Table 6 Transition in the form of esophageal/gastric varix after administration of propranolol hydrochloride at maintenance dose

Form	Baseline	Week 4	Week 16	Week 28	Week 40	Week 52
F0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
F1	0 (0.0)	7(17.5)	9(25.7)	7(25.0)	6(28.6)	5(35.7)
F2	40(88.9)	21(52.5)	22(62.9)	13(46.4)	13(61.9)	9(64.3)
F3	5(11.1)	1 (2.5)	1 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)
No data	0 (0.0)	11(27.5)	3 (8.6)	8(28.6)	2 (9.5)	0 (0.0)
Total number of patients	45	40	35	28	21	14

F0：No varices　F1：Straight varices　F2：Enlarged tortuous varices　F3：Largest-sized varices
(%)

Table 7 Comparison in the form of esophageal/gastric varix between before and after administration of propranolol hydrochloride

Form	Baseline	Final examination after administration	Wilcoxon's signed rank test
F0	0 (0.0)	0 (0.0)	
F1	0 (0.0)	16(40.0)	$p<0.0001$
F2	36(90.0)	24(60.0)	
F3	4(10.0)	0 (0.0)	
Total	40	40	

F0：No varices　F1：Straight varices　F2：Enlarged tortuous varices
F3：Largest-sized varices　(%)

Table 8 Transition in the red color sign of esophageal/gastric varix after administration of propranolol hydrochloride at maintenance dose

Red color sign	Baseline	Week 4	Week 16	Week 28	Week 40	Week 52
--	0 (0.0)	6(15.0)	13(37.1)	12(42.9)	10(47.6)	8(57.1)
+	34(75.6)	20(50.0)	16(45.7)	6(21.4)	8(38.1)	6(42.9)
++	10(22.2)	1 (2.5)	2 (5.7)	1 (3.6)	0 (0.0)	0 (0.0)
+++	1 (2.2)	2 (5.0)	1 (2.9)	1 (3.6)	1 (4.8)	0 (0.0)
No data	0 (0.0)	11(27.5)	3 (8.6)	8(28.6)	2 (9.5)	0 (0.0)
Total number of patients	45	40	35	28	21	14

－：No red color sign　＋：Few localized red color signs
＋＋：Mid between(＋)and(＋＋＋)　＋＋＋：Many sings all around　(%)

肝性脳症が発現した症例はなかった．

b) 臨床検査

試験実施医師が判定した臨床検査値異常変動は，投与前後の検査実施例の41例中19例(46.3%)にみられ，異常変動発現率が10%以上であった臨床検査項目は，Al-p上昇(41例中8例：19.5%)，LDH上昇

Table 9 Comparison in the red color sign of esophageal/gastric varix between before and after administration of propranolol hydrochloride

Red color sign	Baseline	Final examination after administration	Wilcoxon's signed rank test
−	0 (0.0)	20(50.0)	
+	31(77.5)	16(40.0)	P＜0.0001
++	8(20.0)	3 (7.5)	
+++	1 (2.5)	1 (2.5)	
Total	40(100)	40(100)	

−：No red color sign　+：Few localized red color signs
++：Mid between (+) and (+++)　+++：Many sings all around　(%)

Table 10 Transition in the PHG finding of esophageal/gastric varix after administration of propranolol hydrochloride at maintenance dose

PHG finding	Baseline	Week 4	Week 16	Week 28	Week 40	Week 52
−	33(73.3)	16(40.0)	24(68.6)	14(50.0)	12(57.1)	10(71.4)
+	7(15.6)	11(27.5)	7(20.0)	4(14.3)	5(23.8)	4(28.6)
++	5(11.1)	2 (5.0)	1 (2.9)	2 (7.1)	2 (9.5)	0 (0.0)
No data	0 (0.0)	11(27.5)	3 (8.6)	8(28.6)	2 (9.5)	0 (0.0)
Total number of patients	45	40	35	28	21	14

−：No finding　+：Mild lesion　++：Severe lesion　(%)

Table 11 Comparison in the PHG finding of esophageal/gastric varix between before and after administration of propranolol hydrochloride

PHG finding	Baseline	Final examination	Wilcoxon's signed rank test
−	29(72.5)	28(70.0)	
+	7(17.5)	9(22.5)	P＝1.0000
++	4(10.0)	3 (7.5)	
Total	40	40	

−：No finding　+：Mild lesion　++：Severe lesion　(%)

(40例中6例：15.0%)，総胆汁酸上昇(20例中5例：25.0%)および血中アンモニア値上昇(19例中4例：21.1%)であった．これらの異常変動は，基礎疾患(肝硬変)に伴う変動，もしくは臨床上問題のない変動と判断され，有害事象と判定されたものはなかった．

c) 血圧・心拍数および心電図検査

塩酸プロプラノール投与中に血圧上昇傾向を示した症例が4例認められたが，いずれも投与期間中に投与前値付近まで回復した．また，血圧低下を認めた症例は1例(Table 12，症例002-001：心筋梗塞が発現した症例)であった．

本薬投与中に徐脈が3例(それぞれの最少脈拍数：42，48，40拍/分)に認められた．1例で本薬を減量(90 mg/日から60 mg/日)したが，2例(60 mg/日，

Table 12 Adverse events

Case No	Sex Age	Adverse events	Dose at the time of onset	Time of onset (after administration)	Seriousness	Severity	Continuation or discontinuation of investigational drug	Other Interventions	Outcome	Causal relationship with the investigational drug
001-005	Male 65 years old	Hepatic failure(acute aggravation of cirrhosis)	60 mg/day	Day 58	Seriousness	Very severe	Discontinued	Yes	Aggravated (death)	Not related
001-010	Male 50 years old	Dizziness	30 mg/day	Day 15	Not Seriousness	Mild	Continued	No	Relieved	Possibly related
001-016	Male 61 years old	Headache-dull	30 mg/day	Day 23	Not Seriousness	Mild	Discontinued	No	The symptom was remitted after discontinuation	Probably related
002-001	Male 66 years old	Acute myocardial infarction	10 mg/day	Day 2	Seriousness	Very severe	Discontinued	Yes	The symptom was remitted after discontinuation, but the patient died due to aggravation of hepatic failure later.	Possibly related
007 003	Male	Amnesia	60 mg/day	Day 19	Not Seriousness	Moderate	Discontinued	No	The symptom was remitted after discontinuation, and disappeared 1 week later	Definitely related
	64 years old	Tongue-tied	60 mg/day	Day 19	Not Seriousness	Moderate	Discontinued	No		
		Restlessness	60 mg/day	Day 19	Not Seriousness	Moderate	Discontinued	No		
		Insomnia	60 mg/day	Day 19	Not Seriousness	Moderate	Discontinued	No		

30 mg/日）では減量せず，いずれも投与期間中に徐脈は改善した．

心電図所見の異常が2例に認められた．うち1例（症例 002-001）は心筋梗塞を発症した患者で発症時に異常がみられた．もう1例は投与開始1週後に心室性期外収縮（単発）がみられたが，それ以降の心電図検査では心室性期外収縮は認められなかった．

III 考　　察

食道・胃静脈瘤の出現の最も重要な因子は門脈圧亢進であり，食道・胃静脈瘤からの出血にも門脈圧亢進が大きく関与している．したがって，食道・胃静脈瘤の治療目標は，肝循環に悪い影響を与えない程度に門脈圧を低下させることである．心臓非選択性である β 遮断薬は，β_1 遮断作用による心拍出量の減少と β_2 遮断作用による内臓血管収縮により門脈血流量を減少させ，その結果，門脈圧を低下させる．肝硬変患者を対象とした幾つかの海外臨床試験成績のメタアナリシスから，心臓非選択性 β 遮断薬（プロプラノロール，ナドロール）は，静脈瘤初回出血[11]および再出血[12]の予防に有効であることが確認されている．米国学会の診療ガイドライン[13]において，肝硬変静脈瘤初回出血の予防として，大きな静脈瘤のある患者に対して，非選択性 β 遮断薬（プロプラノロール，ナドロール）による薬物療法が第1選択治療法とされ，また，出血の再発予防として，内視鏡的硬化療法，静脈瘤結紮術またはその他の薬物療法（非選択性 β 遮断薬または持続

