993; Botting and Vane, 1989; Harbrecht et al., 1995; Meijer et al., 1996; Manfield et al.,1996; Curtis et al., 1996). The reasons for the variable results are unclear. It could be due to various tissue samples and approaches used in different studies and lack of direct approaches to study the signal pathways of NO and PGs.

IC-iv. FLOW-DEPENDENT FACTOR PROFILES

There are a few published papers reporting the time profile of production of NO and PGs after PHX. These results are summarized in Table 2. Some degree of discrepancy for NO production can be seen from the two different laboratories.

Table 2. Production of flow-dependent factors, NO and PGs, after PHX.

Molecule measured	Time of Increase (hr after PHX)	Site of Detection	Reference
NOS mRNA	2-4 hr	Remnant liver	Hortelano
mRNA	4 hr	Hepatocyte	et al.,1995
Citrulline	4-18 hr	Remnant liver	Hortelano et al.,1995
NO	1, 6 hr	Remnant liver	Obolenskaya et al., 1994a.
Nitrite	3-6 hr	Kupffer cells	Hortelano
	3-18 hr	Hepatocytes (*in vitro*)	et al.,1995
Nitrite	0-12 hr	Hepatocytes	Obolenskaya
	6,37-73 hr	Endothelial cells	et al., 1994b.
	30-73 hr	Kupffer cells (*in vitro*)	
PGE PGE equivalent	2-6 hr	Remnant liver	MacManus and Brace-
PGE synthesis	4-6 hr	Remnant liver	land,1976

Table 2. (continued)

Molecule measured	Time of Increase (hr after PHX)	Site of Detection	Reference
PGE_2	3, 10 hr	Plasma	Tsujii et al.,1993

ID. NITRIC OXIDE

The similarity between NO and EDRF was first suggested by Furchgott and Ignarro in 1986 (Furchgott, 1988; Ignarro et al., 1988), and experimentally confirmed in different laboratories in 1987 (Palmer et al., 1987; Hutchinoson et al., 1987 and Radomski et al., 1987; Ignarro et al., 1987). Since then, NO and NOS have been localized in most cells in the body (Davies et al., 1995). The "magic" gaseous molecule, NO, is involved in a variety of vital biological processes in different systems in the body. It was named as "the molecule of the year" in 1992 (Koshland, 1992). A large amount of literature is published each year describing NO

effects in various physiological and pathophysiological processes. Only related areas of NO study to this project will be discussed below.

ID-i. BIOSYNTHESIS OF NO

NO is a labile gaseous free radical, with a half life of seconds in solution (3-50 sec). NO is synthesized from oxidation of the terminal guanidino nitrogen atom(s) of L-arginine by nitric oxide synthase in the process of converting L-arginine to L-citrulline (Davies et al., 1995; Palmer et al., 1988). NO rapidly forms NO_2 in the presence of oxygen. NO_2 then forms nitrite (NO_2^-) and nitrate (NO_3^-) in solution, which are almost inactive (Moncada et al., 1991). In the vascular system, NO formed in the endothelial cells diffuses into the smooth muscle cells and binds to soluble guanylate cyclase resulting in increased intracellular cGMP and smooth muscle relaxation.

ID-ii. REGULATION OF NITRIC OXIDE SYNTHASE (NOS)

Three types of NOS have been identified and their genes cloned: Type I: brain NOS, bNOS (also referred as c-NOS, bc-NOS or n-NOS, Forstermann et al., 1994); Type II: inducible

NOS, iNOS; Type III: endothelial NOS, eNOS. Type I is a constitutive isoform, type II is inducible and type III regulated. These isoforms have about 50% amino acid homology. The characteristics of these NOSs are summarized in Table 3 (Griendling and Alexander, 1996; Davis et al., 1995). The iNOS has also been found in Kupffer cells and hepatocytes in the liver.

Due to the important roles of NO in various physiological and pathological responses, much work has been focusing on the regulation and expression of NOS genes in recent years. Analysis of the promoter region of the bovine eNOS gene (75% homology with the human gene) showed there were many sequences in the eNOS promoter region for various transcriptional factor binding, CK and shear stress regulation: an AP-1 site, an NF-1 site, a TNF - responsive element, two sterol regulatory elements, three acute-phase response elements, five Sp1 sites, fifteen estrogen half-palindromes and nine shear stress response elements (Venema et al., 1994; Marsden et al., 1993). The presence of shear stress response elements in the eNOS gene could explain the blood flow/shear stress-induced NO production by the vascular endothelium. Also, the TNF-responsive element in the eNOS gene sequence has provided a genetic link for the interaction between TNFα and NO production.

Endotoxin, IFNγ, IL-1 and TNFα have been found to induce

Table 3. Isoforms of NOS

Characteristic	*Type I:* Neuronal	*Type II:* Inducible	*Type III:* Endothelial
Cell Types	Neuronal	Macrophage, Leukocyte, VSMC, etc.	Endothelium only
mRNA	4.3 kb	3.4 kb	3.6 kb
Molecular mass	160 kDa	1300 kDa	135 kDa
Calcium	Dependent	Independent	Dependent
Inducibility	Constitutive	Inducible	Regulated
Subcellular location	Cytosolic	Cytosolic	Membrane-associated
Chromosome	12	17	7
Myristoylation	No	No	Yes

VSMC, vascular smooth muscle cells.

(Adopted from Griendling and Alexander, 1996.)

iNOS expression, while glucocorticoids, TGFβ, IL-4 and IL-10 inhibit the iNOS mRNA expression. Once activated, iNOS synthesizes NO in nanomolar concentration for a sustained period of time. cNOS synthesizes picomolar concentrations of NO for a short period of time (Davies et al., 1995).

ID-iii. GENE EXPRESSION REGULATED BY HEMODYNAMIC FORCES

In addition to eNOS gene, a variety of other genes in the endothelial cells have recently been found to be inducible by shear stress and other hemodynamic mechanical stresses (pressure, stretch). These genes include c-myc, c-fos and c-jun protooncogenes, adhesion molecules ICAM-1 and VCAM-1 genes, TGFβ and tissue factor genes. Shear stress could also increase the transcriptional factor binding of NFκB and AP-1 to genes such as endothelin-1, thrombomodulin and bFGF to modulate their expression in the endothelial cells (Lan et al., 1994; Griendling and Alexander, 1996; Davies and Tripathi, 1993). Increased portal flow *in vitro* in isolated perfused rat liver has also been shown to induce increased c-myc oncogene expression (Isomura et al., 1993).

Hemodynamics has also been found to affect vascular cell growth, leukocyte migration and adhesion, vascular tone and hemostasis (Grindling and Alexander, 1996; Resnick et al.,

1993).

ID-iv. NO SYNTHESIS INHIBITORS AND NO DONORS

A group of molecules with structural analogy to NOS substrate, L-arginine, have been found to be able to inhibit NO generation *in vivo* and *in vitro*. These include: L-NMMA (N^G-monomethyl-L-arginine), L-NIO (N-iminoethyl-L-ornithine), L-NA (N^G-nitro-L-arginine), L-NAME (N^G-nitro-L-arginine methyl ester) and L-NAA (N^G-amino-L-arginine). L-arg analogs inhibit NO generation by competing for NOS with L-arg, but no NO is released since the terminal guanidino nitrogen atom of the L-arg molecule is replaced by substitute groups. These compounds are extremely useful tools to study NO effects in various processes and are extensively used in both *in vitro* and *in vivo* studies (Moncada et al., 1991; Moncada and Higgs, 1993).

Another group of extremely useful agents in NO study is nitrovasodilators or NO donors, which act after their conversion into nitric oxide (Moncada and Higgs, 1993; Feelisch, 1991). Among these agents, commonly used ones include glyceryl trinitrate (GTN), sodium nitroprusside (SNP), S-nitroso-N-acetyl-DL-penicillamine (SNAP), 3-morpholino-sydnonimine (SIN-1), its prodrug molsidomine (SIN-

10), and mesoionic 3-aryl substituted oxatriazole-5-imine derivatives GEA3162 and GEA3175 (Kankaanranta et al., 1996).

ID-v. DUAL EFFECTS OF NITRIC OXIDE ON THE LIVER

Among its multiple biological effects, important functions of NO are its vasodilatory effects in different vascular beds in the body, the regulatory effects on platelet functions, neurotransmitter effects in the central and peripheral nervous systems and its mediator effects in immunological reactions and inflammatory responses (Davies et al., 1995; Moncada et al., 1991; Moncada and Higgs, 1993).

One interesting aspect in the NO effects, which is worth discussing, is the dual effect of NO on the hepatic system. There are indications that the presence of increased NO may be both hepatotoxic and hepatoprotective.

Using *in vitro* coculture of hepatocytes and Kupffer cells, it was found that there was a Kupffer cell mediated decrease in hepatocyte protein synthesis under LPS stimulation in the culture. This decreased protein synthesis did not cause hepatocellular death. It was reversible, associated with nitrates, nitrites and citrulline production and blocked by L-arg analogs (West et al., 1985; 1988). These results suggested a role of NO in the control of hepatic

function (Stark and Szurzewski, 1992). NO may act as an antioxidant and interact with superoxide anion to produce a less toxic radical species. In an endotoxin-induced hepatic necrosis murine model, increased NO production was found to protect the liver from damage and L-arg analogs markedly increased the hepatic injury (Billiar et al., 1990; Harbrecht et al., 1992; Kuo et al., 1994). This observation could be explained by the antioxidant effect of NO (Stark and Szurzewski, 1992). In contrast, it was also shown that NO reaction with superoxide anion could produce a more toxic peroxynitrite anion causing cytotoxicity (Becknman et al., 1990). In an acetaminophen-induced hepatotoxicity model, inhibition of CK-mediated NO production potentiated acetaminophen damage to the liver (judged by increased aspartate aminotransferase level). This hepatoprotective effect of NO was found due to its role in the maintenance of glutathione (a strong antioxidant) homeostasis (Kuo and Slivka, 1994).

These observations were interpreted as: overproduction of NO in the presence of excessive free radical production may be hepatoprotective; Whereas in the presence of normal free radical production, NO may contribute to hepatotoxicity (Davies et al., 1995).

A different type of dual effect of NO has been observed in our experimental studies on liver regeneration, which will

be discussed in a later section.

ID-vi. L-ARGININE

L-arginine is a basic amino acid participating in synthesis of various proteins in the body. In addition, L-arg is an essential component for the synthesis of urea, creatine and creatinine. It can also influence hormone release and pyrimidine synthesis. The effect of hormone release by L-arg is profound, including catacholamines, corticosterone, glucagon, growth hormone, insulin, prolactin and somatostatin, by various mechanisms at different levels in different pathways (Reyes et al., 1994a,b; Alba-Roth et al., 1988; Barbul et al., 1983; Castellino et al., 1987; Mulloy et al., 1982).

Furthermore, L-arg also serves as the substrate of NOS for NO synthesis. These multiple participations have placed L-arg at the centre of the interaction of different metabolic pathways and added more complexity to the interpretation of experimental results where L-arg is involved (Reyes et al., 1994a).

IE. HYPOTHESES OF THE PROJECT

IE-i. Hypotheses:

Based upon evidences in the literature, the hypothesis was first proposed by Dr. W. Wayne Lautt in 1991. After some preliminary results had been obtained, the original hypotheses were further modified and established as follows: 1. Total hepatic blood flow determines hepatic parenchymal cell mass; the immediate increase in blood flow to liver mass ratio that occurs upon PHX serves as the trigger to initiate the onset of a complex cascade of events leading to compensatory hyperplasia; 2. The increased ratio of blood flow to liver mass alters the concentration of one or more key flow-dependent factor (s) that leads to production of proliferative factors (PF, mixture of various growth factors, cytokines and hormones which were increased in the plasma following PHX) reflecting the onset of the regenerative process.

The rationale of the hypotheses is as follows. The liver cannot control the portal blood flow which is the sum of outflows of the splanchnic organs (Lautt and Greenway, 1987). Upon PHX, 2/3 of the liver tissue is removed. Although the

total hepatic blood flow does not increase, the ratio of blood flow to liver mass is immediately increased, with a maximum portal flow and minimum hepatic arterial flow present due to the maximum adenosine washout by the portal flow (the maximal level of the Hepatic Arterial Buffer Response, Lautt, 1985; Ezzat and Lautt, 1987). This hemodynamic response could lead to the trigger of regeneration by two different mechanisms: 1. Adenosine serving as the suppressor of the trigger, the maximal washout of which leads to the initiation of the cascade; 2. Production of regenerative stimulators, possibly NO and PGs, by increased shear stress in the liver due to the increased flow/mass ratio following PHX. NO can be released from one or more liver cell type including the endothelial, Kupffer cells and hepatocytes and PGs can be released from both endothelial and Kupffer cells. NO and PGs trigger the onset of the liver regeneration cascade by inducing PF production in the plasma. The increased PFs prime the quiesent (G_0) hepatocytes to enter the cell cycle (G_1) and proliferate under the control of a large variety of early genes expressed in the hepatocytes.

IE-ii. Objectives:

1. To develop an *in vitro* hepatocyte primary culture bioassay to measure the plasma PF levels *in vitro* to

indicate that the regeneration cascade has been initiated, and to indicate the effects of flow-dependent factors on PF production after the initiation of the cascade.

2. To test the hypothesis that blood flow-dependent factors control the PF production in the 2/3 PHX rat plasma.

3. To confirm the blood flow-dependent nature of PF production by using selective portal vein ligation as an analogous hemodynamic stimulus to that produced by PHX and its ability to generate PF over a similar time frame.

4. To test that the control of PF production by flow-dependent factors leads to the control of the whole process of liver regeneration.

II. MATERIALS AND METHODS

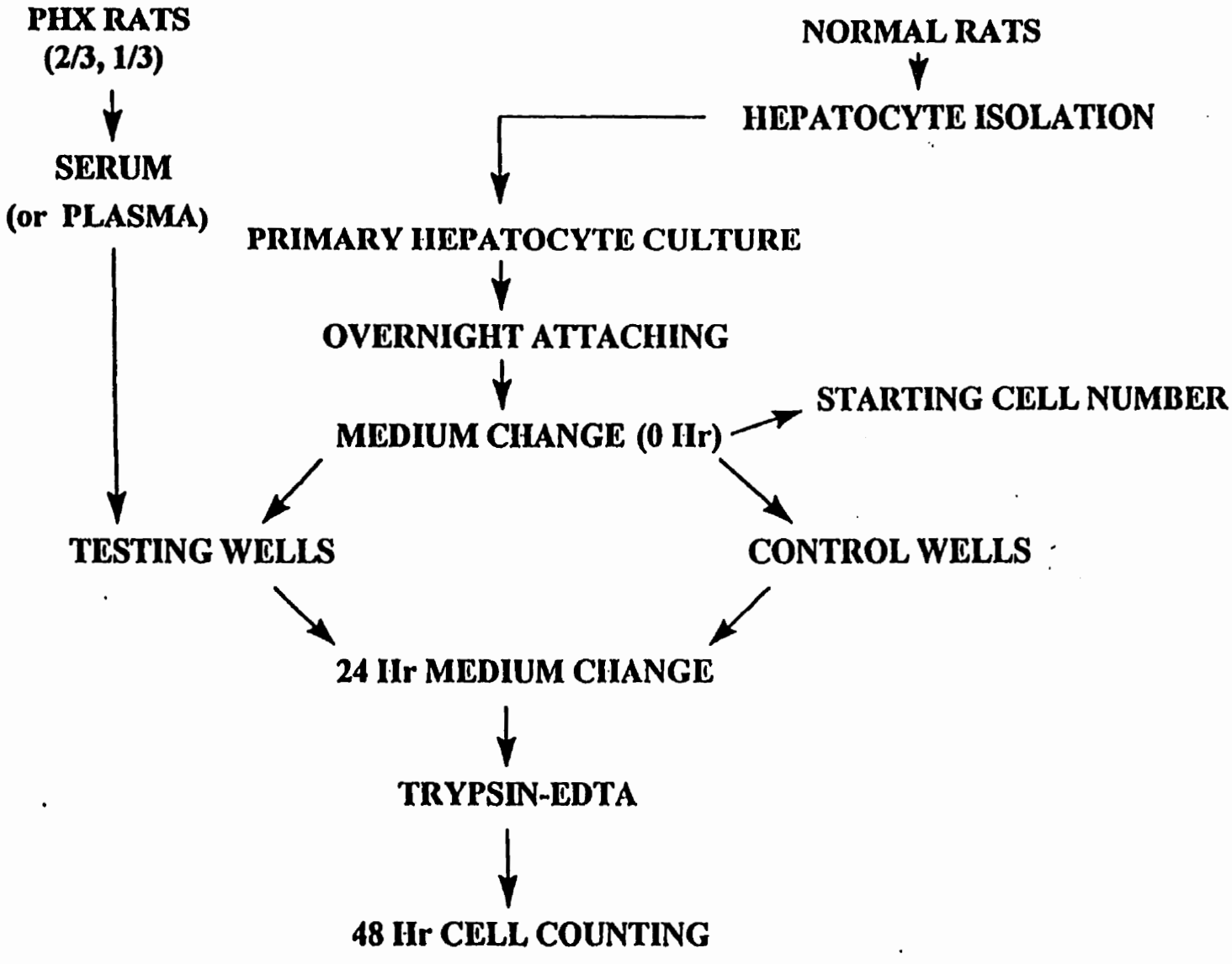
IIA. DEVELOPMENT OF A HEPATOCYTE BIOASSAY *IN VITRO*

An overview of the *in vitro* hepatocyte bioassay, which measures hepatocyte proliferation by counting the net viable cell number increase, is presented in Figure 2.

IIA-i. MATERIALS

Male Sprague Dawley (SD) rats were supplied from a breeding stock maintained by the University of Manitoba (original stock from Charles River Laboratories, Inc. Canada). Sodium pentobarbital (Somnotol) was from MTC Pharmaceuticals (Cambridge, Ontario). Modified Ca^{++}-Mg^{++} free Dulbecco's Phosphate Buffered Saline (DPBS, 2.68 mM KCl,1.47 mM KH_2 PO4, 136.9 mM NaCl, 8.1 mM Na_2 HPO_4) was prepared in our laboratory. Swim's S-77 medium, collagenase (Type IV), Dulbecco's Modified Eagle's Medium (DMEM, D2902), Nutrient Mixture F-12 (Ham, N6760), HEPES, penicillin G, streptomycin sulfate, rat tail collagen (Type VII), epidermal growth factor and trypan blue were from Sigma Chemical Co. (Oakville, Ontario). Six-well plates (Falcol) were from VWR

Figure 2. **Overview of The Hepatocyte Bioassay**



Scientific of Canada Ltd. Insulin-Toronto (regular, CZI) was from Connaught Novo Nordick Inc., Missisauga, Ontario. Trypsin-EDTA was purchased from GIBCO BRL Burlington, Ontario.

IIA-ii. METHODS

a. Partial Hepatectomy and Plasma (or Serum) Preparation

The rat liver is composed of 4 lobes: the left lateral lobe (comprising about 30% of the total liver weight), median lobe (40%), right lateral lobe and caudal lobes (caudal lobes containing 2 omental processes) that together form the remaining 30% of the liver (Steiner and Martinez, 1961).

Male SD rats of 225-250 g body weight were fed *ad libitum* with standard laboratory rat chow. Rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.). Clean but not aseptic technique was applied during the surgery. Tracheotomy was performed and a catheter of PE 240 polyethylene tubing (Intramedic, Clay Adams, Becton Dickinson) was inserted into the trachea to facilitate the respiration. The right femoral artery and vein were cannulated with PE 50 polyethylene tubing. The arterial blood pressure was monitored through the femoral artery (FA)

using an R-611 SensorMedics Dynograph Recorder during the surgery. 0.5 ml saline/100g body weight/hr, containing 1 mg/ml sodium pentobarbital, was infused through the femoral vein (FV) by a Syringe Infusion Pump (Harvard , Model 2720) to supplement fluid loss and maintain the anesthetic level. The catheters were flushed with heparin (200 U/ml) to prevent blood clotting in the catheter. At different times before and after PHX, 0.5 ml saline or drugs were administered by FV injection to the rat to modulate the flow-dependent factor levels and PF production after PHX.

The PHX surgery was adopted according to the method by Higgins and Anderson (Higgins and Anderson, 1931). A median-line incision (3 cm) was made on the abdomen posteriorly from the xiphoid process of the sternum. The left lateral lobe was gently exteriorized and tied around the base of the lobe with one tie at the bottom side and two ties on the top side of the lobe using surgical silk (Ethicon, size 0). The lobe was resected 1 mm above the tie to prevent the tie from sliding off. The wound surface was blotted with clean gauzes. The median lobe was then exteriorized, tied and resected in the same way as the left lateral lobe. The incision was sutured with 2 layers of the muscle and skin. At various time points after PHX, the incision was opened, the diaphragm was cut and a blood sample (5-10 ml per rat) was drawn from the right ventricle of the heart within 2 min to exclude the effects of

hormonal and sympathetic nervous system responses to hemorrhage (Lautt et al., 1982), using a 21 G 1 ½ in needle and a 10 ml sterile syringe. In a tissue culture hood, the blood was transferred to a sterile centrifuge tube and centrifuged at 2500 g for 20 min. Plasma was collected and stored at -20°C before testing (< 1 month storage).

For serum preparation, no heparin was administered during surgery. The blood was taken in the same way and allowed to clot on ice for 10 min, then spun down. Serum was collected into a sterile tube and stored at -20°C before testing. The plasma or serum samples were tested for the PF levels in the hepatocyte primary cuture.

b. Rat Liver Perfusion and Hepatocyte Purification

The two-step collagenase perfusion and hepatocyte purification method was modified from Seglen (Seglen, 1976). Approximately 300 g SD male rats were anesthetized with sodium pentobarbital, i.p., 65 mg/kg. The abdomen was cleaned with 70% alcohol. A "partial sterile" technique was used. The perfusion process was non-sterile. All the procedures after perfusion were sterile.

An 18 G, 1-1/4 in. (32 mm) i.v. catheter (OptiVa™, Critikon) was used as the portal vein catheter tied to the

vein by surgical silk. The perfusion buffer was drained through a catheter (PE 240 polyethylene tubing) inserted into the inferior vena cava via the right atrium of the heart. The liver was first perfused with 400 ml non-recycled oxygenated (O_2 bubbling into the buffer using pure oxygen) Ca^{++}-Mg^{++} free DPBS (pH 7.4), containing 0.49 mM EDTA to reduce Ca^{++} in the liver tissue using a 6-600 RPM Masterflex pump (Cole-Parmer Instrument Co.). 100 ml DPBS without EDTA was perfused to wash out the EDTA in the system. Then the liver was perfused with 100 ml recycled oxygenated collagenase/Swim's 77 (0.25 mg/ml) with 5 mM Ca^{++}, for 10-15 min. The pH of the recycling collagenase solution was monitored using a pH meter and maintained at pH 7.4-7.5 by adding 0.1 M NaOH during the perfusion period to maintain the optimal collagenase activity (Seglen, 1976).

Upon finishing perfusion, sterile technique was strictly applied. The liver was rinsed thoroughly with 10-20 ml sterile DMEM/F-12 (1:1) medium. In the tissue culture hood, the liver was transferred into a sterile petri dish containing fresh medium. The liver capsule membrane was slit using two forceps and the cells were released into the medium by gentle shaking of the liver. No other instruments were used to liberate the liver cells in order to minimize mechanical damage to the cells. The cells were maintained on ice during the isolation and later steps to slow down the

metabolic rate and reduce aggregation of the isolated cells (Seglen, 1976). Two Spectra/Mesh N filters (70 µm, 40 µm, Spectrum), which were sterilized by soaking in 70% alcohol overnight, were used to filter isolated liver cells to exclude tissue chunks and cell debris. The filtered cell suspension was further purified by low speed differential centrifugation at 300 rpm, for 3 min at 4°C in a bench-top centrifuge (Beckman, GS-15R) using a 50 ml graduated sterile tube. The supernatant, which contained mostly nonparenchymal cells, damaged cells and subcellular debris was discarded by aspiration (Seglen, 1976). The cell pellet was gently mixed with freshly added medium (40-50 ml), and the centrifugation procedure was repeated 3 times.

Viable cell concentration and percentage of non-hepatocytes was determined by the trypan blue exclusion, a conventional method to distinquish the dead from the viable cells (viable cells unstained, dead cells stained blue), using a hemocytometer (Fisher Scientific, 35-058) and microscope (Nikon, TMS). 20 µl trypan blue (0.4%) was added into 20 µl cell suspension (dilution factor = 2), which was mixed and loaded onto the hemocytometer. All corner squares (64 squares/chamber) of the 2 chambers were counted (128 squares total; each square is 1 nl, 128 nl x 7.813 = 1 µl). Viable cells/µl = viable cells per 128 squares x 7.813 x 2 (dilution factor). The final concentration was the average

value of 3 separate countings. Viability (%) = viable cells/ (viable + dead cells) x 100%. Nonhepatocyte (%) = nonhepatocyte/total cells x 100%. The hepatocyte viability was usually between 80-90%. Non-hepatocyte contamination ranged from 1-5%, with an average at ~1%, according to the cell size and microscopic morphology. The final cell suspension was an evenly distributed solution containing mostly single cells, couplets, triplets or quadruplets with few large cell aggregates.

c. Primary Hepatocyte Culture

Six-well plates were coated with rat tail collagen (0.8 mg/ml double distilled H_2O with 0.1% acetic acid) at 20 µl / well using a sterile glass bar and allowed to dry in the hood. The culture medium (~2 ml/well) DMEM/F-12, 1:1, supplemented with 25 mM sodium bicarbonate, 10 mM HEPES, 100 U/ml penicillin G and 0.1 mg/ml streptomycin, was added to the wells. 200,000 viable cells were seeded into each well from a cell suspension constantly mixed by agitation. The final cell concentration in the well was 100,000 cells/ml, 2 ml/well (optimized concentration). The plates were left in the humidified incubator (NuAire US Autoflow, CO_2-Water-Jacketed) at 37°C with 5% CO_2 overnight (about 18 hr) to

allow cell attachment to the bottom of the wells. At the end of the attachment period, the medium was changed. The culture plates were mildly shaken and medium was aspirated. The wells were rinsed once with 1 ml medium and aspirated. The counts of the viable starting-cell-number were made from 3 randomly selected wells in the culture as described below. Under our conditions, 10-20% of the seeded viable cells are well attached (calculated from the starting cell numbers), reaching a desired final low density culture of approximately 2000 cells/cm^2 (Michalopoulos et al., 1982). The viability of attached cells was over 90%. Insufficient collagenase digestion (4-6 min) during liver perfusion could generate "sticky" cells resulting in increased cell attaching.

Serum or plasma samples were added with 2 ml fresh medium to wells to a final concentration of 10% (200 µl serum/well, 3 wells/sample). Heparin (35 Units/ml final) was added to plasma containing wells to prevent the medium clotting during the culture. Cells grown in the plain medium (serum free) were used as the negative control. Cells in the plain medium plus 100 ng/ml EGF and 20 mU/ml insulin (final concentration) were used as the positive control (McGowan et al., 1981; Francavilla et al., 1986). The medium was changed by aspiration at 24 hr with no rinse. Our assay assesses the increase of viable cells in each well. Therefore, the low

number of spontaneously detached dead cells were not taken into account. At the end of the 48 hr culture, attached hepatocytes were harvested for cell counting. Contamination of the culture was infrequent with these conditions. In case of contamination, the contaminated wells were discarded and the sample was retested in a different culture.

d. Hepatocyte Harvesting and Counting by Hemocytometer

Attached viable cells at the end of the attaching period and 48 hr cultures were counted using the hemocytometer. The medium in each well was aspirated. Serum or plasma-containing wells were rinsed once with saline and aspirated to wash off various plasma proteins. A minimum amount of trypsin-EDTA (GIBCO BRL, 25200-072) of 400 µl was added to each well to just cover the bottom of the well. The plate was covered and left in the incubator for approximately 1 min, then removed from the incubator and checked under the microscope. The trypsin digestion was assessed and digestion time was adjusted based on microscopic examination. The plate would be put back for longer digestion when necessary. When the majority of the cells became rounded and ready to detach but still remained attached, the digestion was stopped. The plate was placed on ice immediately and

serum (SD rats , 40 µl , final concentration 10%) was added into each well to inhibit further trypsin digestion of the cells (440 µl total volume per well).

With the plate maintained on ice, the cells were detached using a pasteur pipet to gently blow the cells from the bottom of the well. The cell detaching was also monitored using the microscope. After all the cells were lifted, the cell suspension was transferred into a 1.5 ml Eppendorf tube and kept on ice for an immediate counting with no centrifugation or trypsin removal in order to minimize handling artifacts leading to cell number variation. With the low temperature and serum present, further trypsin digestion of the cells was prevented. The completeness of cell removal from each well was checked under the microscope. If some remaining cells were found, the cell suspension was put back into the well and the pipeting process was repeated until all cells had been removed. Cell aggregation was minimized by keeping the cells on ice and pipetting the cell suspension.

In general, 1-2 minutes of trypsin digestion was sufficient. The growth status of the cells affects the length of digestion required, with well stimulated cells needing longer digestion and poorly stimulated cells needing less.

The Eppendorf tube containing the cell suspension was well mixed before counting. 40 µl of cells + 10 µl of 0.4%

trypan blue were well mixed in a plate (dilution factor = 1.25) and loaded onto the hemocytometer. Under the microscope, the even distribution of cells on the hemocytometer was first verified. Then large viable hepatocytes in all of the 128 corner squares (sq) were counted (dead cells and small but rarely seen nonhepatocytes were not counted). At the end of the counting, the bioassay was finished and left-over cells in the Eppendorf were discarded. Total viable cells per well was calculated as: Cells / well = cell no. per 128 sq (nl) x 7.813 (128 nl x 7.813 = 1 µl) x 1.25 (dilution factor) (= cells / µl) x 440 µl (total volume in each well). The increased viable cell counts at the end of the culture indicated that the morphologically viable cells (by Trypan blue) exibited functional viability of cell proliferation.

e. Data Expression

Our experiments were designed to test the growth stimulatory effects of various serum (or plasma) samples from PHX rats using the cultured normal rat hepatocytes *in vitro*. Each sample was tested in triplicate wells. The mean of 5 separate cell counts was used as the count for each well. The mean of the triplicate wells was the final cell number of

each sample tested. The net cell proliferation was calculated by subtraction of the starting attached cell number from the final number of each sample after 48 hr culture. The data were compared by ANOVA followed by Tukey's honestly significant difference (HSD) test for multiple comparisons.

IIB. *IN VIVO* METHODS

The 2/3 PHX rats (male SD, 225-250 g) were allowed to recover. Liver weight increase was measured at 48 hr. Rats were fed with the laboratory rat chow *ad libitum* before surgery. The surgery was performed under sterile conditions between 10:00 and 14:00 hr. Rats were anesthetized using sodium pentobarbital (6.5 mg/100g body weight,i.p.) anesthesia. 15 min before PHX, 0.5 ml of saline (control) or drug (L-NAME) was given, i.v. (tail vein). PHX was at time 0. The procedure of PHX was the same as described in Section IIA-ii.a. for the PHX rat serum preparation. No cannulation of any blood vessel nor tracheotomy was performed. An osmotic pump (preactivated in saline in the 37°C incubator overnight according to the Manufacture's instruction, Alzet, Model 2ML1, 10.0 µl/hr) containing saline or drug was implanted s.c. on the back of each rat following PHX. 1 hr after PHX,

the second i.v. injection (0.5 ml saline or drug) was given. Banamine (analgesic, 2.5 mg/kg/rat) was injected s.c. to both controls and testing rats after surgery.

Post surgical care was given (Surgical Guidelines of the Central Animal Care Services at the University of Manitoba and the Canadian Council on Animal Care; Protocols were approved by the Animal Use Committee of the University of Manitoba). The rats were fed *ad libitum* and the cages were maintained on 37°C heating pads (Solid State T/pump, Gaymar) postsurgically.

With the rats anesthetized at 48 hr, the liver was removed, the surface slashed to blot the blood and the liver mass weighed. Liver regeneration (%) = (48 hr liver wt - Remnant wt) / Resected wt x 100%. Remnant liver wt = Standard total liver wt corrected for body wt (control rats, n=43) - Resected wt. (See Results section IIIC-i and Fig. 12).

IIC. PHARMACOLOGICAL MANIPULATION OF FLOW-DEPENDENT FACTORS

The levels of the flow-dependent factors, adenosine, NO and prostaglandins were pharmacologically manipulated in both *in vitro* and *in vivo* experiments by administering adenosine, L-NAME, L-arg and indomethacin (an NSAID, cyclooxygenase

inhibitor) via different routes (All purchased from Sigma). Their roles in triggering liver regeneration were tested through their effects on PF generation in the plasma and on liver weight increase after PHX. Sham operated rats were incorporated in the PF tests. The sham-rats underwent the same surgical procedures (as PHX rats) with liver manipulation only without PHX and drug administration.