性硝酸薬)が推奨されている．英国[14]，イタリア[15]の診療ガイドラインも米国のそれとほぼ同様の治療法を推奨している．

今回，我々は基礎疾患が主に肝硬変(43/46例)の門脈圧亢進症患者を対象にして，塩酸プロプラノロールの上部消化管出血に対する予防効果および安全性について検討した．

本臨床試験においては，安静時心拍数のおよそ25％低下を目標にして，塩酸プロプラノロールを1日30 mgから投与を開始し，1週間の間隔を開けて1日量30 mgずつ増量を行い，患者ごとに維持用量を設定することとした．しかし，30 mgずつ増量すると高度の徐脈が引き起こされることを危惧して，10～20 mgずつ増量した症例が3例あった．また，1日30 mgで目標心拍数を達成し，1日30 mgを維持用量としたが，投与中，徐脈による可能性を否定できないめまいが発現したため，1日10 mgに減量した症例が1例あった．このように維持用量の設定にあたっては，患者の状態を注意深く観察し患者ごとに適した維持用量を決める必要がある．本臨床試験における維持用量は48.4±20.6 mg/日で，維持用量開始1週後の心拍数の低下率は23.7±8.4％で，目標とした心拍数の低下が得られた．同様の方法で設定したLebrecらおよびAndreaniらの臨床試験における塩酸プロプラノロールの維持用量は，それぞれ159±83* mg/日[16]，91±10** mg/日[17]であり，金沢らの臨床試験では78(30～120) mg/日[7]，73.6±38.1* mg/日[8]，森瀬らの臨床試験では31.1±19.5* mg/日[5](*：平均値±標準偏差，**：平均値±標準誤差)と報告されている．これら維持用量は個人差が大きいことに留意する必要があるが，森瀬らは塩酸プロプラノロールの投与量は20～60 mg/日が適当であると考察しており，本試験の結果もこれを支持するものであった．

有効性解析対象45例における維持用量投与期間は33.5±19.6週で，上部消化管(食道・胃静脈瘤およびPHG)出血例は3例(6.7％)であった．この出血率は投与12カ月後の初回出血の累積出血率が4.3％であった森瀬らの報告[5]とほぼ同じであった．食道静脈瘤患者の自然経過群での結果としては，Inokuchiらの報告があり[18]，1年間の初回出血率は19.1％であった．また，海外で実施された食道静脈瘤患者を対象としたプラセボコントロール試験における結果ではAndreaniらの報告があり[17]，1年間のプラセボ群での初回出血率は30％であった．この試験のプラセボ群における対象患者には，Child分類Cの患者が10％含まれていたがこれを考慮しても，これらの報告と比較して本臨床試験における出血率は低かったといえるであろう．塩酸プロプラノロール投与4週時点で食道・胃静脈瘤の形態，発赤所見が改善した例が認められ，投与後最終検査時では，形態および発赤所見はともに投与前と比較して有意に改善した．出血率および内視鏡所見からみて，塩酸プロプラノロールは食道・胃静脈瘤からの出血の予防に有効であると考えられた．

わが国においては，食道・胃静脈瘤の初回出血の予防的治療として内視鏡的硬化療法を施行することが一般的であるが，その有効性や施行に伴う合併症の発現等に関する問題点も報告されている．金沢らの肝硬変食道静脈瘤初回出血の予防に関する内視鏡的硬化療法と塩酸プロプラノロールとの比較試験[8]において，両治療法ともほぼ同程度の効果が認められている．これに対して，Andreaniらの報告[17]では，静脈瘤の初回出血予防としての内視鏡的硬化療法は塩酸プロプラノロールに劣り，プラセボと有意な差が認められていない．また，The Prova Study Group[19]は静脈瘤出血の予防では内視鏡的硬化療法は塩酸プロプラノロールおよびコントロールと変わらず，生存率ではわずかに劣ったと報告している．最近のメタアナリシス[20]の結果では，塩酸プロプラノロールと内視鏡的効果療法では静脈瘤の再出血率や生存率に有意差は認められなかったものの，前者では有害事象の発現件数が有意に低く，塩酸プロプラノロールは患者にとっても簡便で，かつ苦痛に感じることなく服用可能な経口剤であるので，再出血防止の第一選択とするべきだと考察されている．内視鏡的硬化療法を予防的治療として行う場合は，硬化療法実施直後の出血，拡張術を必要とする食道狭窄，縦隔炎，胸水などの合併症が問題となる．欧米の成績におけるこれらの合併症の発現率は，平均9％であったと報告されている[21]．わが国において1,000例の食道静脈瘤患者を対象として内視鏡的硬化療法が実施された試験における合併症の発現率は13.4％で，その多くは拡張術を必要とする食道狭窄であった[22]．

このように，塩酸プロプラノロールの有効性と内視鏡的硬化療法を予防的治療に用いた場合の手技による合併症を考えあわせると，塩酸プロプラノロールによる薬物療法(保存的治療法)の有用性は高いと考えられる．

本臨床試験において，副作用が4例(8.7％)7件に

発現し，うち，3例が副作用（頭重感，急性心筋梗塞，健忘症，舌がもつれる，不穏動作，不眠）のため投与中止となった．

海外の臨床試験では，塩酸プロプラノロールの長期投与による副作用として，一過性の無力症および心機能障害[16]，インポテンス，気管支拡張症，完全心ブロックおよび肝性脳症の誘発[23]が報告されている．

本臨床試験では，本薬投与開始8週後に肝機能が急激に悪化し，急性肝不全の状態になり，投与を中止した症例が1例あった．中止後も肝機能は悪化の一途をたどり，中止2週後に肝不全のため死亡した．試験実施医師は臨床経過から判断して，肝不全は基礎疾患である肝硬変症の自然経過によるもので，塩酸プロプラノロールとの因果関係はないと判定した．肝不全の発症に関連して，塩酸プロプラノロールによる血中アンモニア値の上昇（12 μM以上）により臨床的に肝性脳症を発現するリスクが高いとの報告[24]があるが，最近の報告では，塩酸プロプラノロールの投与と肝性脳症の発現には関連はないとの報告[25]や，肝静脈圧力勾配の反応性の低い患者では肝性脳症の発現のリスクが高いとの報告[26]もある．本臨床試験においても血中アンモニア値が異常変動（高値）を示した例が4例あったが，特に症状は認められなかった．

本試験において，対象患者の多くは基礎疾患が肝硬変であったが，塩酸プロプラノロールとの関連性が示唆される他の肝機能障害を認めた症例はなかった．

急性心筋梗塞が投与2日目に発現し，本薬投与2日で中止した症例が1例（Table 12，症例番号002-001）あった．投与開始時に発熱があり，脱水状態にあったことも誘因となったと考えられるが，本薬との因果関係は明らかでなく，否定もできないと判定された．本症例と投与1週後に一過性の心室性期外収縮（単発）が認められた症例を除いて，他には，異常心電図所見が認められた例はなかった．心筋梗塞発症例を除き，血圧が異常低下した症例はなかった．徐脈が3例に認められたが，1例で本薬の減量を実施し，他の2例は継続投与としたが，いずれも投与期間中に改善し，特に臨床上問題はなかった．

IV 結　　語

門脈圧亢進症患者46例を対象として，塩酸プロプラノロールの上部消化管出血に対する予防効果および安全性について検討し，以下の結果を得た．

(1) 有効性解析対象45例における塩酸プロプラノロールの維持用量は48.4±20.6 mg/日で，維持用量投与1週後の心拍数の低下率は23.7±8.4%であった．維持用量の投与期間は33.5±19.6週であり，上部消化管出血は45例中3例（6.7%）にみられ，食道・胃静脈瘤からの出血例が2例，PHGからの出血例が1例であった．Kaplan-Meier法により算出した52週後の累積非出血率は90.9%であった．

(2) 投与後最終検査時の食道・胃静脈瘤の形態および発赤所見はともに投与前と比較して，有意に改善した．

(3) 投与前からPHG所見の（−）の症例が多く，PHG所見は投与前後で有意な変化は認められなかった．

(4) 安全性解析対象46例中5例（10.9%）8件に有害事象が発現した．うち，4例が有害事象のため本薬投与を中止された．

(5) 臨床検査値異常変動は，投与前後の検査実施例41例中19例（46.3%）に認められたが，ほとんどが基礎疾患（肝硬変）に伴う変動，もしくは臨床上問題のない変動と判断され，有害事象と判定されたものはなかった．

(6) 本薬の副作用によると思われる死亡例はなかった．

(7) 本薬との関連性が示唆される他の肝機能障害を認めた症例はなかった．

以上の結果，塩酸プロプラノロールは門脈圧亢進症における食道・胃静脈瘤の血行動態を改善することにより，上部消化管出血の予防に有効な薬剤であると判断された．また本試験の結果から，1日最大投与量120 mgまでの範囲での安全性に特に問題は認められておらず，本疾患に対する有用性も示唆された．

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Assessment of the prophylactic effect and safety of propranolol in upper gastrointestinal bleeding associated with portal blood circulation disorder (portal hypertension)

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A prospective long-term study was conducted to evaluate the prophylactic effect and safety of propranolol in upper gastrointestinal bleeding for 46 patients with portal hypertension untreated by sclerotherapy. The duration of administration at the maintenance dose, which was to decrease the resting heart rate by 25%, was 33.5±19.6 weeks. Of the 45 patients, 3 patients experienced bleeding from upper gastrointestinal tract and cumulative non-bleeding ratio was 90.9%. Significant improvement was observed in the form and red color sign of the varices compared with the baseline by endoscopy. A total of 8 adverse events were reported in 5 of 46 patients evaluable for safety. Of them, 4 patients were withdrawn from the study due to the events, however, there was no clinically problematic cases. Propranolol was considered as a useful drug in prevention of upper gastrointestinal bleeding for the patients with portal hypertension, in terms of the efficacy and safety.

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Original Paper

Portal and Parenchymal Alterations of the Liver in Idiopathic Portal Hypertension: A Histological and Immunochemical Study

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Summary

Idiopathic portal hypertension (IPH) is characterized by presinusoidal portal hypertension owing to the intrahepatic, presinusoidal portal venous block, whereas the primary cause and initial vascular lesions(s) remain only speculative. In this study, a total of 97 IPH livers were histopathologically and immunohistochemically examined, placing emphasis on hepatic parenchymal fibrosis and atrophy as well as on portal tract fibrosis. Alcoholic cirrhosis and normal livers were used as controls. When compared with normal livers, the expression of connective tissue growth factor (CTGF) in periductal mononuclear cells was significant. Matrix metalloproteinase (MMP)9-positive mononuclear cells were fewer in number in the portal tract of IPH liver, when compared with alcoholic cirrhosis. These findings suggest a possible pathogenesis of collagen and elastin deposition because of increased CTGF expression and decreased MMP-9 expression in portal tracts of IPH. Sinusoidal dilatation associated with hepatocellular atrophy and apoptosis occurred frequently, but focally in 20% of the IPH cases. These changes were most often found in hyperplastic hepatocellular areas and in the perivenular areas of hepatic lobules. In these areas, pericellular fibrosis and thin fibrous septa were also frequently seen. In these fibrotic areas, there were deposited not only collagen fibers, but also elastic fibers, in which α-smooth muscle actin-positive sinusoidal cells, reflecting activated hepatic stellate cells, were frequently detected. It is possible that in IPH cases, continuing portal venous blood insufficiency may be responsible for hepatic parenchymal damage, which may be followed by hepatocellular apoptotic dropout and then by hepatic parenchymal atrophy and fibrosis.

Key words: Idiopathic portal hypertension – Hepatocellular apoptosis – Portal venous insufficiency – Perisinusoidal cells

Abbreviations: ABS = acidophilic bodies, α-SMA = alpha-smooth muscle actin, CTGF = connective tissue growth factor, EVG = elastica von Gieson, HSPG = heparan sulfate proteoglycan, IPH = idiopathic portal hypertension, MMP = matrix metalloproteinase

Introduction

It is generally believed that intrahepatic microcirculation disturbance is more or less involved in the progression of chronic hepatobiliary diseases, irrespective of etiologies [17, 18]. In experimental animals, hepatic ischemia, in particular portal venous insufficiency, is known to cause hepatocellular atrophy and apoptosis, followed by hepatic parenchymal atrophy [6, 19]. In fact, in human livers, atrophy of hepatocytes or hepatic lobules is found in cases of portal vein obliteration [17]. For example, focal portal venous obstruction is associated with focal hepatic atrophy, called infarct of Zahn [18]. Recently, Wanless et al. [17] suggested that hepatocellular apoptosis and atrophy as well as hyperplasia caused by ischemia might be the consequence of vascular obstruction in normal or cir-

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rhotic human livers. They also suggested that hepatocellular apoptosis is a transient response to acute ischemia, that atrophy and nodular hyperplasia of hepatocytes constitute a chronic response to ischemia, and that vascular obstruction is an important cause of atrophy of hepatocytes and hepatic lobules in chronic liver diseases [17].

Idiopathic portal hypertension (IPH) presents with sustained presinusoidal portal hypertension because of intrahepatic portal venous block, and it is believed that dense portal fibrosis and portal venous phlebosclerosis are probably related to the immune-mediated process and coagulative factors are responsible for the portal venous block [1, 7, 9]. Thrombotic processes of the portal venous system eventually occur after splenectomy in particular, possibly leading to aggravation of portal hypertension and death of IPH patients [18]. IPH is also frequently associated with intralobular fibrosis, such as slender hepatic fibrosis, and with parenchymal damage, such as hepatocellular atrophy and hyperplasia. While many authors believe that these parenchymal changes represent secondary developing changes in IPH [9], it is unclear how these changes occur, and whether these changes are progressive or not.

In this study, we first tried to clarify the fibrotic mechanism of IPH, placing emphasis on the immunohistochemical expression of connective tissue growth factor (CTGF), which is one of the strongest fibrotic factors of the liver, and on that of matrix metalloproteinase (MMP)9, which plays an important role in the degradation of elastin [5, 8, 11, 16]. Alpha-smooth muscle muscle actin (α-SMA)-positive sinusoidal cells, reflecting activated hepatic stellate cells, were also immunohistochemically examined. The hepatic parenchymal changes were surveyed histopathologically.

Materials and Methods

Histopathological analysis

IPH is characterized by the presence of chronic presinusoidal portal hypertension and the absence of cirrhosis [1, 10]. The well-known causes of portal hypertension, such as extrahepatic portal venous obliteration and congenital hepatic fibrosis, are excluded in the diagnosis of IPH. The liver specimens from the cases fulfilling these criteria were obtained from the files of hepatobiliary diseases collected in our laboratories and affiliated hospitals: a total of 20 needle and 27 wedge biopsies as well as 50 autopsy liver specimens were used for this study.

As controls, we used 10 cases of normal livers, and 10 cases of alcoholic cirrhosis (wedge biopsy or surgically resected liver specimens) with an age and sex distribution similar to that in IPH cases. It is known that inflammatory cell infiltration in portal tracts and fibrous septa is minimal to mild in alcoholic cirrhosis, and comparable to that of IPH.

Tissue preparation

The liver tissues were fixed in neutral formalin and embedded in paraffin. More than 20 serial sections, 4 μm thick, were cut from each paraffin block, some of which were processed for H&E, elastica van Gieson (EVG), Mallory's azan, Gomori's reticulin and orcein stains. The remaining slides were used for immunohistochemistry as described below.

Histopathological evaluation of IPH livers

Fibrotic portal tracts were defined as dense collagen and elastin deposition in the enlarged portal tracts. In addition, the following six histopathological changes in hepatic parenchyma were examined: atrophy of hepatic lobules, hepatic parenchymal fibrosis, sinusoidal dilatation, hepatocellular atrophy, hepatocellular nodular hyperplasia, and apoptotic hepatocytes. Atrophy of hepatic lobules was characterized by an unusual approximation of medium to large portal tracts or hepatic vein tributaries to the hepatic capsule or to each other [1, 10]. Hepatic parenchymal fibrosis can be subdivided into three types: intralobular slender fibrosis, pericellular fibrosis, and slender fibrous septa from the portal tracts. Intralobular fibrosis was defined as slender fibrous septa in the parenchyma, and pericellular fibrosis was found around the hepatocellular cords. Hepatocellular atrophy was characterized by small or slender hepatocytes. Round acidophilic bodies (round ABs) and stellate-shaped acidophilic bodies (stellate ABs) were considered to represent 2 types of hepatocellular apoptosis [17]. The round ABs were characterized by round and shrunk hepatocytes with pyknotic or fragmented nuclei or without nuclei and detached from surrounding hepatocytes. The stellate ABs were rhomboid or had condensed cytoplasm and pyknotic nucleus; they were shrunk and still firmly attached as compared with adjacent hepatocytes. Hepatocellular nodular hyperplasia of IPH was characterized by predominant nodular hyperplasia of the hepatic parenchyma.