IIC-i. MANIPULATION OF ADENOSINE

For PF effects, adenosine (0.4 mg/kg/min/0.02 ml) was infused intraportally from 5 min before to 4 hr after PHX, using a 24 G i.v. catheter (OpTiVa™, Critikon), sealed to the portal vein by a Histoacryl tissue glue (Braun Melsungen AG, Germany), and connected to a 10 ml syringe driven by an infusion/withdrawal pump (Harvard Apparatus) at 0.4 mg/Kg/min/0.0206 ml. Blood samples were taken at 4 hr after PHX to be tested in the bioassay for the PF levels.

IIC-ii. MANIPULATION OF NO

The level of NO was manipulated by the NOS inhibitor and substrate: L-NAME and L-arginine. L-NAME was given by 3 i.v. (FV) bolus injections, 2.5 mg/kg in 0.5 ml saline each, at

0 (before PHX), 1 and 2.5 hr after PHX. L-NAME + L-arg [(2.5 mg/kg L-NAME + 75 mg/kg L-arg) in 0.5 ml saline] (1:30) was administered by the same route and time interval as L-NAME alone. L-arg alone (75 mg/kg in 0.5 ml saline) was given in the same way. Blood was drawn at 4 hr after PHX from these rats. Plasma was tested in the bioassays for PF levels.

For liver weight regeneration effects, 2 x L-NAME 2.5 mg/kg in 0.5 ml saline were injected i.v.(tail vein) at 15 min before and 1 hr after PHX. Osmotic pumps contained 2 L-NAME doses: low dose, 15 mg/kg/24 hr; high dose, 30 mg/kg/24 hr.

The PHX control group received 2 i.v. injections of saline at the same time as the L-NAME group. Osmotic pumps contained saline only. Liver weight was measured for all rats at 48 hr after PHX.

IIC-iii. MANIPULATION OF PGs

Indomethacin was administered i.v., 7.5 mg/Kg in 0.2 ml saline, single dose, at 30 min before PHX. Plasma samples were taken at 4 hr and tested in the *in vitro* bioassay to assess the PG effects on PF production.

The results were compared by ANOVA followed by Tukey's

honestly significant difference (HSD) tests for all groups.

IID. SUMMARY OF THE PHX METHODOLOGY

The overall methodology of the *in vitro, in vivo* methods and manipulation of flow-dependent factors in the system is summarized in Figure 3.

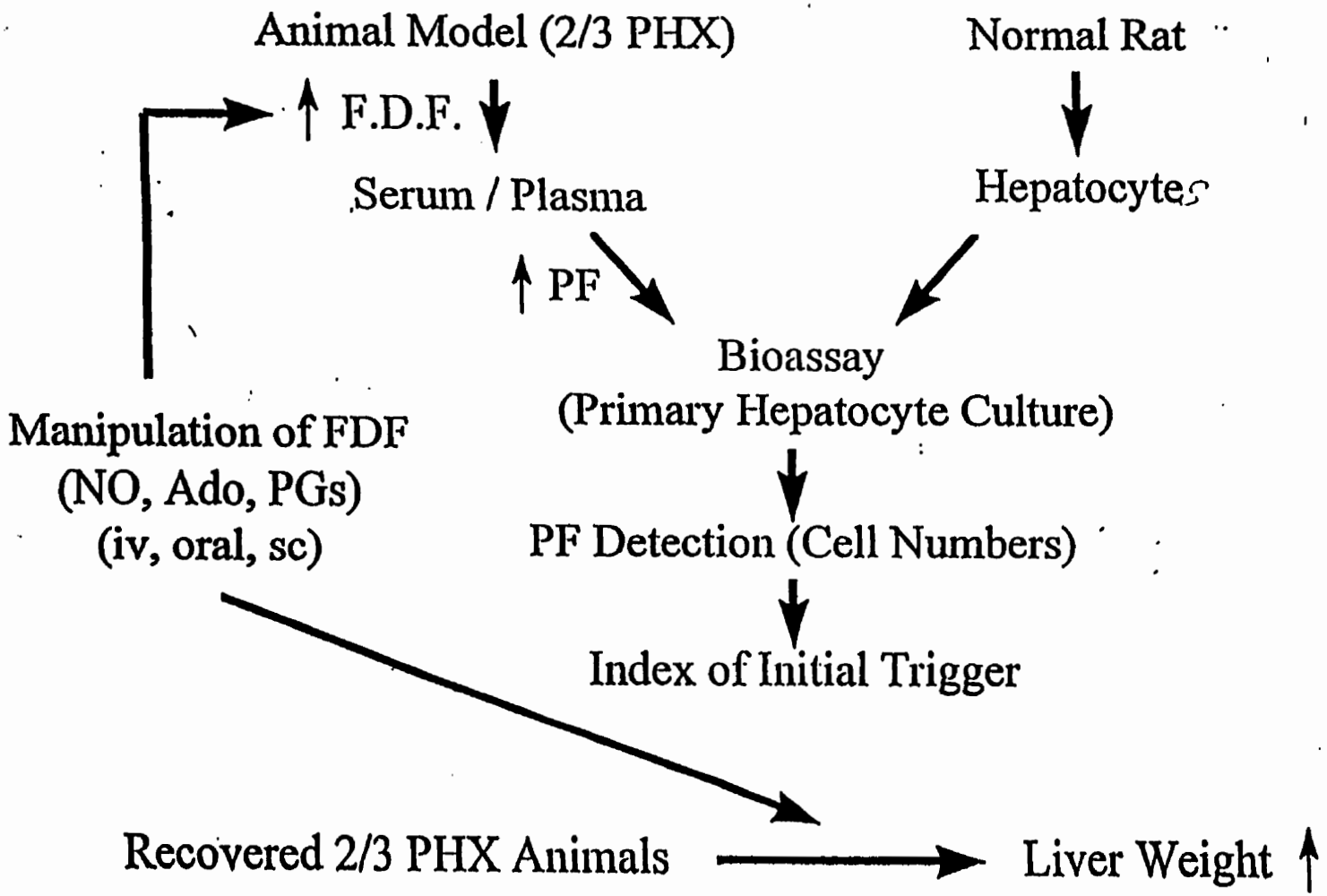
IIE . PORTAL VEIN LIGATION (PVL)

IIE-i. PVL FOR PF MEASUREMENT

As mentioned in Section IA-iii.c., PVL is an analogous model to PHX, which is less often used in liver regeneration studies, compared with the PHX model. The PVL procedure was initially developed for various purposes (see Section IA-III.c.). Systemic and comprehensive morphological studies at the gross and cellular levels in rabbits and rats have been reported (Rous and Larimore, 1920; Steiner and Martinez, 1961).

No reports are available on using PVL model to compare the initial triggering process of liver regeneration with the PHX model. The detailed description of the surgical procedure of PVL is also lacking in the literature.

Figure 3. **IID. Summary of The PHX Methodology**



We have used the PVL model to study the PF production at 4 hr after PVL in order to test if our hypotheses on the PHX model also apply to the PVL model. Our method of PVL was modified from that of Steiner and Martinez (Steiner and Martinez, 1961), as described below.

Fed rats (225-250g) were anesthetized with sodium pentobarbital, 6.5 mg/100g, i.p., between 10:00 - 15:00 hr. Tracheotomy and cannulation of the femoral artery and vein was performed to facilitate breathing, monitor the arterial blood pressure and provide fluid supplement. The catheters were flushed with heparin (200 U/ml saline). A median incision (4-5 cm) was made. The left lateral and median lobes of the liver were carefully flipped out of the abdominal cavity and rested on the thoracic wall during the surgery. The rat was allowed to stabilize for a few minutes before ligation started. After the arterial pressure was back to normal, the left branch of the portal vein was carefully dissected from the connective tissue, nerve bundles and hepatic artery under a microscope (Carl, Zeiss), using a pair of small curved-tip forceps. After ~ 1 mm in length of the left branch was cleared out, a thread was inserted on top of the branch to pull the connective tissue aside, which contains the hepatic artery (on the left of the branch), bile duct (on the right of the branch) and hepatic nerves. Another thread was inserted under the left branch and one tie

was made, excluding the bundle of hepatic artery, bile duct and hepatic nerves.

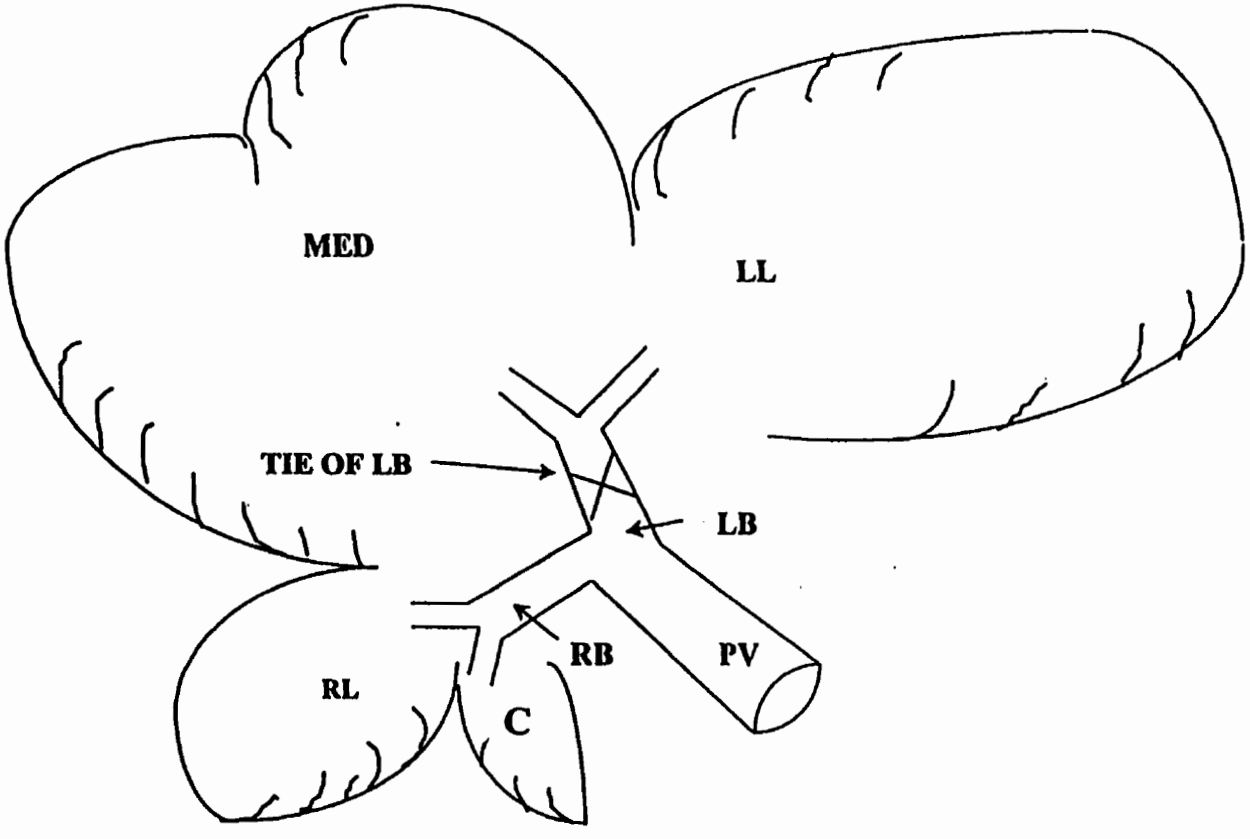
The left lateral and median lobes were returned back into the abdominal cavity without resection. The incision was closed using stainless steel wound clips (MikRon, Autoclip, Clay Adams). Fluid and anesthetic supplement (1 mg/ml pentobarbital in saline) were given via the femoral vein at 0.5 ml/100g/hr. The arterial pressure was monitored on an R-611 Sensormedics Dynograph Recorder during the surgery. The anatomic location of the portal vein left branch ligation is shown in Figure 4.

At 4 hr after the ligation of the portal vein left branch, the diaphragm was cut, blood was drawn from the right ventricle of the heart, and spun at 2500 g in a GS-15R bench-top centrifuge (Beckmen). Plasma was collected and filter-sterilized and kept at -20°C for the hepatocyte bioassay test. ~ 1 ml of India ink was injected into the portal vein to examine the proportion of ligated (unstained) vs unligated (stained) lobes. The ligated (left lateral and median lobes) and unligated (right lateral and caudate lobes) were cut, surface slashed and blotted and weighed separately.

IIE-ii. LIVER HYPERPLASIA IN RECOVERED PVL RATS

The surgery was conducted under sterile conditions as for the recovery PHX rats. No tracheotomy nor cannulation was performed. 15 min before PVL, 0.5 ml saline was injected i.v. (tail vein). PVL surgery was the same as described above. The incision was sutured using Dexon II, 3-0, C-6 sutures (Davis + Geck, 26 mm). An osmotic pump (containing saline) was implanted s.c. on the back of the rat as in PHX. Analgesic was given (Banamine, 2.5 mg/kg, s.c,) after PVL. 1 hr after PVL, 0.5 ml saline was injected. The rats were allowed to recover under the same condition as for recovered PHX rats. 48 hr after PVL, rats were anesthetized. The abdomen was opened, blood was drawn from the right ventricle of the heart to facilitate the drainage of the blood from the liver. The portal vein was punctured with a 26 G needle to inject 1 ml India ink. The ligated and unligated lobes were cut, surface slashed and blotted and weighed separately. The liver lobe regeneration or atrophy (%) was expressed as the percentage weight of the lobe to the total liver weight: Ligated (or unligated) lobe wt / total liver wt x 100 %.

Figure 4. Anatomy of PVL



PV: Portal vein LB: PV Left Branch RB: PV Right Branch
LL: Left Lateral Lobe MED: Median Lobe RL: Right Lateral Lobe C: Caudate Lobe

III. RESULTS

IIIA. OPTIMIZATION OF THE HEPATOCYTE BIOASSAY

A series of experiments was conducted to optimize the *in vitro* assay conditions in the hepatocyte primary culture in order to test the index of the initiation of liver regeneration cascade, the PF production.

IIIA-i. OPTIMAL CULTURE TIME

Isolated normal hepatocytes were cultured as described. 3 samples were tested: negative control (plain medium), positive control (medium plus EGF and insulin) and pooled serum (10% final concentration) from 2/3 PHX rats (n=4) at 2.5 hr post PHX. Three wells of each sample were harvested for cell counting at various time points (0-96 hr), as indicated in Figure 5. The results showed a peak of cell proliferation at 48 hr culture in both EGF/insulin and PHX rat serum stimulated wells, suggesting 48 hr is the most sensitive time for hepatocytes to show stimulatory effects present in the culture medium. Normal serum did not show a clear pattern for optimal culture time (data not shown). The results indicate that EGF and insulin can directly stimulate

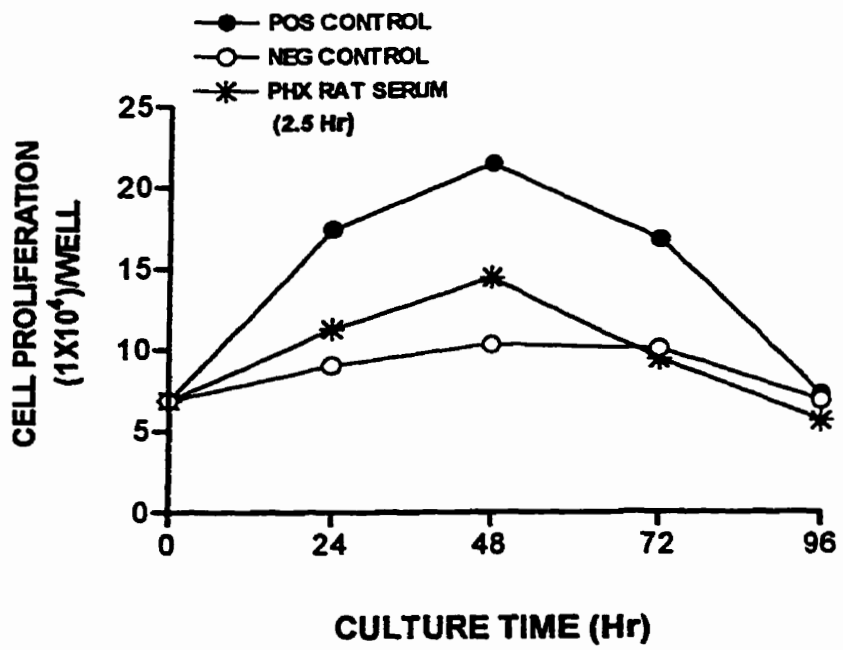


Figure 5. Optimal culture time titration. The PHX rat serum and hepatocytes were prepared as described in IIA-ii. a and b sections. Cells were initially seeded at 200,000/ml, 2 ml/well (non optimized concentration). Triplicate wells were used for each time point per sample. Positive control: medium plus EGF (100 ng/ml) and insulin (20 mU/ml). Negative control: medium only. PHX serum collected at 2.5 hr after PHX (n=4, pooled) was used at 10% final concentration. Medium was changed at each 24 hr interval. 3 wells/sample were harvested for counting at 0, 24, 48, 72 and 96 hr of the culture. The mean of the 2 closer counts were plotted in the curve. The experiment was repeated (0-72 hr) with the same pattern of proliferation observed.

hepatocytes to proliferate in the absence of serum. The serum from PHX rats had stimulating effects on hepatocyte proliferation.