Immunohistochemical analysis for portal tract and parenchymal fibrosis

Of 97 IPH specimens, 16 wedge biopsies, which showed parenchymal hepatic fibrosis and optimal fixation, were used for immunohistochemical analysis. Ten cases of normal livers and 10 cases of alcoholic cirrhosis (wedge biopsy and needle biopsy) with similar age and sex distribution to the IPH cases were used as controls.

After deparaffinization and standard microwave treatment [2], the sections were incubated in serum-free protein blocking reagent (DAKO, Carpinteria, CA) for 30 minutes, and then incubated with a goat polyclonal antibody against CTGF, mouse monoclonal antibody against MMP-9 (Fuji Chemical, Toyama, Japan), or mouse monoclonal antibody against α-SMA (DAKO, clone 1A4) overnight at 4 °C. After a phosphate-buffered-saline (PBS) wash, Envision-PO or Envision-alkaline phosphatase (Envision-AP) (DAKO) was applied. Specimens were incubated with Envision solution for 1 hour. After PBS wash, DAB was applied as the substrate for Envision-PO, and Fast Red (Vector Laboratories, Burlingame, CA) with Levamisol (Vector) for Envision-AP. Replacement of the primary antibody with PBS resulted in negative staining.

Statistics

Intergroup comparison was done by Fischer's extact test and the χ^2 test; the difference was considered significant when p values were less than 0.05.

Results

Histopathologic evaluation in IPH

Dense portal fibrosis and portal venous obliteration were found in all IPH cases. In addition, the following histopathological changes were found (Table 1):

Atrophy of hepatic lobules occurred in 45 cases with different degrees.

As for *hepatic parenchymal fibrosis*, intralobular slender fibrosis was seen in more than 50% of IPH cases, and was occasionally linked to the pericellular fibrosis or slender fibrous septa from portal tracts, which developed afterwards. Pericellular fibrosis was frequently seen in the perivenular areas of the hepatic parenchyma, and between hyperplastic hepatocellular nodules. In these areas, hepatocytes were atrophic, and sinusoids were usually dilated, resulting in the linkage to neighboring central veins. Pericellular fibrosis was also seen in the perivenular areas without sinusoidal dilatation. In two cases, there were clear-cut thin fibrous septa around these areas. These septa appeared to be linked to intralobular fibrosis, which is unrelated to portal tracts. Central veins frequently showed obliteration or thickening of their wall with focal sinusoidal dilatation.

Sinusoidal dilatation was seen in 35 cases. Of these, hepatocellular atrophy and nodular hyperplasia occurred in 3 and 6 cases, respectively. In some cases, sinusoidal dilatation was closely related to fibrous cord (probably obliterated central veins). Some of the dilated sinusoids were related to abnormally dilated vasculatures in the hepatic parenchyma.

Hepatocellular atrophy was most often seen in perivenular areas and between hyperplastic hepatocytic nodules.

Hepatocellular nodular hyperplasia was found in 35 cases.

As for *hepatocellular apoptosis*, stellate and round ABs were usually found focally or in clusters in the hepatic parenchyma. They were more or less frequent in the areas with sinusoidal dilatation.

In addition, focal hepatocellular necrosis was occasionally associated with pigmented macrophages in 20 of the 97 cases.

Table 1. Incidence and degree of the histopathological changes in IPH livers

Hepatic parenchymal change	
– Atrophy of hepatic lobules	45 (46.4%)
– Parenchymal hepatic fibrosis	
Intralobular slender fibrosis	49 (50.5%)
Pericellular fibrosis	90 (92.8%)
Slender fibrous septa from the fibrotic portal tracts	71 (73%)
– Sinusoidal dilatation	35 (36.1%)
– Hepatocellular atrophy	20 (20.6%)
– Hepatocellular nodular hyperplasia	35 (36.1%)
– Apoptosis of hepatocytes	
Round acidophilic bodies	60 (61.9%)
Stellate acidophilic bodies	50 (51.5%)
Other hepatic and portal changes	
– Focal hepatocellular necrosis (with pigmented macrophages)	20 (20.6%)

Immunohistochemical analysis

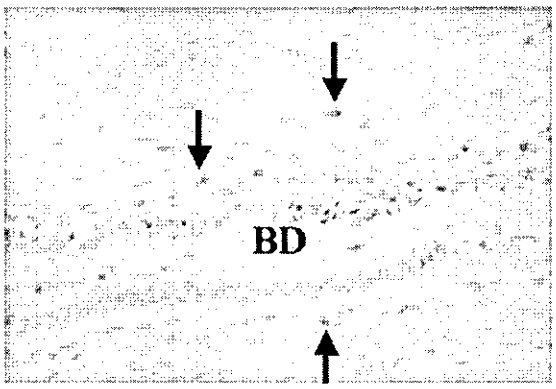
CTGF expression in portal tracts

CTGF was expressed in mononuclear cells and in the extracellular matrix in portal tracts, while there was no clear expression in hepatic parenchyma except for a few sinusoidal mononuclear cells (Fig. 1). The mononuclear cells were frequent around the bile ducts. Expression of CTGF on the biliary epithelial cells was vague and variable in 13/16 cases (81%) of IPH, 7/10 cases (70%) of alcoholic cirrhosis, and in 9/10 (90%) cases of normal liver. The results are shown in Table 2. The number of CTGF-positive mononuclear cell infiltration in portal

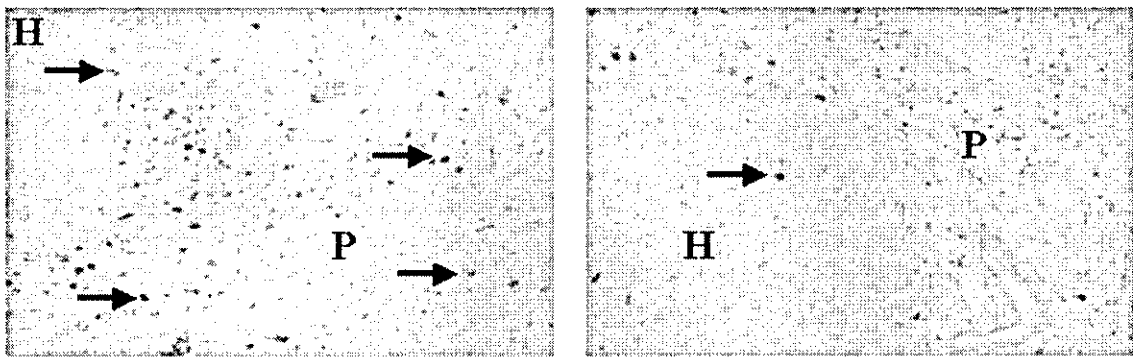
Table 2. Expression of connective tissue growth factor (CTGF) in portal tract in idiopathic portal hypertension (IPH), alcoholic cirrhosis (AC), and normal livers (NL)

Diseases	Frequency of CTGF expression on biliary epithelial cells	CTGF expression in mononuclear cells in portal tracts and their distribution pattern[1] (PD type: Diffuse type)	CTGF expression in extracellular connective tissue matrix
IPH (16)	13 (81%)	7.3 ± 10.5* (11:5)	8 (50%)
AC (10)	7 (70%)	8.3 ± 6.7*,[a] (2:8)	8 (80%)
NL (10)	9 (90%)	2.8 ± 2.3* (2:8)	1 (10%)

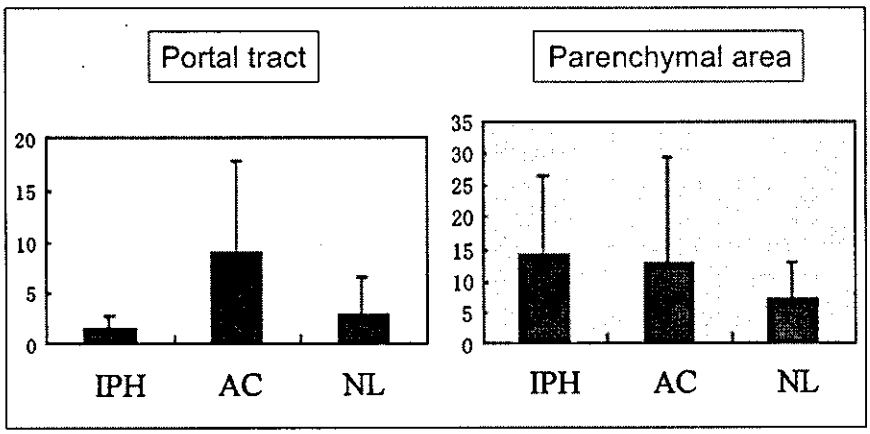
[1] Distribution pattern of CTGF-positive mononuclear cells are subdivided into two types: PD type, CTGF-positive cells are relatively dense around the bile ducts; diffuse type, CTGF-positive cells are diffusely located in portal tracts.
* The number of positive cells per one visual field of ×200 magnification. [a] $p < 0.05$ when compared to normal liver