IIIA-ii. VIABILITY OF CELLS IN CULTURE

To assess how well the cultured hepatocytes can survive under various conditions, the viability of trypsin-detached cells was calculated as described in the Materials and Methods (Section IIA-ii.b.). It was shown that cell viability remained above 80% (80-95%) in the serum (normal and PHX rats), EGF/insulin containing medium and medium alone for up to 72 hr in culture. By 96 hr, the viability dropped to below 65% in control wells, whereas it remained at 85% in serum-containing wells, suggesting a longer survival of cells could be maintained in the presence of serum than in growth factor and insulin only (Figure 6).

IIIA-iii. OPTIMAL CELL AND SERUM CONCENTRATION

Using the 48 hr optimal culture time and final 10% pooled PHX rat serum collected at 0 (non-PHX rats), 1, 2, 4, 6 and 48 hr after PHX ($n \geq 4$), 3 plating cell concentrations of 0.5 x 10^5/ml, 1 x 10^5/ml and 2 x 10^5/ml were tested. Cell

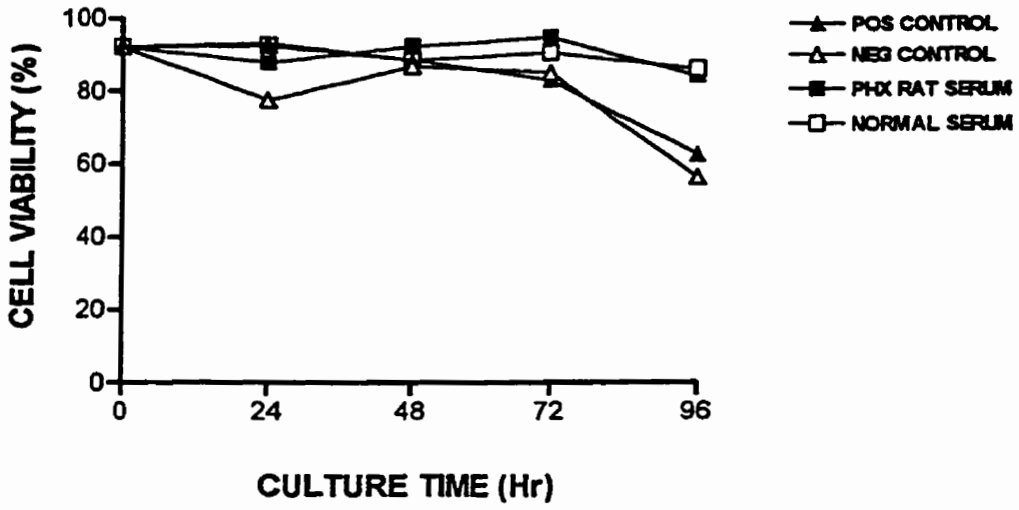


Figure 6. Viability of hepatocytes in culture. Culture conditions were the same as in Fig. 5. The total counts of detached cells from all 3 wells in each sample were used to calculate the viability of the sample at the given time point.

concentration of 0.5 x 10^5/ml had cell counts too low to indicate responses to stimulation. 2 x 10^5/ml wells did not give optimal responses to various serum samples, presumably due to the high density of cells in each well, which prevented cells from further proliferation. However, at 1 x 10^5/ml plating concentration, there was a detectable and significant peak of cell proliferation in the 2 hr and 4 hr serum stimulated wells. This stimulatory effect did not exist in the normal serum (0 hr) and disappeared by 6 hr and thereafter (48 hr). The results suggested that there were some proliferative factors (PF) present in the serum between 2-4 hr following PHX surgery in rats and that their proliferative effects could be detected in the culture with a plating concentration of 1 x 10^5/ml (data not shown, see more details later).

Using both the optimal culture time (48 hr) and cell concentration (1 x 10^5/ml), the optimal serum concentration was determined, using the same pooled serum taken at 0, 1, 2, 4 and 6 hr post PHX. The final concentrations of 5%, 10%, 20% and 30% serum were tested. 5% serum did not show significant stimulation at 2 and 4 hr (2 hr: 1.67 x 10^4/well; 4 hr: 1.1 x 10^4/well). 30% serum showed predominantly inhibitory effects on cell proliferation (cell counts below the starting cell count at all time points tested), the reason for which is unclear at present (1 hr: -0.5 x

10^4/well; 2 hr: 0; 4 hr: -0.15 x 10^4/well; 6 hr: -1.1 x 10^4/well). Both 10% and 20% serum from the 2 and 4 hr time points showed stimulatory effects on hepatocyte proliferation (10%, 2 hr: 2.55 x 10^4/well; 4 hr: 1.19 x 10^4/well; 20%, 2 hr: 2.45 x 10^4/well; 4 hr: 2.9 x 10^4/well). Since only half the amount of serum is needed for assays at 10%, compared with 20%, 10% was chosen as the optimal serum concentration for future experiments.

IIIA-iv. STARTING CELL VARIATION

The variation of starting cell numbers within the same culture and among different cultures was examined. From 5 separate cultures, 15 samples of starting cell numbers were counted (triplicate wells per sample). The mean starting cell number was 23780 ± 1900 cells / well, with the variance between cultures greater than the variance within cultures ($p < 0.05$).

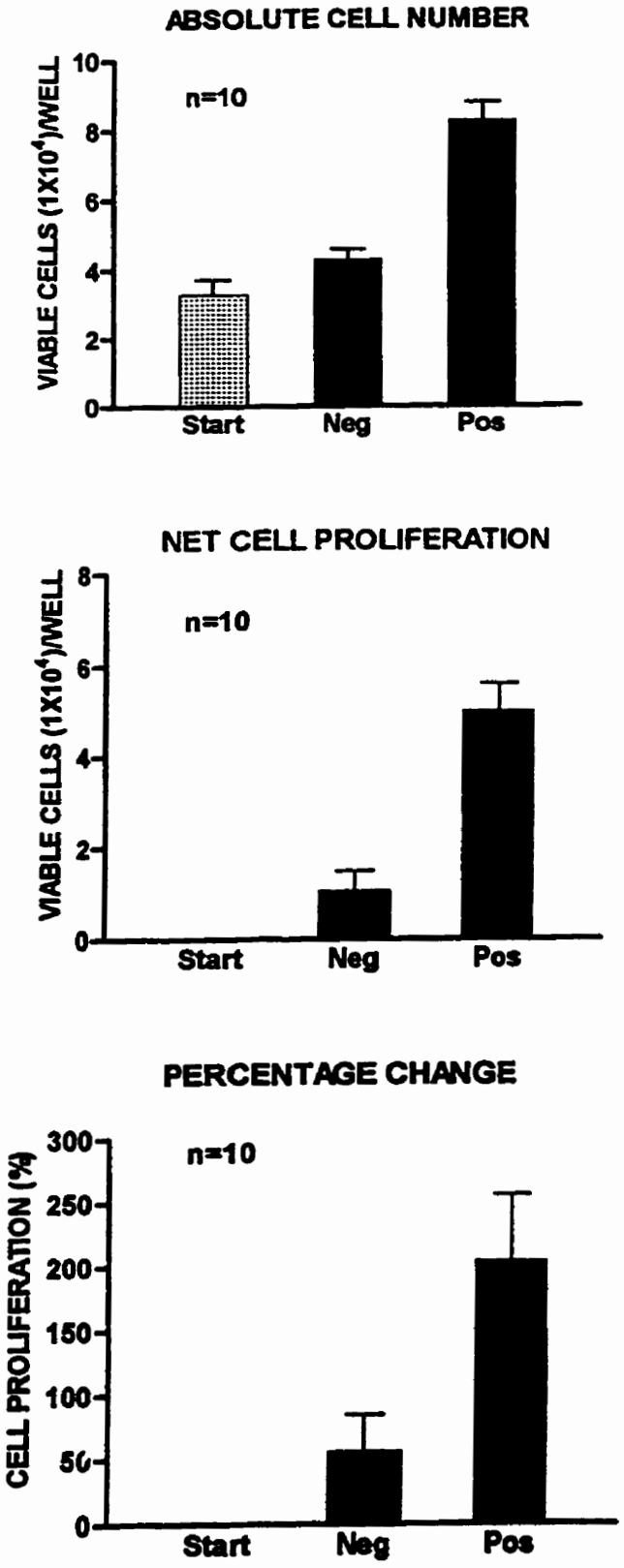
IIIA-v. DATA EXPRESSION

With the optimal culture conditions and starting cell variation determined, we further explored the best way of expressing our final results. Using the data of starting

cell counts, negative and positive controls from 10 different cultures, the absolute viable cell numbers, net increase of viable cells versus starting cells and percentage of cell increase were expressed as shown in Figure 7. The absolute cell number gives the highest cell counts and shows both the basal level and cell proliferation after culture under different conditions. With the starting level subtracted , this expression demonstrates the net viable cell increase, directly reflecting the stimulatory (or inhibitory) effects in the culture. When the same data were expressed as a percentage increase, the cell proliferation was still clearly demonstrated but with larger variation. Comparing the 3 different ways, the net cell number increase demonstrates the culture effects most directly and clearly. Another way was to express the cell number increase as a percentage of the increase in the positive control in the same culture. This expression could also directly demonstrate the stimulatory effects in culture. It has the advantage of standardizing for inter-culture variability but it relies on both the starting count and positive cell count, thus requiring more data manipulation compared to the net cell increase (data to be shown later). Therefore, our final results were all expressed using the net cell number increase.

Figure 7. Data expression comparison. The starting cell counts, negative (medium only) and positive (EGF + insulin) control results from 10 different cultures were used. The absolute cell number: the actual cell count at 48 hr of the culture in each sample. Net cell proliferation: cell count at 48 hr - cell count at 0 hr (start.). Percentage change: % increase of each sample in comparison with the starting count. Data were expressed as mean + S.E..

Figure 7


ABSOLUTE CELL NUMBER
n=10
VIABLE CELLS (1X10⁴)/WELL
10
8
6
4
2
0
Start
Neg
Pos
NET CELL PROLIFERATION
n=10
VIABLE CELLS (1X10⁴)/WELL
8
6
4
2
0
Start
Neg
Pos
PERCENTAGE CHANGE
n=10
CELL PROLIFERATION (%)
300
250
200
150
100
50
0
Start
Neg
Pos

IIIB. *IN VITRO* DETECTION OF PROLIFERATIVE FACTORS IN PHX RAT SERUM (OR PLASMA)

IIIB-i. PROLIFERATIVE FACTOR PROFILE

The proliferative effect of PHX rat serum was examined using the optimized hepatocyte primary culture bioassay. Sera collected from normal, sham operated (plasma) and 2/3 PHX rats (unpooled individual samples) were tested in hepatocyte cultures at final 10% concentration with 1 x 10^5 cells/ml for 48 hr. The results showed that following PHX, an early and transient stimulatory phase on cultured hepatocytes appeared in the serum from 1 to 4 hr, with a peak activity at 4 hr. The stimulatory effect disappeared by 6 hr. This effect was not shown in the normal serum (0 hr) and sham-operated time controls (4 hr), as demonstrated in Figure 8a. The same data were also expressed as the percentage increase in comparison with the positive controls in each culture in Figure 8b. This expression did not change the PF profile compared to the net cell proliferation in Figure 8a. Also, it did not improve the data expression compared to the net cell proliferation. In another experiment designed to more precisely locate the peak of activity, pooled plasma samples (n=5) from 3-6 hr after PHX

were tested. Both 3 hr and 4 hr samples showed significantly higher proliferative effects than the sham, and the peak activity was confirmed to occur at 4 hr (Figure 9)..

Figure 8. Proliferative factor (PF) detection *in vitro*.

(a). 36 rats underwent PHX, sham operation or no surgery (0 hr) as described in the Materials and Methods. Serum samples were collected at the time indicated. 0: normal serum. 0-6 hr groups: n=5 rats/point. S: sham operation (4 hr plasma, n=11) with liver manipulation without PHX. A transient increase of PF was detected in the rat serum from 1-4 hr, peaking at 4 hr, after PHX. Mean ± SE were from 11 separate cultures. *: $P < 0.01$.

(b). The same data were used as in (a) except for 2 normal serum and 2 sham control samples, which were excluded because of the loss of the positive control wells in the same culture by contamination. The bars represent the mean ± SE of all data points at the same time, expressed as the percentage of net cell increase of the positive controls. 0: n=3; 1-6 hr: n=5/point; S: n=9. * : $p<0.05$.

Figure 8 a.

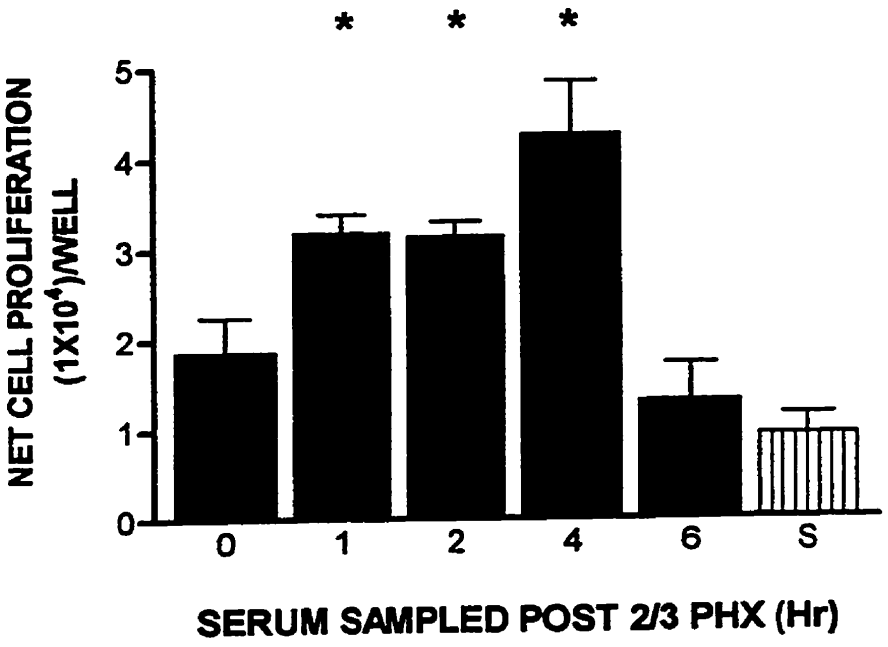
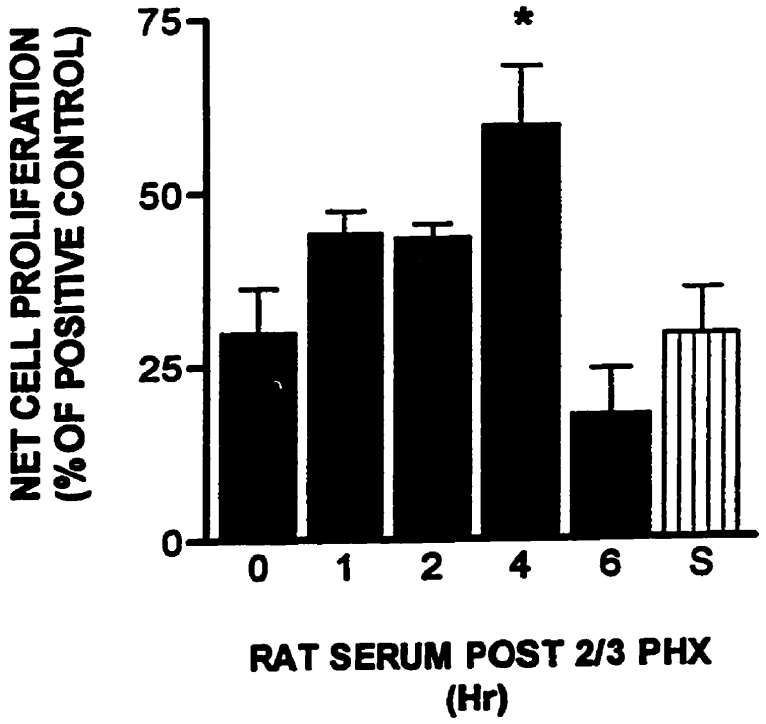


Figure 8 b.



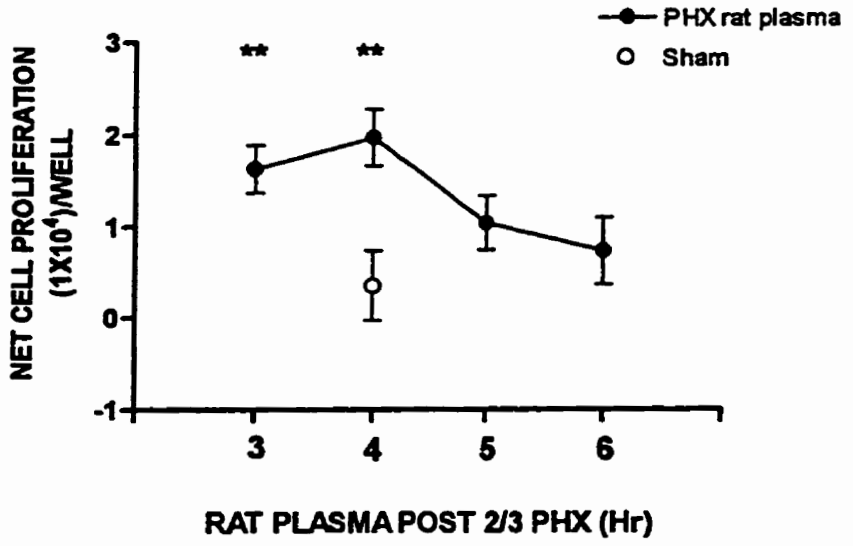


Figure 9. PF detection in pooled PHX rat plasma. Plasma was collected and pooled from rats at 3,4,5,6 hrs after PHX and 4 h after sham operation (n=5 rats/point). The pooled plasma samples were tested in 3 separate bioassays. The results are expressed as mean ± SE of 5-7 wells in the 3 assays. Both 3 and 4 hr samples showed significantly increased level of PF ($p < 0.01$) compared with the sham.

IIIB-ii. BIOASSAY SENSITIVITY

Figure 10 shows the PF assay results from 1/3 PHX rat plasma sampled at 4 hr, compared with a group of 2/3 PHX rat plasma samples. The results showed that the PF level in 1/3 PHX rat plasma was lower than that in the 2/3 PHX plasma and higher than the sham control. The differences were statistically significant in both cases.

The presence of heparin on PF activity was also tested. Heparin added to the culture did not change PF effects on hepatocytes in the paired serum samples tested (Figure 11). The effect on PF level by plasma filtration using 0.2 μm syringe filters (Nalgene) for sterilization (in case of plasma sample contamination) was further tested. The paired samples showed no significant decrease of PF activity by filtration (Figure 11).

The 4 hr peak PF production in the PHX rat plasma was used in the following experiments as the indicator that liver regeneration cascade has been initiated after PHX.

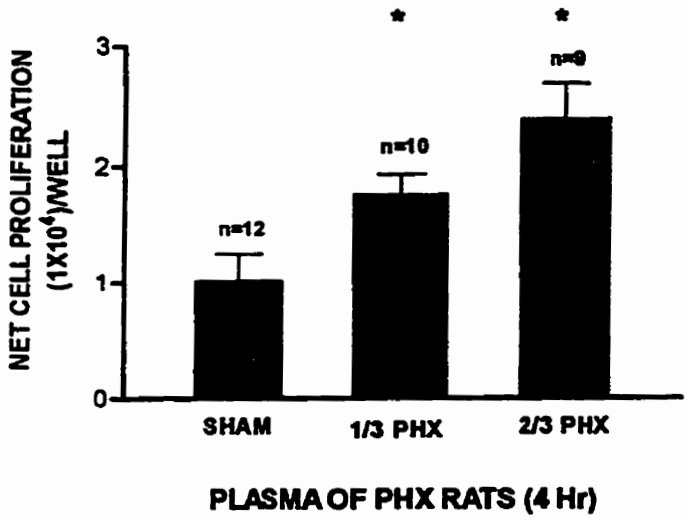


Figure 10. Bioassay sensitivity. Plasma samples were collected from rats subjected to 1/3 , 2/3 PHX and sham operation at 4 hr. The results are expressed as mean ± SE. 1/3 vs sham: $p<0.05$. 2/3 vs 1/3: $p<0.05$. 2/3 vs sham: $p<0.01$.

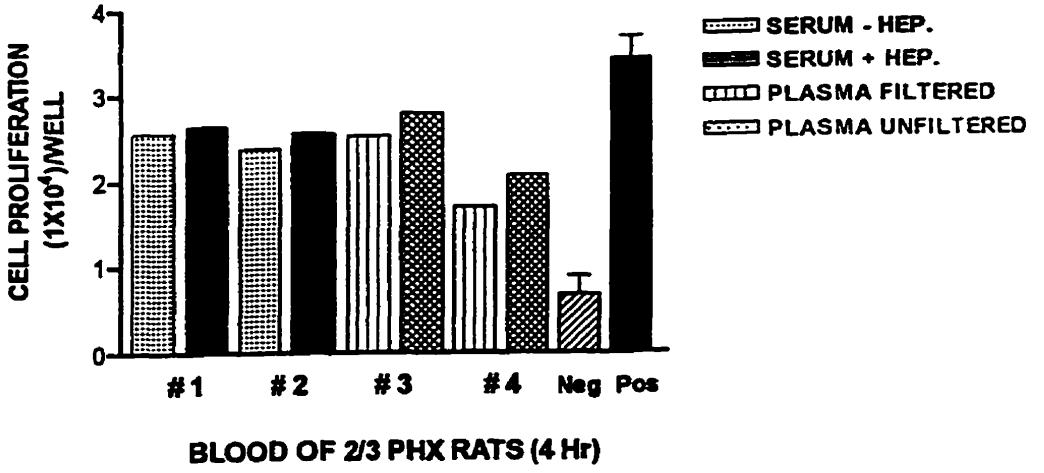


Figure 11: Effects of heparin and filtration on PF activity levels. Serum samples from 2 separate 2/3 PHX rats (#1, #2) were each divided into 2 samples, one with heparin added (35 Units/ml), the other without . Plasma from 2 other 2/3 PHX rats (#3, #4) was each split into 2 samples, one filtered, the other not. Positive and negative controls are shown. Neither the presence of heparin nor the bacterial filtration produced significant impact on the PF activity.

IIIC. EFFECTS OF PHARMACOLOGICAL MANIPULATION OF FLOW-DEPENDENT FACTORS

IIIC-i. STANDARDIZATION OF LIVER WEIGHT

Liver weight regeneration was calculated as follows: Liver regeneration (%) = (48 hr liver wt - Remnant liver wt at 0 hr) / Resected wt x 100 %. Remnant liver wt = Standard total liver wt - Resected wt. The standard total liver weight was calculated from a regression analysis of the total liver weight vs body weight in control rats (non PHX and 4 hr post PHX rats, n=43). A positive correlation of liver weight vs body weight was observed within these control rats, as seen in Figure 12a.

The percentage liver weight to body weight was obtained from a regression analysis of the data from the same group of rats. Figure 12b shows a negative correlation between the percentage liver weight to total body weight. In the 250-350 g body weight range, it shows an insignificant correlation between the percentage liver weight and total body weight, suggesting the total liver weight is a better parameter to use in these rats than percentage liver weight.

Figure 12. Standard liver weight.

(a). The total liver weight vs body weight of non PHX and 4 hr post PHX rats (n=43) were plotted. Regression analysis demonstrated a positive correlation between liver weight and body weight. $P < 0.0001$.

(b). The percentage liver weight was calculated from the same data as in (a) and plotted. Regression analysis shows a negative correlation between the percentage liver weight and body weight. $P < 0.01$.

Figure 12 a.

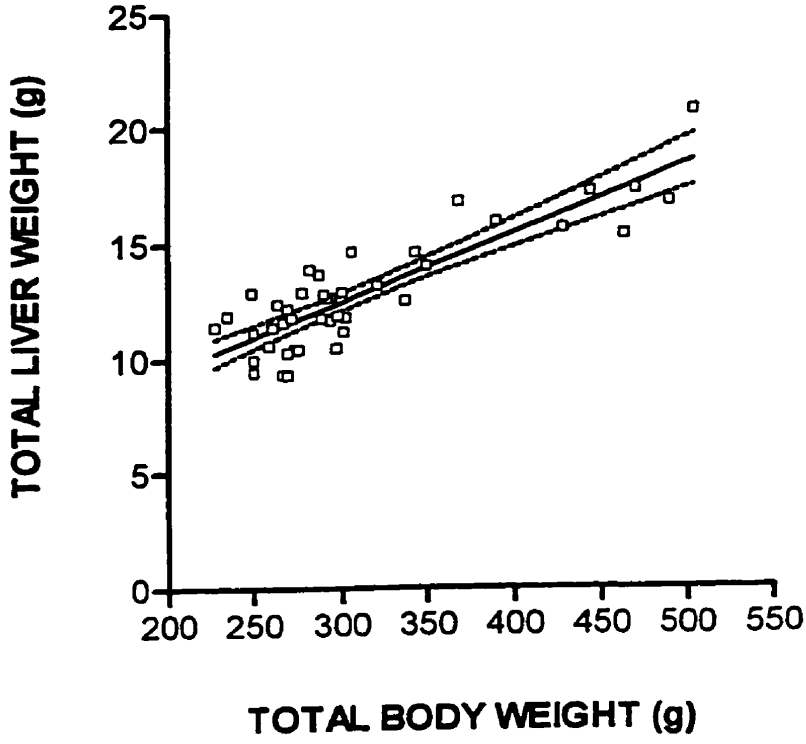
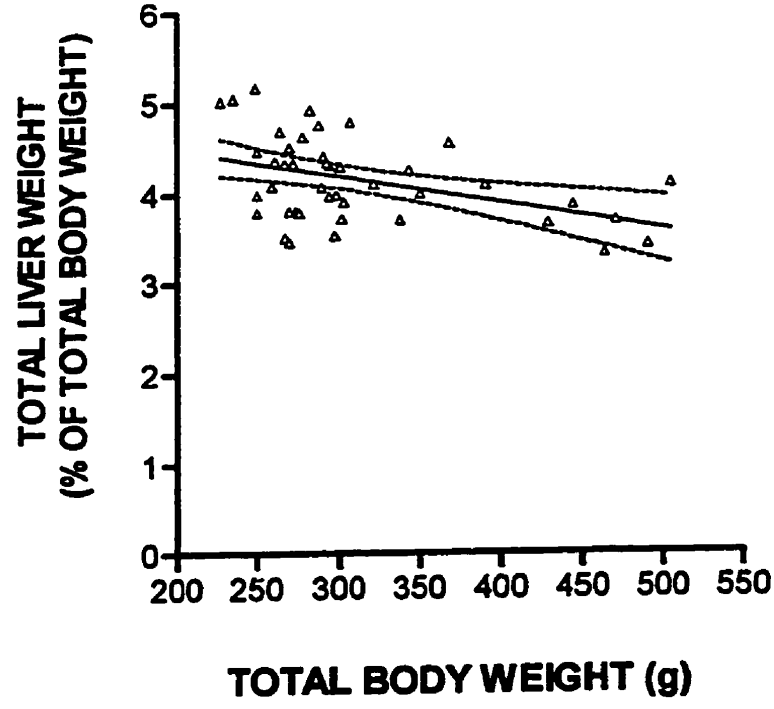


Figure 12 b.



IIIC-ii. EFFECTS OF MANIPULATION OF FLOW-DEPENDENT FACTORS ON PF PRODUCTION AND LIVER REGENERATION

To test our hypothesis and demonstrate that flow-dependent factors play a critical role in the initiation of liver regeneration, pharmacological agents, which could modulate flow-dependent factor levels, were administered to PHX rats to examine changes in the PF generation and liver weight regeneration in these rats. Three flow-dependent factors were tested.

a. Effect of adenosine

The first flow-dependent factor tested was adenosine. It was postulated that an increase in portal flow/mass would decrease adenosine levels due to the washout mechanism of adenosine (Ezzat and Lautt, 1987; Lautt 1985). The decreased adenosine may serve as the trigger. To prevent activation of this putative trigger, adenosine (maximal dose) was infused into the portal vein in a concentration shown to produce maximal dilation of the hepatic artery without significant recirculation (albeit in cats) (Zhang and Lautt, 1993).

By using the hepatocyte bioassay, plasma from 2/3 PHX

rats with intraportal infusion of adenosine (0.4 mg/Kg/min/0.02 ml) for 4 hr showed a slightly higher level of PF in the plasma than PHX controls, but the difference did not reach statistical significance, as shown in Figure 13. The adenosine data thus excluded the possibility that reduced intrahepatic adenosine secondary to increased portal flow washout following PHX initiates the regeneration cascade.

b. Effects of NO

We then tested the role of NO in the triggering process. The increased flow/mass ratio was anticipated to cause a shear stress dependent elevation of NO production (Macedo and Lautt, in press). The role of NO in the initiation was indirectly tested by administering L-NAME to block the NO production *in vivo*. Three intravenous injections of L-NAME into 2/3 PHX rats at early time points after PHX blocked the PF generation in the plasma to the same level as seen in the sham PHX control group, as shown in Figure 12. The sham PHX control was subjected to all procedures except resection. This PF production was maximally inhibited with the 2.5 mg/Kg L-NAME dose since the 5.0 mg/Kg dose did not inhibit the PF any further.

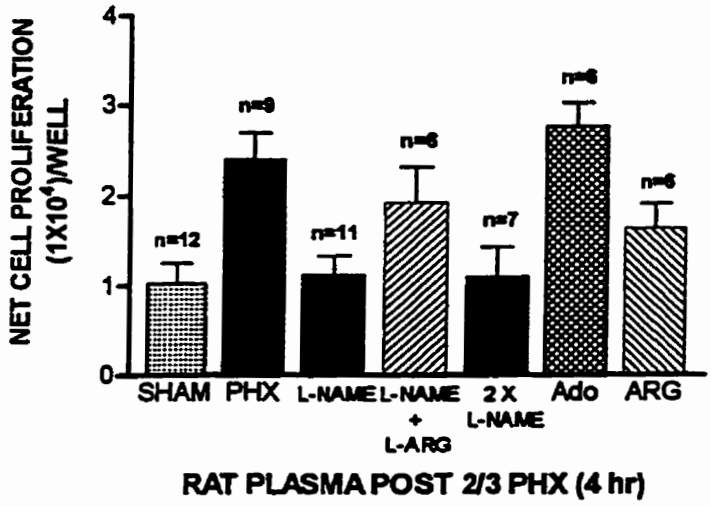


Figure 13. Manipulation of Plasma PF production at 4 hr after PHX. Hepatocyte primary culture bioassays were set up at our optimized conditions (1 x 10^5 cells/ml plating concentration, 10 % plasma, 48 hr culture). Each sample was tested in triplicate wells. Sham: plasma from rats with liver manipulation without PHX. PHX: 2/3 PHX rat plasma. L-NAME: plasma of 2/3 PHX rats with 3 x 2.5 mg/Kg in 0.5 ml saline, i.v. at 0 (before PHX), 1 and 2.5 hr after PHX. LN + L-arg: plasma of PHX rats with 3 x (2.5 mg/Kg L-NAME + 75 mg/Kg L-arg) in 0.5 ml saline, i.v. at the same time as L-NAME group. Ado: plasma of rats with adenosine infusion (0.4 mg/Kg/min) from 5 min before to 4 hr after PHX. L-arg: plasma of rats with 3 x 75 mg/Kg in 0.5 ml saline, i.v. by the same schedule. Bars represent the mean ± SE of cell numbers in each group from repeated cultures (n=12). PHX leads to significant elevation of PF compared to sham ($p<0.01$) which was fully prevented by L-NAME. L-arg reversed the inhibition of PF by L-NAME ($p<0.05$). L-arg alone caused a suppression of PF ($p<0.05$).