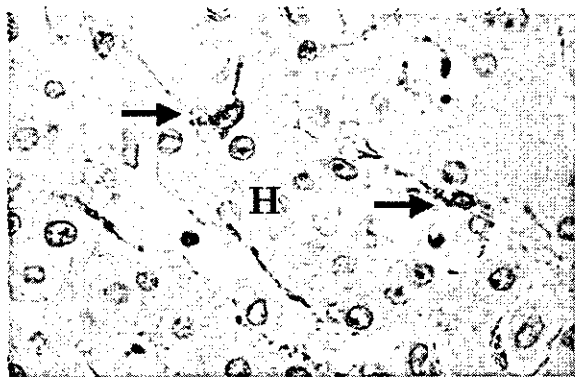
◀ **Fig. 1.** Immunostaining for connective tissue growth factor (CTGF) in portal tract of idiopathic portal hypertension. Biliary epithelial cells faintly expressed CTGF in their cytoplasm. CTGF expression was evident in mononuclear cells near or beside the bile duct (arrows). Diffuse expression of CTGF was also seen in extracellular matrix. BD, bile duct. (Envision-AP method with Fast Red. Counterstained with hematoxylin.)



▲ **Fig. 2.** Immunostaining for matrix metalloproteinase (MMP)-9 in the liver of alcoholic cirrhosis (A) and idiopathic portal hypertension (B). **A:** MMP9-positive mononuclear cells (arrows) were seen not only in hepatic parenchyma (H), but also in portal area (P). **B:** MMP9-positive mononuclear cells (arrows) were seen in hepatic parenchyma (H), while MMP-9 expression was not evident in mononuclear cells in portal tract (P). (A, B: Envision-PO method with DAB. Counterstained with hematoxylin)



◀ **Fig. 3.** The number of MMP9-positive mononuclear cells per one visual field of 200 times magnification was evaluated in portal tract and hepatic parenchymal area (sinusoids). In sinusoidal area, the number of MMP9-positive mononuclear cells in idiopathic portal hypertension (IPH) and alcoholic cirrhosis was increased compared to that of normal liver ($p < 0.05$). In the portal tract, the number of MMP9-positive mononuclear cells in IPH was not increased compared to that in normal livers; however, there was a significant increase in the number in alcoholic cirrhosis. IPH = idiopathic portal hypertension, AC = alcoholic cirrhosis, NL = normal liver



◀ **Fig. 4.** Immunostaining for α-smooth muscle actin (α-SMA) in hepatic parenchyma of idiopathic portal hypertension (IPH). α-SMA-positive activated hepatic stellate cells (myofibroblasts, arrows) were evident and increased around atrophic hepatocytes (H). (Envision-PO method with DAB. Counterstained with hematoxylin.)

tracts was more increased in IPH (7.3 ± 10.5 per one visual field of 200 times magnification) and alcoholic cirrhosis (8.2 ± 6.7) than in the normal liver (2.8 ± 2.3) ($p < 0.05$). There were two distribution patterns for CTGF-positive mononuclear cells. One was the periductal pattern, in which CTGF-positive mononuclear cells were located near and/or besides bile ducts; the other pattern showed diffusely located CTGF-positive cells, there being no association with bile duct. In IPH, the "periductal pattern" was seen in 11/16 (69%) cases (Figs. 1A–C), and was significantly more frequent than in alcoholic cirrhosis (2/10) and the normal liver (2/10). CTGF was also expressed on extracellular connective tissue matrix in 8/16 (50%) of IPH cases, in 8/10 (80%) cases of alcoholic cirrhosis, and in 1/10 (10%) normal livers (Fig. 1).

MMP-9 expression in portal tracts and hepatic parenchyma

MMP-9 was expressed in mononuclear cells of portal tracts and sinusoids, but not in the hepatocytes or bile ducts. The number of MMP9-positive mononuclear cells per one visual field (200×) was evaluated in the sinusoidal and portal tracts. In the sinusoidal area, the number of MMP9-positive cells of IPH (14.1 ± 12.3 per one visual field, 200×) was similar to that in alcoholic cirrhosis (12.9 ± 16.5), and was higher than in the normal liver (7.1 ± 5.6). Although the number of MMP9-positive cells of alcoholic cirrhosis (8.9 ± 8.8) was higher in the portal tracts, the number of IPH (1.5 ± 1.3) was almost equal to that of normal liver (2.9 ± 3.7) (Fig. 2A, B, Fig. 3).

α-SMA in portal tracts and hepatic parenchyma

In hepatic parenchyma, α-SMA-positive activated perisinusoidal cells (myofibroblasts) were increased in the perivenular areas and also around atrophic hepatocytes in almost all IPH livers (Fig. 4). Such activated perisinusoidal cells were also frequent in alcoholic cirrhosis, but were rare or occurred only occasionally in normal livers. In portal tract, α-SMA was expressed on the smooth muscle layer of portal veins and hepatic arteries in IPH, alcoholic cirrhosis, and normal livers.

Discussion

The present study disclosed that IPH livers constantly showed not only portal tract dense fibrosis and portal venous obliteration, but also hepatic parenchymal changes, such as atrophy of hepatic lobules and hepatic parenchymal fibrosis, with high frequency. Sinusoidal dilatation with hepatocellular atrophy and apoptosis were most often found in the perivenular areas of hepatic lobules and between hyperplastic hepatocellular nodules. Hepatic atrophy and lobular distortion were frequent in the IPH livers, as already reported in previous studies [1, 7, 10]. All of these findings, including those obtained in this study, suggest that the hepatocellular dropout from the hepatic lobules persists, leading to atrophy of hepatic lobules and hence to lobular distortion in IPH.

Interestingly, pericellular fibrosis was frequent in the perivenular areas and also around atrophic hepatocytes in hepatic parenchyma. Slender fibrous septa were also found in these areas, and these septa and pericellular fibrosis merged sometimes. These findings allow us to hypothesize that microcirculatory changes are related to pericellular fibrosis. Interestingly, in these areas, α-SMA-positive activated hepatic stellate cells (myofibroblasts) were increased or accumulated in these parenchymal fibrotic areas. It is assumed that perisinusoidal cells, known to produce extracellular matrix proteins, including collagen, laminin, and fibronectin [3, 4, 12], are involved in the hepatic fibrosis, caused by several diseases, such as alcoholic fibrosis and chronic hepatitis [13]. It seems likely that these cells were increased and activated in the parenchymal areas showing ischemic change. Taken together, these intralobular fibroses, such as intralobular thin fibrous septa and pericellular fibrosis, might have been caused by increased and activated perisinusoidal cells in the hepatic parenchyma, probably secondary to ischemia. This suggests that such parenchymal changes are related to portal venous insufficiency, and contribute to the development and progression of parenchymal fibrosis during the long course of IPH.

Hepatocellular hyperplasia, often occurring with vague nodule formation, was also frequently seen. It remains unclear why hepatocellular hyperplasia is predominant in some cases and absent in other cases. It is known that almost all chronic hepatocellular, biliary and hepatic venous obstructive diseases may lead to liver cirrhosis. However, it still needs to be clarified whether or not the chronic effect of intrahepatic portal venous blocks on the liver results in liver cirrhosis.