The question raised at this point was whether PF generated in the PHX rat plasma really represented, or was related to, the onset of the liver regeneration cascade. If PF does reflect activation of the regeneration cascade, inhibition of appearance of plasma PF by L-NAME should also inhibit liver mass regeneration *in vivo*. We then investigated the effects of L-NAME, given to recovered PHX rats, on liver weight changes, which is the end result of liver regeneration. Pilot studies showed an approximately 5, 10 and 50% liver weight regeneration at 12, 24 and 48 hr respectively after PHX (data not shown). The present *in vivo* study was conducted at 48 hr for all groups. Liver weight regeneration was significantly inhibited in the high dose L-NAME (2 x 2.5 mg/kg in 0.5 ml saline i.v. + 30 mg/kg/24 hr s.c. infusion) group (33 ± 4.7% regeneration), compared with the PHX control (47 ± 3.8 % regeneration, $p<0.01$) as shown in Figure 14. 6/14 rats died in the high dose group between 36-44 hr. These animals had only 18 ± 4.7 % regeneration. The data showed that when PF production was blocked in the PHX rat plasma by inhibition of NO, liver mass regeneration was also inhibited. The results suggested that PF is closely related to liver regeneration and this early appearance in the PHX rat plasma could represent the onset of the regeneration cascade. The data strongly suggest

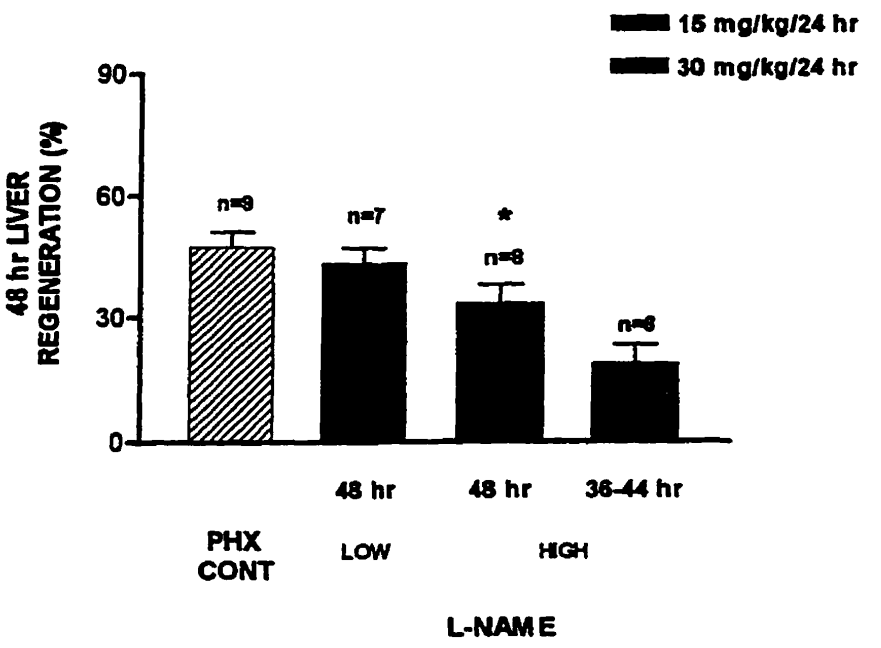


Figure 14. Inhibitory effect of L-NAME on liver weight regeneration. PHX control: 2/3 PHX + 2 x 0.5 ml saline, i.v. at 15 min before and 1 hr after PHX. Osmotic pump contained saline. L-NAME low dose: 2/3 PHX + 2 x 2.5 mg/Kg L-NAME in 0.5 ml saline, i.v. at 15 min before and 1 hr after PHX. Osmotic pumping rat: 15 mg/Kg/24 hr. L-NAME high dose: 2/3 PHX + 2 x 2.5 mg/Kg L-NAME in 0.5 saline, i.v. at the same time. Osmotic pumping rate: 30 mg/Kg/24 hr. 36-44 hr: rats survived for 36-44 hr in the high dose L-NAME group. Liver weight increase was significantly inhibited in the L-NAME high dose group. *: $p<0.01$ (compared to control). The mortality rate (36-44 hr) was 6/14 in the high dose group.

that NO could be a key factor at the initiating step of the regeneration cascade.

In order to further identify that it was NO that played the role in the initiation of regeneration, L-arginine (L-arg), a substrate for NOS, was introduced. Using the *in vitro* bioassays, we investigated if L-arg could reverse the PF inhibition caused by L-NAME. PHX rats were given L-arg together with L-NAME and plasma was sampled at 4 hr. L-arg significantly reversed the PF inhibition by L-NAME (Figure 13), indicating it was NO that was playing the role of trigger for the cascade. L-arg alone was also given to PHX rats. Although L-arg was able to reverse the inhibitory effect of L-NAME, it produced a significant inhibition of PF production when administered by itself to 2/3 PHX rats (Figure 13). The experiments on L-arg reversal of L-NAME effect on liver weight changes were hindered by the limitation of the various hormone-inducing effects of L-arg *in vivo* at the continuous (48 hr) high dose required for reversal. Animals either died earlier or showed increased liver weight compared to the L-NAME group. But, the increased liver mass regeneration could be induced by other hormones than L-arg itself (See ID-vi).

c. Direct effect of L-NAME

Because suppression of liver regeneration coincided with increased mortality, the L-NAME toxicity to normal rats was also tested. The same L-NAME high dose regime was used in 6 normal rats without PHX. All rats survived, suggesting the rats in the L-NAME high dose group died of impaired liver regeneration, not L-NAME toxicity. The results are shown in Figure 15. Different routes and doses of L-NAME have been tested in the lab without significant improvement in the survival of the PHX rats.

The direct effect of L-NAME on cultured normal hepatocytes was further tested over a range of *in vitro* concentrations covering the estimated *in vivo* concentrations administered. In the presence of epidermal growth factor (EGF) and insulin, L-NAME further stimulated hepatocyte growth rather than suppressing it, even at the highest dose tested, as shown in Figure 16.

d. Effects of PGs

The role of another group of flow-dependent factors, prostaglandins, on PF production was also tested using the *in vitro* hepatocyte bioassay. Indomethacin (Indo), an inhibitor of cyclooxygenase which is the rate limiting enzyme for production of PGs, was administered i.v. 30 min before PHX to

the rat. Results showed that Indo also inhibited the PF production in the PHX rat plasma, shown in Figure 17.

IIID. STUDIES USING PORTAL VEIN LIGATION MODEL

PF production was detected in the 4 hr plasma samples of PVL rats at about the same level to the 4 hr plasma from PHX rats, as shown in Figure 18. The ligated lobes undergo atrophy and unligated lobes undergo hyperplasia shortly after PVL. The control data are shown in Figure 19. The PVL model generated a much higher sensitivity to the inhibition of NO production *in vivo* by L-NAME in the rats. The same L-NAME dosing regime to PHX rats became intolerable in the PVL rats and killed virtually all of the rats tested (data not shown). This observation may indicate the hepatoprotective effect of cytokines mediated NO production. When NO production was inhibited, the oxidative stress may be overloaded and kill the animal (Kuo and Slivka, 1994).

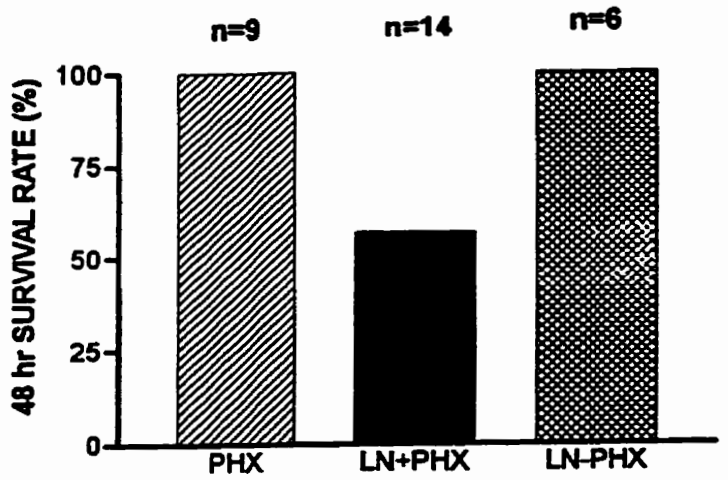


Figure 15. L-NAME effect on normal rats. The 48 hr post-surgery survival rate was compared among PHX control (PHX + saline infusion), L-NAME + PHX (L-NAME high dose + PHX) and L-NAME - PHX (high dose L-NAME to noirmal rats without PHX) rats. Survival rates: PHX = 9/9; L-NAME + PHX = 8/14; L-NAME - PHX = 6/6.

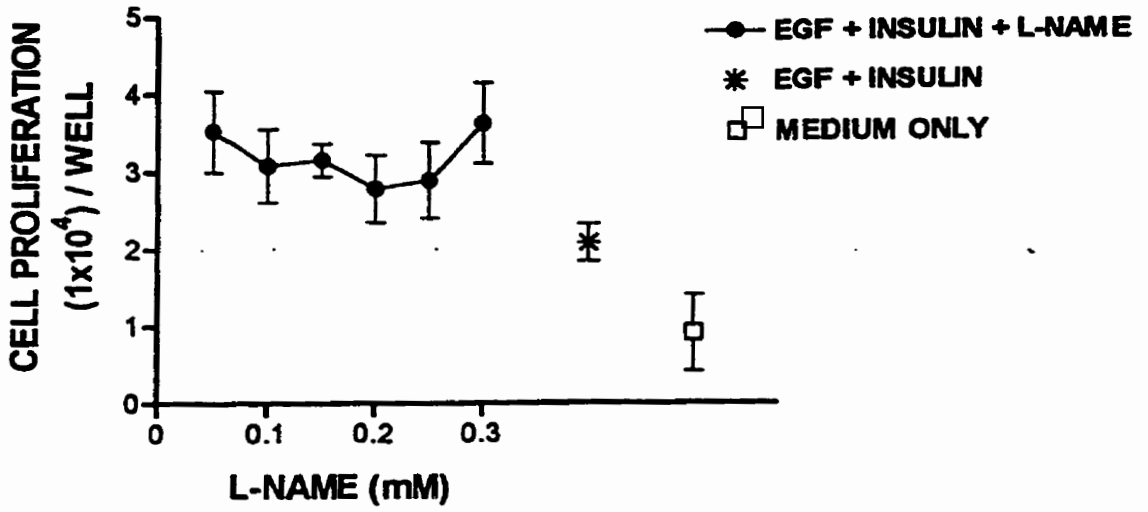


Figure 16. Direct effect of L-NAME on cultured hepatocytes. A series of L-NAME concentrations representing the estimated concentrations reached in the liver *in vivo* was tested in 2 separate cultures in the presence of EGF (100 ng/ml) and insulin (20 mU/ml). The results are expressed as the mean ± S.E. of the 4-6 wells tested from the 2 cultures. L-NAME consistently increased the stimulatory effects of EGF and insulin on hepatocytes at all concentrations tested.

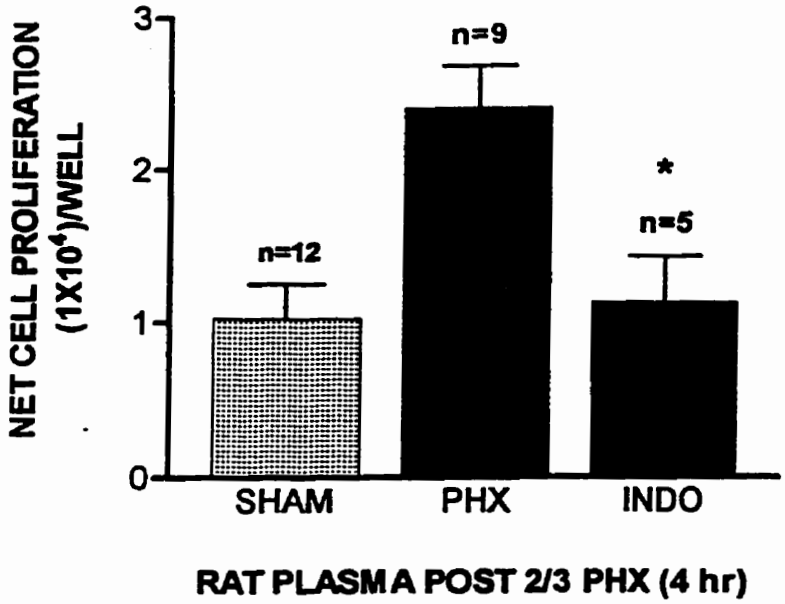


Figure 17. Indomethacin mediated modulation of PF production. A single dose of indomethacin, 7.5 mg/Kg in 0.2 ml saline was given (i.v.) to the rat 30 min before PHX. 4 hr plasma samples were assayed in the primary culture with 3 wells/sample. Indomethacin inhibited the PF production to the sham level. *:$p < 0.01$.

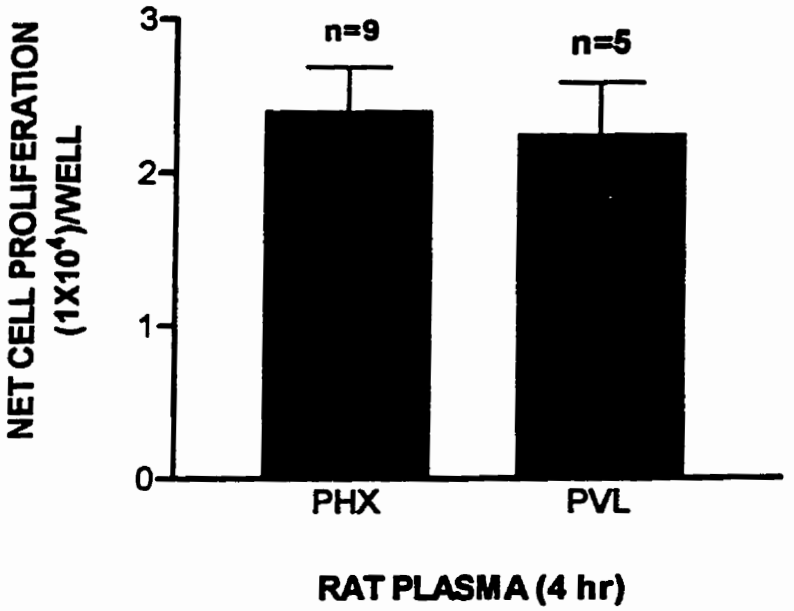


Figure 18. PF production after PVL. Plasma from PVL rats at 3-4 hr after PVL was assayed in the hepatocyte bioassay. PF was produced at the same level as seen in the 4 hr PHX rat plasma.

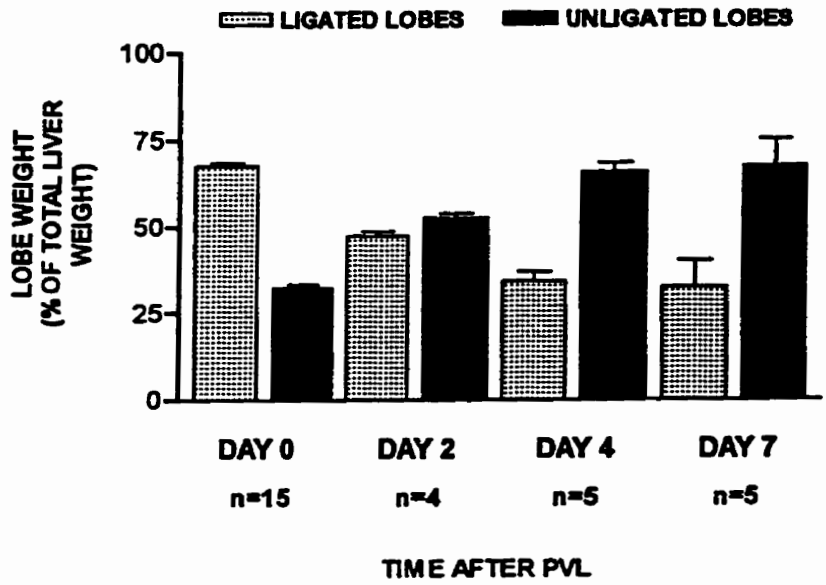


Figure 19. Liver hypertrophy and atrophy after PVL. The weights of ligated and unligated liver lobes of recovered PVL rats at different time periods after PVL are expressed as the percentage of the total liver weight. The bars represent mean ± SE.

IV. DISCUSSION

IVA. ADVANTAGES OF THE HEPATOCYTE BIOASSAY

An *in vitro* hepatocyte primary culture bioassay was developed and optimized to 1 x 10^5 cells / ml plating concentration, overnight attachment, 10% final serum concentration and 48 hr culture period. Cell counting was performed at the end of attachment (starting hr) and after 48 hr culture. Final results are expressed as the difference of viable cell counts between the 48 hr and the starting counts, i.e. the net cell proliferation. The flow chart of the method is shown in Figure 2. This non-radioactive method was shown to be an effective way of measuring hepatocyte proliferation in culture from the present study.

Although liver research has been carried out extensively and intensively, a most satisfactory method of measuring hepatocyte proliferation is lacking. In the past decade, a considerable effort has been directed to determination of the most representative indices of hepatocyte proliferation in order to monitor the liver regeneration more directly and accurately. Various cell proliferation markers and experimental approaches have been identified and developed (Theocharis et al., 1994; Miyachi et

al., 1978; Mathews et al., 1984; Gerlyng et al., 1992; Alisen et al., 1994; Masaki et al., 1994; Gratzner et al., 1982; Jezequel et al., 1991). These markers still do not, however, show complete correspondence with the actual cell proliferation (Gerlyng et al., 1992; Bucher, 1963).