Dense portal fibrosis is a characteristic and important feature of IPH liver [9]. In the present study, CTGF was expressed in portal mononuclear cells, particularly around the bile ducts, while MMP9-positive mononuclear cells in portal tracts were fewer in number in IPH liver. CTGF, a cystein-rich peptide with heparin-binding modules, can enhance fibroblastic proliferation and chemotaxis [11, 16]. In the present study, although bile ductal expression of CTGF was vaguely seen in IPH, alcoholic cirrhosis and also even in normal liver, CTGF expressing mononuclear cells around the bile ducts was frequent in IPH liver. CTGF, which is produced by these periductal mononuclear cells, may play an important role in progressing periductal fibrosis in IPH liver [10]. CTGF was also expressed on the extracellular matrix of portal tract in alcoholic cirrhosis and IPH. CTGF has heparin-binding modules and can bind to heparan sul-

fate proteoglycan (HSPG), which exists in newly formed fibrotic areas of the portal tracts [14, 15]. As HSPG was frequently seen in the portal tract of alcoholic cirrhosis and IPH (data not shown), CTGF may bind and be stored in extracellular matrix.

MMP9-positive mononuclear cells were not increased in the portal tract of IPH, but they were significantly increased in alcoholic cirrhosis. As MMP-9 can degrade not only collagen but also elastin [5], it seems to play a role in maintaining the fibrotic balance in the portal tract in normal and pathological livers. Decreased expression of MMP-9 positive mononuclear cells, despite fibrosis, may cause disturbance of elastin and collagen degradation, thus inducing excessive elastin and collagen deposition in IPH.

In IPH, dense portal fibrosis and periductal fibrosis are frequently seen; however, cirrhosis does not develop afterwards [9, 10]. In IPH, portal venous insufficiency is the most common cause of hepatocellular damage, although in other progressive diseases, such as chronic viral hepatitis and alcoholic liver disease, active hepatocellular damage, such as immune reaction and metabolic injuries, are associated with active or reparative hepatocellular regeneration and eventually followed by cirrhosis [20]. This fact suggests that portal venous insufficiency alone does not necessarily lead to liver cirrhosis [9]. The expression patterns of CTGF and MMP-9 in portal tracts and hepatic lobules in alcoholic cirrhosis and IPH were different. Increased CTGF-positive mononuclear cells, particularly around the bile ducts, and non-increased MMP9-positive mononuclear cells in portal tracts may explain the periductal and portal fibroses in IPH, although the expression patterns of CTGF and MMP-9 in alcoholic liver disease do not explain the development of cirrhosis. Fibrogenetic factors other than CTGF and MMP-9 as well as persistent and active hepatocellular damage with regeneration play a more important role in the development of liver cirrhosis.

In conclusion, portal tract fibrosis and elastosis may be due to unbalanced activation of CTGF and MMP-9 in the portal tract in IPH. The long-standing portal venous insufficiency caused by portal fibrosis and portal venous obliteration may be responsible for the occurrence and progression of several hepatic parenchymal changes in IPH.

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Early development of cavernomatous vasculatures in portal venous thrombosis: morphometric kinetics in rabbit model

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Abstract

We tried to establish an animal model of portal venous thrombosis in order to analyze the ensuing pathological changes of the liver. An emulsion with *Escherichia coli* endotoxin and Lipiodol-Ultra-Fluide was injected into the portal vein of the left anterior lobe of the rabbit liver. The target lobe and portal vein were time-sequentially examined. In the experimental groups, it was found that fibrin thrombi were formed in the portal vein within 48 h after the injection and thrombi persisted for over 7 days. In an association with thrombus formation, numerous tortuously dilated vasculatures developed in the portal tract (cavernomatous vasculature) within 48 h. Both the number and the total area of the cavernomatous vasculatures increased from 2- to 3-fold more than in the control group at 72 h. The majority of proliferated vasculatures were positive for α-smooth muscle actin, thus suggesting that they derived from the portal venous branches. In conclusion, portal endotoxemia may be one of the pathogenetic factors of portal venous thrombosis and, in this model, the cavernomatous vasculatures rapidly developed from the portal venous branches.

Keywords: Portal venous thrombosis; Cavernomatous vasculature; Cavernomatous transformation; α-Smooth muscle actin

1. Introduction

Extrahepatic portal obstruction (EHO) is a disease characterized by portal hypertension due to the obstruction of the portal venous trunk near the hepatic hilum [1,2]. Portal venography demonstrated a complete obstruction of the extra-hepatic portal trunk with tortuously dilated hepatopetal venous channels and this development is called cavernomatous transformation [3,4]. EHO is divided into two types, namely, idiopathic or secondary. The onset of most cases of the idiopathic EHO occurs in the pediatric age group. The etiology of the idiopathic EHO remains unestablished, however, umbilical catheterization [5], sepsis [6], exchange transfusion [7], abdominal operation [8], peritonitis [9], congenital anomaly of the portal vein [10] and anticoagulant deficiency [11,12] are suspected. On the other hand, secondary EHO is the sequela of hepatic fibrosis [13,14], idiopathic portal hypertension (IPH) [15], collagen disease [16], biliary tract infection [17], pancreatic disease [18], myeloproliferative disorder [19] and malignancies [20,21]. While an animal model for idiopathic as well as secondary EHO are mandatory for an analysis of their etiopathogenesis, such models are not available so far.

Several clinical experiences have raised the possibility that intra-portal coagulopathy due to intra-abdominal infection may play an important role in the initiation of EHO, as described above. However, the pathophysiology of thrombus formation localized to the extrahepatic portal trunk has not been clearly settled, so far. In addition, numerous abnormal vasculatures characteristically develop around the occluded portal veins in the portal tracts [22,23], which is also a hallmark of EHO. The nature of these vasculatures and the sequences of vasculature development subsequent to the portal venous thrombus formation also remain unclarified. Furthermore, Ohno et al. [24] reported that massive hepatic necrosis was able to be induced by the administration of *Escherichia coli* endotoxin. This phenom-

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enon is known as the Shwartzman mechanism [24]. Interestingly, portal venous thrombosis was frequently seen in the portal tracts of the liver in their study.

Such clinical as well as experimental evidence has prompted us to hypothesize that portal venous endotoxemia is an important causative factor for portal venous thrombosis and the resultant EHO. As a result, in this study, we tried to determine whether or not portal venous endotoxin administration can induce the portal venous thrombosis and EHO in rabbits. Such an approach has not been reported so far.

2. Materials and methods

All experimental protocols were reviewed and approved by the Nagasaki University Research, Animal Resources and Animal Care Committees and met the institutional and national guidelines. The experiments were performed at the Animal Center of the Nagasaki University School of Medicine.

2.1. Animals

Japanese albino adult rabbits weighing between 2.2 and 3.2 kg were used for the experiments. All were female, but none had ever previously been pregnant.

2.2. Chemicals

E. coli endotoxin (0111:B4, Difco Laboratories, MI) was used as the agent. Lipiodol Ultra-Fluide (ethyl ester of the fatty acid of poppy-seed oil, Andre-Gelbe Laboratory, France), an oily contrast medium for lymphography, was used as the carrier for endotoxin. Endotoxin (3 mg/ml) was dissolved in a mixture of 60% Urografin (Schering AG, Berlin/Bergkamen, Germany) with one-fifth volume of distilled water. This solute was mixed with three times volume of Lipiodol just before the injection (termed EX-LPD emulsion).

2.3. Experimental procedures

The rabbits were anesthetized with intravenous pentobarbital sodium (Abbott Labs., Abbott Park, IL), 30 mg/kg of body weight. The abdomen was opened through a midline incision. A needle (No. 26 gauge) was inserted directly into a branch of the portal vein of the left anterior lobe of the liver, which was the target lobe. The EX-LPD emulsion was then injected manually. The dose of the emulsion was about 0.4–0.8 ml per body.

The animals were divided into four groups. Groups II–IV were experimental groups treated with EX-LPD emulsion as described above. The animals were autopsied at varying intervals: group II ($n = 10$) after 48 h, group III ($n = 10$) after 72 h and group IV ($n = 10$) after 7 days. The rabbits in group I ($n = 3$) which underwent a sham operation served as a control.

2.4. Biochemical evaluation

Blood samples were collected via the marginal ear vein before and at 6, 12, 24, 48, 72 and 168 h after the EX-LPD emulsion injection. The samples were analyzed for serum chemistry analyses, including aspartate aminotransferase, alanine aminotransferase and total bilirubin.

2.5. Pathological and immunohistochemical evaluation

The animals were sacrificed by an intravenous overdose of sodium pentobarbital and were then autopsied. All the organs were grossly examined and representative sections, including the liver, portal vein trunk and spleen, were fixed in formalin and embedded in paraffin. More than 20 thin sections, 4 μm in thickness, were cut from each block and some were stained with hematoxylin-eosin and phosphotungustic acid hematoxylin for fibrin. The remaining sections were used for the following immunohistochemical examination.