The most commonly used method in the literature to monitor hepatocyte proliferation both *in vivo* and *in vitro* is measuring DNA synthesis by the incorporation of [3H]thymidine into DNA or the nuclei. Our initial experiments were also carried out using this method as the cell proliferation index. Technical limitations exist in measuring DNA synthesis from the specific activity of incorporated [^{3}H]thymidine using the conventional acid precipitation and other DNA extraction methods. Morley and Kingdon reported nonspecific binding of [^{3}H]thymidine in hepatocytes (Morley and Kingdon, 1972). They suggested incorporating a modified phenol DNA extraction procedure and a proper control system in the DNA extraction to overcome the thymidine nonspecific binding. Using acid precipitation, significant radioactivity was incorporated into non-DNA synthesizing hepatocytes and was bound to unknown proteins (Morley and Kingdon, 1972). They proposed the use of hydroxyurea (DNA synthesis inhibitor) as a control to allow for "protein bound" [^{3}H]thymidine. Glycogen was also reported to bind with

[^{3}H]thymidine (Counts and Flanim, 1966). Many studies do not incorporate these controls. The nonspecific binding of [^{3}H]thymidine in hepatocytes may account for the variation in specific radioactivities of DNA seen in different studies.

Other limitations of using [^{3}H]thymidine as a radioactive precursor were described by Bucher in 1963 (Bucher, 1963). [^{3}H]thymidine is not on the main pathway but on the "salvage" pathway to DNA synthesis. It needs to be converted to d-TMP to be used in DNA synthesis by thymidine kinase, which is low in some species (e.g. in humans). Radioactivity can cause direct DNA injury and depress DNA synthesis and mitosis. It has been shown that a dose of 1 microcurie per gram of body weight of [^{3}H]thymidine into partially hepatectomized rats significantly delayed liver restoration and depressed the mitotic rate to 25-35% of the control level (Grisham, 1960). Furthermore, whether DNA synthesis in a hepatocyte leads to cell proliferation or produces a polyploidy cell is still unclear (Gerlyng et al., 1992; Bucher, 1963).

In addition to DNA synthesis, other conventional or newly developed methods such as mitotic index, nuclei-counting and PCNA (proliferating cell nuclear antigen) detection have also been frequently used in estimating hepatocyte proliferation. The major disadvantages of mitotic index are its low reading (about 0.01% in normal adult liver

and 3-5% in regenerating liver) and sampling errors (different regions in the liver have different mitotic indices) (Bucher, 1963). The limitation of nuclei-counting is that it does not directly reflect cell number and a correction for the percentage change of binucleated cells must be applied (Bucher, 1963). PCNA detection is an immunohistological method and technically complex to use (Theocharis et al., 1994). It does not correspond completely with the liver regeneration process *in vivo* (Shiratori, 1996).

The most direct measurement of cultured hepatocyte proliferation is the net increase of viable cell number, which is also the most commonly used measurement of growth for various other mammalian cell types (Patterson, 1979). Nevertheless, sufficiently detailed information on applicable methodology to enumerate hepatocytes *in vitro* is lacking in the literature, although some groups did use cell number as one of their indices of the hepatocyte proliferation (Leffert et al., 1977; Hasegawa et al., 1982). Leffert et al. published a comprehensive methodology of fetal and adult liver cell isolation, plating and cell counting in 1979 (Leffert et al., 1979). The significance of their report is that it gives a technically detailed description on counting cultured hepatocytes by the Coulter counter. The drawbacks

of their method are the complexity of the buffer solutions used to detach hepatocytes from culture dishes and the long trypsin digestion time involved. With our bioassay, the detaching solution of hepatocytes was simplified to one single trypsin-EDTA solution and the digestion time was shortened to within three minutes.

In comparison with our earlier measurements (unpublished observations Wang & Lautt) using [^{3}H]thymidine incorporation into DNA and total protein content change in the cultured hepatocytes stimulated by PHX rat serum, the present viable cell number counting method produced more reproducible results with much smaller variations among different experiments. Figs 7, 10, 13, 17 and 18 expressed the combined means and SEs of both different animals as well as different experiments. However, the data were more difficult to compare between different experiments using the DNA synthesis and total protein content measurements, although the later produced less variable results. In addition, the results using cell counts are more directly interpretable than those using DNA and protein synthesis methods. This method is more time consuming than the DNA synthesis method, but it is a more economic method in the long run, since it is easy to use, highly reproducible, non-radioactive and much less expensive than DNA synthesis and other methods. Also, intuitively, the best index of cell

proliferation is the appearance of more cells.

IVB. HEPATOCYTE BIOASSAY AS A TOOL TO ASSESS THE INITIATION OF LIVER REGENERATION

Using the bioassay, it was demonstrated that serum from PHX rats produced a transient, early proliferative response in cultured hepatocytes, which indicates that there are proliferative factors (PF) occurring or increasing early in the serum following PHX that can be used as a tool to indicate that the regeneration cascade has been triggered. The PF appeared by 1 hr post PHX, reached a peak at 3-4 hr and was not detected at 6 hr and beyond. The same level of PF was also detected in PVL rat plasma at 3-4 hr after PVL using the bioassay.

The exact nature of the PF is unclear at present. PF may or may not be exactly equivalent to GF, CK or hormones detected in the PHX rat plasma according to the literature (see Introduction). The appearance of some of these factors correlated with the PF appearance in our hypotheses. The important candidates of PF include the complete hepatocyte mitogen, hepatocyte growth factor, (hepatopoietin A), which is the most potent hepatocyte mitogen and is reported to increase sharply from 2-6 hr after 2/3 PHX (Lindroos et al.,

1991; Fausto et al., 1995). Norepinephrine, the strongest hepatocyte comitogen (see IB-ib), increases at 2-4 hr after PHX (Lindroos et al., 1991). Heparin-binding epidermal growth factor-like growth factor (HB-EGF), which has recently been shown to be a hepatotrophic factor *in vitro,* shows increased mRNA level from 1.5-6 hr after 2/3 PHX (Kiso et al., 1995). Other hepatocyte comitogens, such as insulin and glucagon, may also play a role in the hepatocyte proliferation (McGowan et al., 1981; Diehl and Rai, 1996). It is likely that there are multiple factors involved in this early stimulatory phase in the PHX rat serum. Regardless of the chemical identity of the PF, appearance of PF following PHX is a clear indication that the triggering signal for initiating a whole cascade of regenerative events has occurred.

Comparing the sensitivity of our optimized bioassay with the DNA synthesis method, the bioassay could detect PF produced in the 1/3 PHX rat plasma and differentiate it from the PF levels in the 2/3 PHX rat plasma. In contrast, there was little DNA synthesis detected in 1/3 PHX regenerating liver using the DNA synthesis method, and therefore, 1/3 PHX was only regarded as a "priming stimulus" for hepatocytes to respond to other growth factors (Webber et al., 1994). Another report showed that there was no difference in the increase of DNA synthesis in cultured hepatocytes stimulated

by the sera collected from two different strains of mouse at 14-48 hr after PHX, whereas there was a significant difference in liver weight regeneration at that time between the two strains (Shiratori et al., 1996). Therefore, the sensitivity and reliability of measuring DNA synthesis by [^{3}H]thymidine incorporation is worth questioning. Our optimized hepatocyte bioassay allows for identification of the presence of PF in the serum or plasma of rats subjected to PHX, which serves as a tool to assess the initiation of the liver regeneration cascade.

IVC. EVIDENCE OF FLOW-DEPENDENT FACTOR(S) BEING THE TRIGGER OF THE REGENERATION CASCADE

In testing our hypotheses by using pharmacological manipulation, three flow-dependent factors have been tested for their roles in triggering the onset of the liver regeneration cascade in PF production and affecting liver regeneration.

IVC-i. IS ADENOSINE A TRIGGER?

As a key intrinsic metabolic flow regulator for the hepatic arterial blood flow (Lautt, 1985; Ezzat and Lautt,

1987), adenosine was the first flow-dependent factor that has been tested. The hypothesis was that adenosine accumulation in the Space of Mall served as an inhibitor of the initiation of the liver regeneration. Therefore the decreased adenosine concentration in the Space of Mall after PHX due to the increased washout of adenosine by the increased portal flow should initiate the regeneration cascade. The inhibitory effect of adenosine on cell proliferation has been demonstrated in cultured neuronal cells (Huffacker et al., 1984).

However, the results do not support this hypothesis. With a dose of 0.4 mg/Kg/min intraportal infusion to maintain the intrahepatic inhibitory adenosine level for 4 hr, which was shown to cause the maximum vasodilation in the hepatic artery (Zhang and Lautt, 1993), there was no sign of inhibition of the PF in the plasma, but instead, PF was slightly increased compared to PHX controls. The liver has been shown to be a potent extracting organ for adenosine in the circulation. Since the adenosine dose used had been shown not to affect the circulating adenosine concentration, it was, therefore, postulated that the majority of the infused adenosine has been uptaken by the liver (Zhang and Lautt, 1993). The data showed an opposite direction (stimulatory) of the role of adenosine in the triggering process to the hypothesis (inhibitory) without showing statistical

significance. Therefore the possibility of reduced adenosine being a trigger of the regeneration cascade was excluded and no other doses were tested.

IVC-ii. THE ROLE OF NITRIC OXIDE AS THE TRIGGER OF LIVER REGENERATION

NO was the second flow-dependent factor tested. The NOS inhibitor, L-NAME, was the primary tool to assess the effects of NO. The results showed that intravenous administration of L-NAME was able to inhibit the PF production in the plasma following PHX and inhibit the liver weight regeneration at 48 hr after PHX. The NOS substrate, L-arg, was able to reverse the inhibition of L-NAME on PF production in the PHX rat plasma. These results have indicated a key role of NO in the initiation of the liver regneration cascade.

As mentioned in the introduction, NO possesses both hepatotoxic and hepatoprotective effects in the liver. Dual effects of L-NAME, NO and L-arg have been observed in our studies, presumably caused by their direct and indirect actions in the liver. Intravenous injections of L-NAME at early time points after PHX could block the PF production in the PHX rat plasma to the sham control level. Similar dose of

L-NAME also significantly inhibited liver weight increase compared to PHX controls, with an increased mortality rate. Nevertheless, L-NAME given to normal rats produced no mortality and stimulated hepatocyte proliferation in culture when added to the culture medium in concentrations covering the *in vivo* dose administered. The data are consistent with the finding of an antiproliferative effect of NO *in vitro* on cultured vascular smooth muscle cells in the literature (Garg and Hassid, 1989).

These observed dual effects indicate that L-NAME did not act directly on hepatocytes *in vivo* to cause the inhibition of liver regeneration, but rather, L-NAME acted through the inhibition of production of NO, causing a blockade of the initial trigger for the regeneration cascade. As a result, the liver regeneration was inhibited. These observations also indicate that NO did not act directly on the liver to cause the inhibition of regeneration (due to its antiproliferative property), but rather, NO acted through other events (i.e. as a trigger) to initiate the regeneration, since blockade of an antiproliferative molecule should increase hepatocyte proliferation, but instead, we saw an inhibition of liver regeneration.

In addition, L-arg, one function of which is to serve as the substrate of NOS for NO synthesis, could partially

reverse the inhibitory effects of PF generation produced by L-NAME, thus promoting liver regeneration. But when given alone, L-arg produced a significant inhibition of PF production. The data were interpreted as that NOS was a substrate limiting enzyme; when substrate was a limited factor, L-arg could reverse the inhibition of L-NAME on NO production; when there is enough substrate, additional L-arg will act through its other effects (protein synthesis, hormonal release and others) indirectly causing the inhibition of PF. The exact explanation is unclear at present (Noeh et al., 1996; Moncada and Higgs, 1993).

Interpretation of the observed dual effects of L-NAME, NO and L-arg has further confirmed the key role of NO as a trigger to initiate the liver regeneration cascade.

IVC-iii. THE ROLE OF PROSTAGLANDINS IN THE TRIGGERING PROCESS

We further tested the role of another group of flow-dependent factors, prostaglandins, in the triggering process. As described in Section IC-iii, there is a large body of literature reporting various interactions between NO and PGs: corelease of NO and PGs, synergistic effects of each other, inhibitory effects of each other, NO acting through PGs or vise versa, or NO and PGs acting on separate pathways (Kaley

and Koller, 1995; Salvemini et al., 1995a, 1995b; Sautebin et al., 1995; Klein-Nulend et al., 1995; Salvemini et al., 1993; Botting and Vane, 1989; Xu et al., 1995; Harbrecht et al., 1995; Meijer et al., 1996; Manfield et al.,1996; Curtis et al., 1996).

However, more reports are in favour of the co-release of NO and PGs by the same stimuli and of the increased production of PGs by NO. A mechanism of NO enhancing COX activity through a cGMP independent pathway has been suggested (Salvemini et al., 1993). Our results showed that a single dose of indomethacin (i.v.) could also block the PF production in PHX rat plasma to the sham level.

The data support the point of view that NO and PGs act through a common pathway after their respective second messenger (NO → ↑ cGMP; PGs → ↑ cAMP) to cause the generation of PF, since our data do not show reciprocal effects on the PGs and NO production. PGs may be involved in the initiation of liver regeneration cascade as a mediator of NO effects.

Increase of blood flow can also increase PGs release from the endothelium. Tissue specific flow-dependent vascular responses through PGs or PGs and NO production have been reported (Kaley and Koller, 1995). Shear stress has been shown to induce vasodilation by PG production only in rat cremaster microvessels (Koller et al., 1993). In rat gracilis

muscle, the shear-induced vasodilation was found to be mediated 50% by NO and 50% by PGs (Kalley and Koller, 1995). Therefore, the evidence in the literature suggests that NO and PGs can be co-released by increased shear stress, and both NO and PGs are involved in maintaining the vascular shear stress within normal range.

The involvement of PGs in the triggering process suggested to us a possible mechanism of the effect of NO in the initiation of liver regeneration. The NO effect may be mediated through PGs.

IVD. PORTAL VEIN LIGATION MODEL IN THE STUDY OF LIVER REGENERATION

As an analogous model to PHX in the rat, the PVL was selected in the study of the present project. The rationale of using the PVL model include: 1. If one branch of the portal vein is ligated, the hemodynamic change in the unligated lobes will be similar to the PHX model. The unligated lobes recieve total portal blood flow and the ratio of blood flow/liver mass will be immediately increased, resulting in an increased shear stress and production of flow-dependent factors, NO and PGs, which then trigger PF production and liver hypertrophy; 2. The ligated lobes

recieve only hepatic arterial blood with no portal blood and the lobes will quickly undergo atrophy. The majority of the hepatocytes undergo apoptosis and are cleared by Kupffer cells between day 4-7 (Ikeda et al., 1995). Adenosine has been reported to be able to induce apoptosis in neurons (Wakade et al., 1995). Whether adenosine can also induce apoptosis in hepatocytes is unclear. The PVL model has provided an approach to test this hypothesis.

The *in vitro* results of PF production in PVL rat plasma support the hypothesis that a similar initiating mechanism is involved in the PVL model, compared with PHX, to induce hypertrophy in unligated lobes, suggesting the same role of triggering that flow-dependent factors play in this model.

However, the difference between the PVL and PHX models is also remarkable, presumably depending on whether the ligated 2/3 of the liver is resected or retained. With the 2/3 liver removed (PHX model), the model only induces cell proliferation in the remnant lobes without the presence of cell injury , death and inflammatory responses (see Section IA-iii). In the PVL model, cell proliferation is induced in unligated lobes, and severe cell injury, apoptosis and inflammatory responses are also induced simutaneously in the ligated lobes, which creats a much higher level of TNF, CKs

and NO production and sensitivity to the inhibition of NO production *in vivo* in the PVL model than PHX.

With this feature of the PVL model, the inhibitory effect of L-NAME on liver hyperplasia in unligated lobes could not be tested, since rats could hardly survive after L-NAME administration. Although negative, these results do support the finding of the hepatoprotective effect of NO production, and that of L-arg analog-induced hepatic injury in the endotoxin-mediated hepatic necrosis model in the literature, suggesting a critical role of NO in maintaining liver functions (Billiar et al., 1990; Harbrecht et al., 1992; Kuo et al,. 1994) (see Section ID-v).

IVE. ORIGINALITY OF THE HYPOTHESES

A relationship between blood flow and liver regeneration has been studied since 1930s (Stephenson, 1932, Mann, 1940; Weinbren, 1955), but the studies were conducted from different points of view on the blood flow/liver regeneration relationship. It was generally accepted that the most important limiting factor in liver regeneration was the portal blood supply. The early "Vascular Theory" only emphasized the importance of the filling of the liver by venous blood in liver regeneration (Sigel, 1969).

In comparison with the early Vascular Theory, we hypothesize for the first time: 1. The hemodynamic change of an immediate increase in blood flow/liver mass ratio (i.e. increased shear stress) upon PHX serves as the trigger to initiate the regeneration cascade; 2. The increased flow/mass ratio alters the concentration of flow-dependent factors that lead to production of PF representing the onset of the regeneration process. The novelty of our hypotheses reside in: 1. emphasizing the increased flow/mass ratio causing increased shear stress in the liver as the trigger; 2. emphasizing the key role of flow-dependent factors in the initiation of the cascade by inducing PF production. 3. The possibility that NO as the initiating signal can also serve as the trigger of regeneration following cell damage but with NO release caused by a different, non-flow dependent, mechanism. This latter possibility requires further study.

IVF. LIVER REGENERATION IS CONTROLLED UNDER A REDUNDANT SYSTEM

In the April 1997 issue of Science, Michalopoulos and DeFrances concluded in their review on liver regeneration that "In analogy to studies of the big-bang theory of the universe, research in liver regeneration still needs to sort

out the earliest signals associated with triggering the origin of the regenerative response". Collectively, our results using both the generation of PF as an index of initiation of the regeneration cascade, and the 48 hr liver weight restoration following 2/3 partial hepatectomy as an end point of the regeneration cascade, have provided a first line of evidence for $NO,^{\cdot}$ a flow-dependent factor, being the initiating trigger of the liver regeneration process. PGs may be the mediator of the NO effects.

The exact site and cell type of NO production in the liver after PHX is still unclear at present. Multiple cell types in the liver have been reported to be able to produce NO: endothelial cells (eNOS), Kupffer cells (iNOS) and hepatocytes (iNOS) (Obolenskaya et al., 1994b). Evidence in the literature suggests that shear stress and CK can induce eNOS gene expression (Venema et al., 1994; Marsden et al., 1993). The most likely sources of NO after PHX are endothelial and Kupffer cells, which are located in the lumen of sinusoids, directly sensing the shear stress increases following PHX. Regarding the mechanism of NO mediated PF production, it is still unknown at this stage.

There are reports indicating that NO was produced at early time after PHX (Obolenskaya et al., 1994a; Hortelano et al., 1995). It was also postulated that NO may prevent the blood-flow collapse resulting from the liver resection,

facilitating vascular readaptation after PHX, modulate endothelial permeability and stimulate specific metabolic responses required for hepatic function recovery (Hortelano et al., 1995; Kubes, 1992; Pipilisynetos et al., 1993).

Our data also indicate that liver regeneration at 48 hr was suppressed but not completely prevented. It appeared that rats in which regeneration was severely suppressed, survival was imperiled. Attempts to alter dose levels, routes and formats (data not shown) continue to support the impression that suppression beyond the present level is associated with very high mortality. It is possible that complete suppression of regeneration would be incompatible with life but it is not clear from this study if the significant regeneration remaining after L-NAME is due to incomplete block of the sole trigger or if, in the absence of a primary trigger, some other redundant control, independent of the presence of PF, is still able to produce a slow restoration through a different mechanism.

V. SPECULATION

Taken together, the findings of this project can be added to the research on initiation of liver regeneration and as well be speculated into the areas described below.

VA. TERMINATION OF LIVER REGENERATION

As mentioned in Section IA-ii. b., very little is known about how liver regeneration terminates. Flow-dependent factors (NO, PGs and adenosine), however, can be a good candidate to stop the regeneration by two different mechanisms: 1. PF mechanism: As liver mass increases, the initiation signal disappears: the ratio of blood flow/liver mass decreases to normal, and shear stress is reduced to normal, stopping production of flow-dependent factors, which then stop the induction of PF and terminate the entire series of liver regenerative events; 2. Adenosine mechanism: The increased liver size and decreased flow/mass ratio reduces portal flow washing-out of adenosine, leading to adenosine accumulation in the Space of Mall. Adenosine may prevent further liver mass increase by inducing hepatocyte apoptosis. Our PVL data support this hypothesis. The PVL control group showed a progressive liver atrophy in ligated lobes (left lateral and median lobes) from day 0-7, where massive adenosine can be produced and accumulated due to ischemia and reduced washout in the ligated lobes. The increased adenosine level may have caused hepatocytes to undergo apoptosis and resulted in liver atrophy in the ligated lobes.

VB. INITIATION OF LIVER REGENERATION FOLLOWING CELL INJURY

The role of NO as an initial trigger of the regeneration cascade in the PHX model can also be extended to other cell injury models.

VB-i. CCl_4 MODEL

As described in Section IA-iii.b., liver regeneration can be induced after carbon tetrachloride administration by causing cell necrosis. Early gene expression, DNA synthesis and growth factor production patterns are very similar, but with different timing, in the PHX and CCl_4 models. The CCl_4 model is a more complex model in that cell proliferation is induced in the presence of cell death, fibrosis and inflammatory responses.

Dual effects of NO of hepatoprotection and hepatotoxicity, presumably due to its direct and indirect effects on hepatocytes, have been well documented (see Section ID-v). Evidence in the literature has suggested a link between NO and the protection against hepatotoxicity by toxins such as phalloidin, CCl_4 and acetaminophen by the fact that there is a parallel between the proliferation of hepatocytes and protection against these toxins (Barriault et

al., 1997). PHX rats and newborn animals were found to be resistant to phalloidin toxicity (Wieland, 1986; Tuchweber et al., 1972). Non-lethal doses of CCl_4 at 24 hr before a lethal dose of it could provide protection of the lethality by CCl_4 (Thakore and Mehendale, 1991). Our data have provided the direct evidence of the underlying link of NO to these reports, i.e. through triggering liver regeneration, NO can promote hepatocyte proliferation and, therefore, increase the liver resistance and hepatoprotection to these toxins. Evidence is also available in the literature that Kupffer cell activation was observed after administration of these toxins. NO is a product of the activated Kupffer cells which can both increase hepatoprotection or hepatic injury from these toxins (Barriault et al., 1997; Agostimi et al., 1980).

Combining the literature and our own findings, it is reasonable to postulate that NO is also a key triggering factor in CCl_4 induced liver regeneration.

VB-ii. PVL MODEL

A similar pattern of PF production to the PHX model has been detected in the PVL rat plasma at 4 hr after PVL, using our *in vitro* hepatocyte bioassay. In addition, our data from recovered PVL rats demonstrate that the hepatoprotective

effect of NO is critical, since blockade of NO by L-NAME produced an 100 % lethality within a few hours after PVL (See Section IIID).

These findings strongly suggest that NO may be both a key trigger for the liver hypertrophy in the unligated lobes and a key hepatoprotective molecule in the ligated lobes of the liver in PVL rats

VI. CONCLUSIONS

1. A rat hepatocyte primary culture bioassay has been developed and optimized to be used as an effective tool to measure hepatocyte proliferation *in vitro* and to assess the initiation of the liver regeneration cascade based on counting the net viable cell number increase in the culture.

2. The bioassay can qualitatively detect the proliferative factor increase in the PHX rat plasma between 1-4 hr after PHX, with a peak activity detected at 4 hr.

3. The bioassay can also quantitatively distinguish the plasma PF levels generated by 1/3 PHX and 2/3 PHX, indicating a high sensitivity of the assay.

4. This early and transient increase of PF (presumably composed of GFs, hormones and CKs) in the plasma following PHX is closely related to the liver regeneration process and can be used as an indicator of the initiation of the regeneration cascade.

5. Nitric oxide, a flow-dependent, flow-regulatory factor, has been demonstrated to be a key triggering factor for the

liver regeneration cascade.

6. Adenosine, an intrinsic hepatic blood flow regulator, has been demonstrated not to play a role in the initiation process.

7. Flow-dependent factors, prostaglandins, have also been demonstrated to be involved in the triggering process, possibly as the mediator of the trigger by NO.

8. It is still unclear how NO and PGs increase PF production in the PHX rat plasma.

9. PF is also produced in the PVL rat plasma, indicating a similar initiation of the liver regeneration cascade to PHX.

10. Collectively, our data support the hypothesis that increased blood flow/liver mass ratio upon PHX increases shear stress in the liver and alters the concentration of the flow-dependent factors, NO and PGs, leading to production of PF that reflects the onset of the regeneration cascade. The change in NO and PG levels could reflect the blood flow change, indirectly serving to both initiate and terminate the complex regeneration process as the flow/mass ratio re-establishes.

11. The overall picture of our hypotheses and liver regeneration cascade can be illustrated in Figure 20.

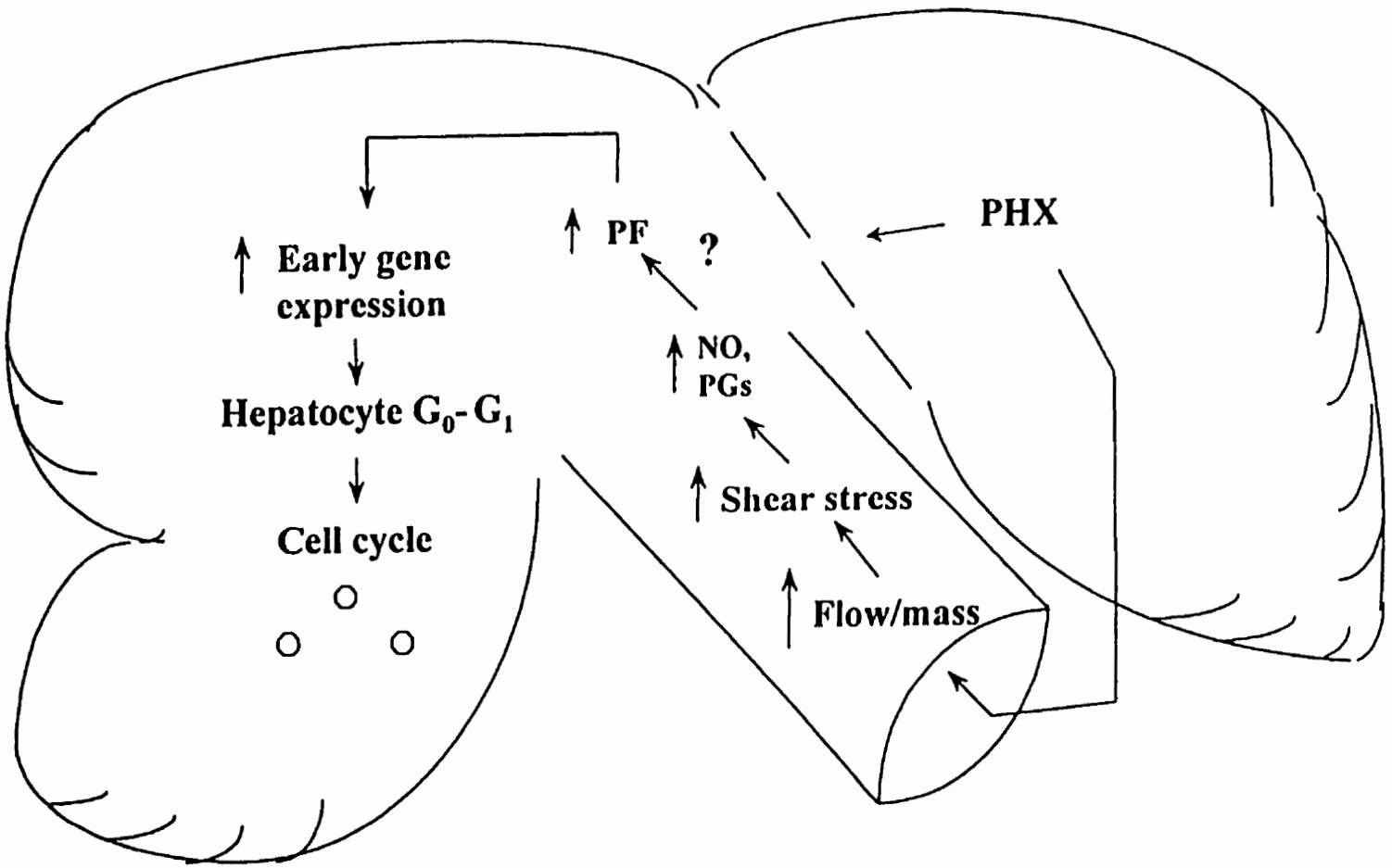


Figure 20. Initiation of Liver Regeneration

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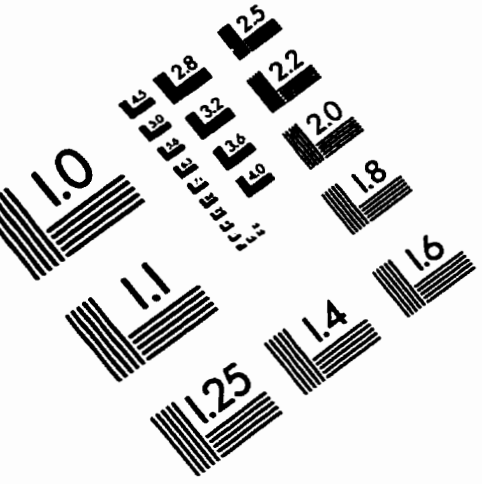

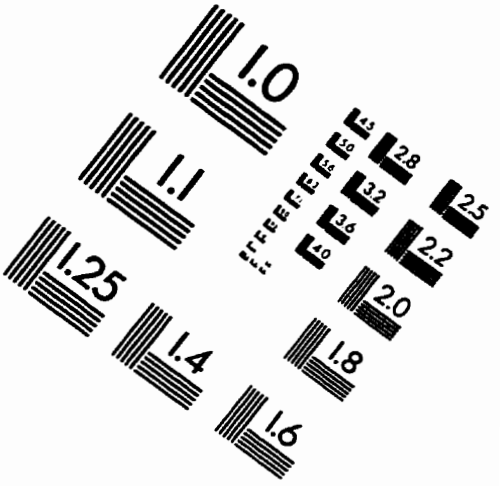

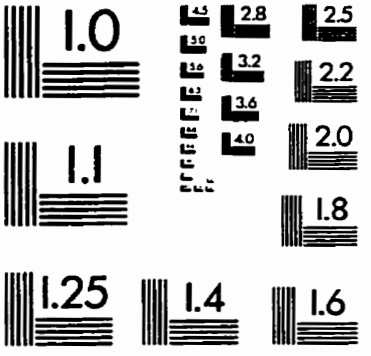

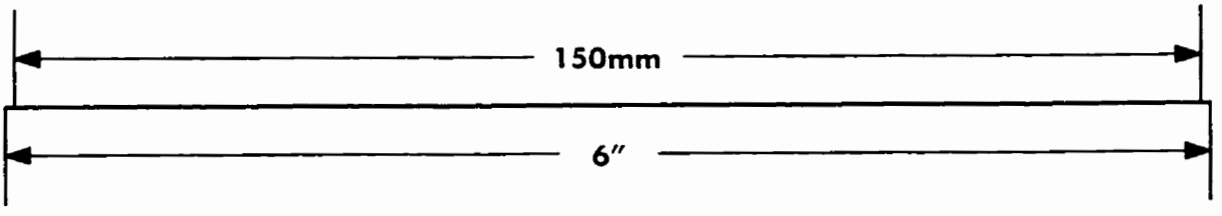

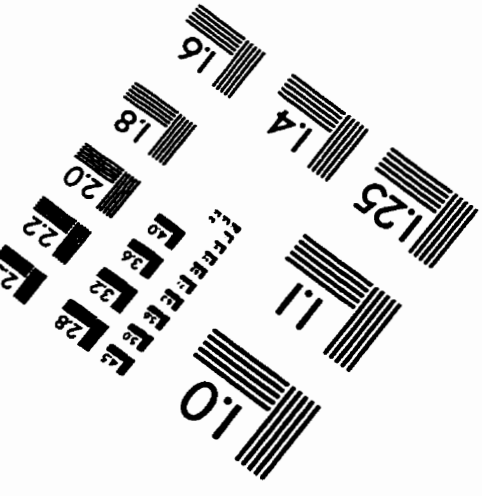

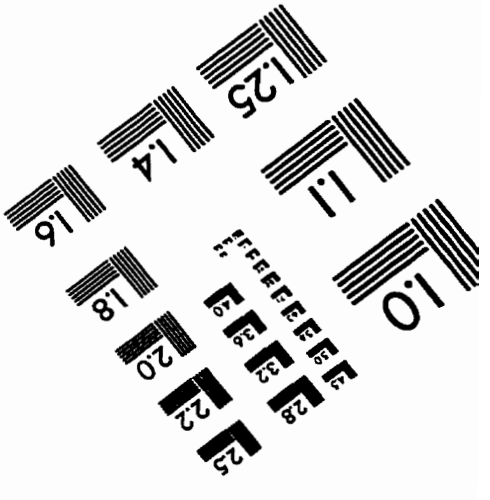

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