Serial tissue sections were stained immunohistochemically for α-smooth muscle actin (α-SMA). Anti-human α-SMA monoclonal antibody (1A4, Dako, Glostrup, Denmark) was used as a primary antibody and the water soluble polymer conjugates (ENVISION K1490, Dako) was used as a secondary antibody. After deparaffinization, the sections were immersed in 3% H_2O_2 solution for 5 min to block endogenous peroxidase activity. Each step was followed by repeated washing in phosphate-buffered saline adjusted to pH 7.4. The sections were incubated with primary antibody for 1 h at room temperature. Next, these sections were incubated with secondary antibodies for 1 h at room temperature. The reaction products were visualized in 0.001% H_2O_2-3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical, Osaka). The sections were counterstained with hematoxylin.

In the histological evaluation, portal venous thromboemboli and the formation of cavernomatous vasculature were focused. In the present study, the cavernomatous vasculatures were defined as the thin-walled, dilated vessels in the portal tract in the liver. Main portal vein branches were not included in the cavernomatous vasculatures. The cavernomatous vasculatures increased in number in the experimental groups in comparison to the small channels in the portal tract in the normal liver and were rather concentrated around the portal vein. It is difficult to clearly identify their origin, either in the portal venous branch or lymph vessel, based on routine histological examinations. α-SMA staining was positive on smooth muscle cells of the

blood vessels, including the portal vein, hepatic artery and central vein of the liver. In this study, the cavernomatous vasculatures were divided into two groups according to whether or not they were positive immunohistochemically for α-SMA (Fig. 1).

2.6. Computerized analysis

In each group, at least 20–30 tissue blocks including various levels of portal tracts were obtained. To minimize the sampling error, the portal tracts containing bile duct with 0.1–0.2 mm diameter were selected for the measurements. A digital microphotograph was obtained using the Olympus HDTV system (AX80T and OHD-200D, Olympus, Tokyo). The identifiable vessels were manually traced on the monitor by means of a computer-tracking device. An image analysis was performed using the public domain NIH Image program (developed at the US National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov) on a Power Macintosh 9500/150 computer.

Both the number and total area of cavernomatous vasculatures were measured and calculated per portal tract. The number and total area of the cavernomatous vasculatures positive or negative for α-SMA were measured and compared between them.

2.7. Statistical analyses

The data were expressed as the mean ± S.D. Differences among the groups were tested for statistical significance with the use of the Student's t-test and ANOVA post hoc test. A P value of $< 5\%$ was considered to be significant.

3. Results

3.1. Serum biochemical analysis

The results of a serum biochemical analysis in experimental groups II–IV are summarized in Fig. 2. The serum levels of aspartate aminotransferase, alanine aminotransferase and total bilirubin rose within 24 h and then returned to basal levels within 2–7 days.

3.2. Histopathological findings

In the target lobe of the liver in the control (group I), there were no abnormal changes. In the target lobe in group II, there were marked thrombus formations in all rabbits examined. The fresh fibrin thrombi were mainly present in the middle-sized portal branches, while they also both extended to near the portal trunk and the subcapsular peripheral portal venous branches. Hepatocellular necrosis was focally seen in five of ten rabbits. The subcapsular peripheral branches were filled with inflammatory cell infiltrations around the Lipiodol droplets and the infiltrative cells were composed of lymphocytes, neutrophils and histiocytes. Eosinophil infiltration was also seen.

In the target lobe of groups III and IV, the histological findings were fundamentally similar to those of

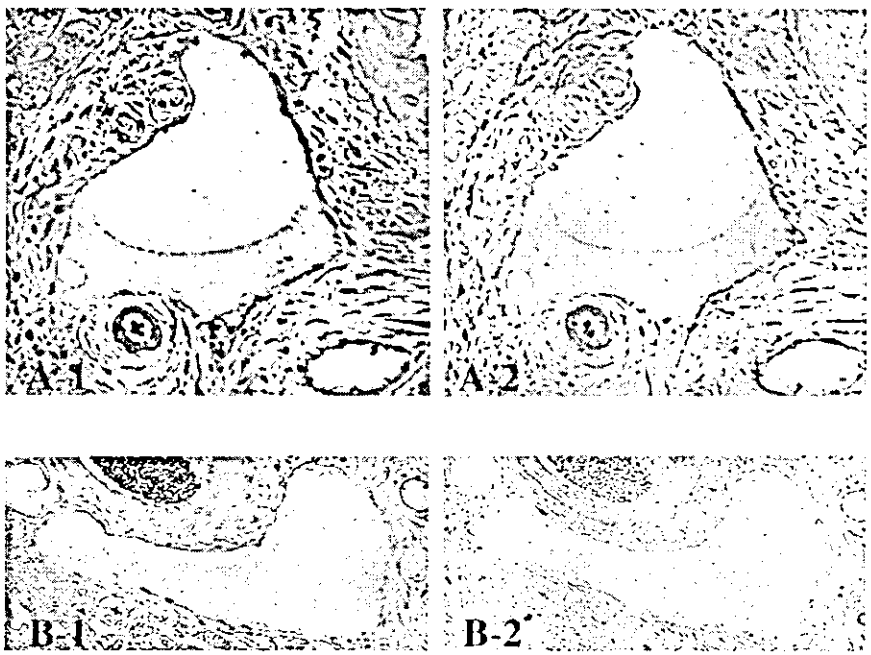

Fig. 1. The different immunoreactivities of the cavernomatous vasculatures for anti-α-SMA. (A) α-SMA positive vasculature. A-1; hematoxylin-eosin (× 80), A-2; anti-α-SMA staining. (B) α-SMA negative vasculature. B-1; hematoxylin-eosin (× 60), B-2; anti-α-SMA staining.

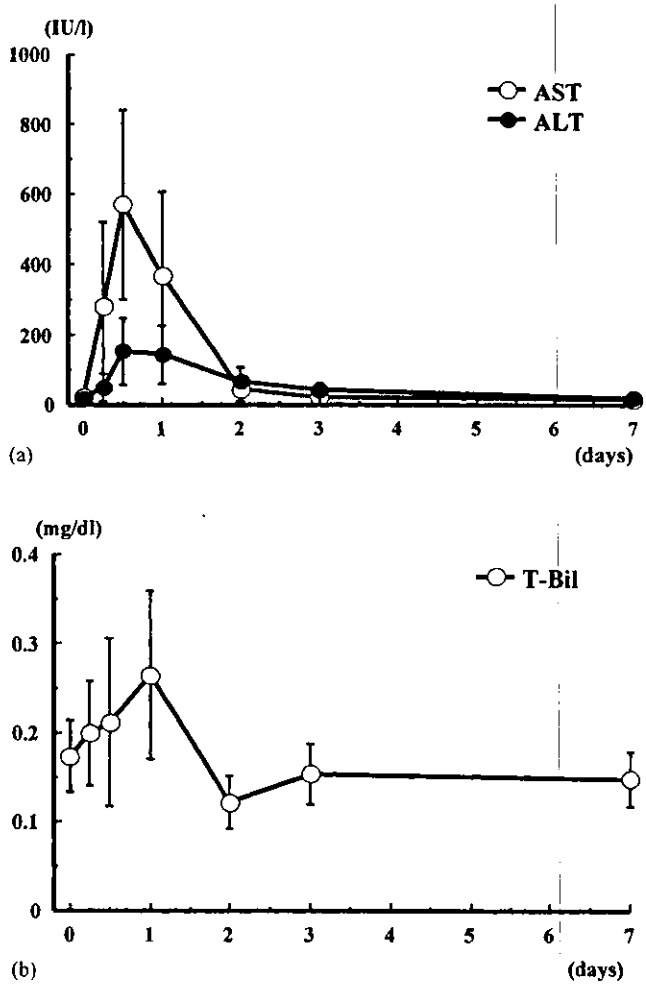

Fig. 2. (a, b) The results of the serum biochemical analysis. AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin.

group II. In group III, all rabbits showed marked thrombus formations in the distal to middle-sized portal branches (Fig. 3). In group IV, the absorption figure of the organized thrombi with multinucleated giant cells was frequently seen. In the hepatic lobes, other than the target lobe in groups II–IV, no histological changes were evident.

3.3. Image analysis of cavernomatous vasculatures

The outstanding feature in group III was a significant increase in both number and area of cavernomatous vasculatures, which were mainly distributed around the large to middle-sized portal branches (Fig. 4). In group IV, the cavernomatous vasculatures tended to decrease in both number and area.

In the normal livers (group I), the mean number of cavernomatous vasculatures was 13.9 ± 5.6 per portal tract. It was 12.5 ± 7.5 in group II and increased up to 25.7 ± 8.6 in group III, respectively. However, it was 20.5 ± 7.9 in group IV (Fig. 5). The mean number of

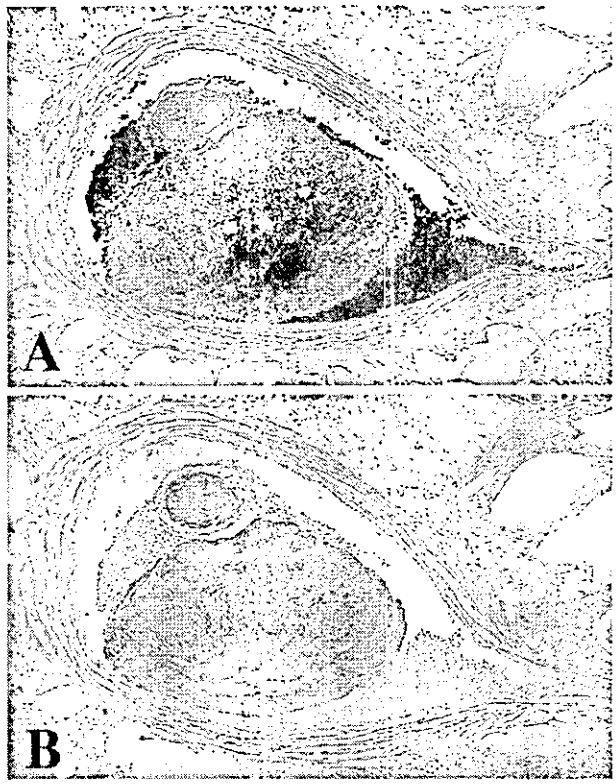

Fig. 3

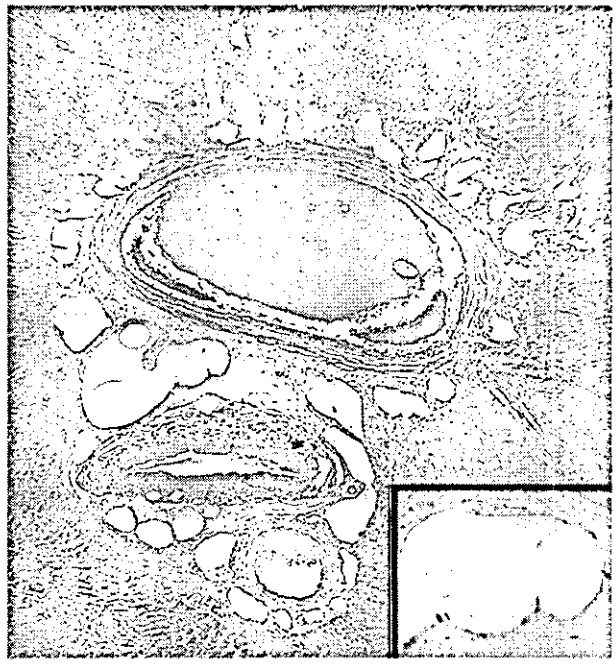

Fig. 4

Fig. 3. Portal venous thrombosis in group III (× 40). (A) Hematoxylin-eosin; (B) phosphotungustic acid hematoxylin.
Fig. 4. Cavernomatous vasculatures in group III. Hematoxylin-eosin (× 20); (inset) magnification view of a cavernomatous vasculature.

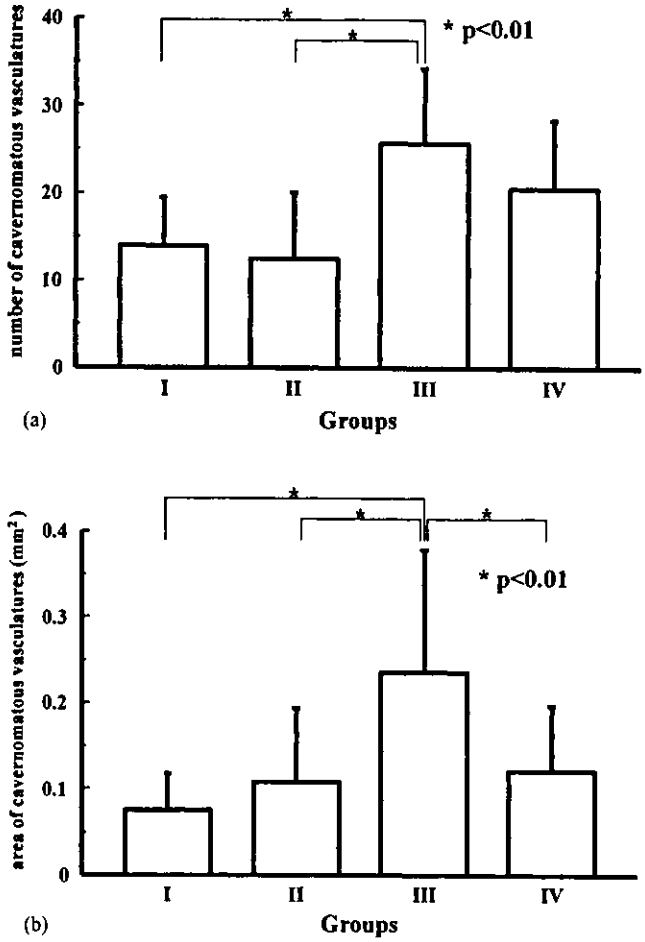

Fig. 5. (a, b) Changes in the number and total area of the cavernomatous vasculatures per portal tract.

cavernomatous vasculatures in group III was significantly higher than in groups I and II ($P < 0.01$), respectively.

The total area of cavernomatous vasculatures per portal tract was 0.075 ± 0.042 mm^2 in group I. It increased up to 0.108 ± 0.035 mm^2 in group II and 0.236 ± 0.143 mm^2 in group III, respectively. However, it was 0.120 ± 0.076 mm^2 in group IV. The total area of cavernomatous vasculatures in group III was significantly larger than in groups I, II and also group IV ($P < 0.01$).

3.4. α-SMA immunohistochemistry

In group I, the mean number of cavernomatous vasculatures per portal tract was 13.9, as described above. Of these, 1.8 (12.9%) were positive for α-SMA, but 12.1 (87.1%) were negative (Fig. 6). On the other hand, in group III, the mean number of cavernomatous vasculatures was 25.7, which was the largest among the experimental groups. Of these, 17.6 (68.5%) were positive for α-SMA, but 8.1 (31.5%) were negative.

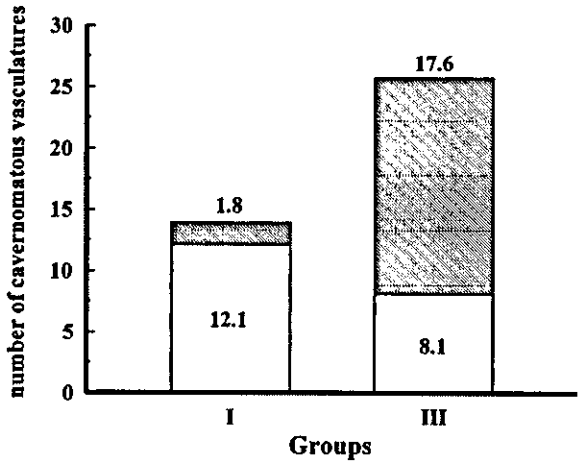

Fig. 6. Anti-α-SMA staining and changes in the number of cavernomatous vasculatures per portal tract. α-SMA: ■, positive; □, negative.

From the standpoint of the total area of cavernomatous vasculatures, which was 0.075 mm^2 in group I, 0.018 mm^2 (24.0%) were positive for α-SMA but 0.057 mm^2 (76.0%) were negative (Fig. 7). In group III, the total area of the cavernomatous vasculatures was 0.236 mm^2. Of these, 0.179 mm^2 (75.8%) was occupied by positive vasculatures for α-SMA staining and only 0.057 mm^2 (24.2%) of area was negative for α-SMA.

4. Discussion

The present study established a novel animal model of portal venous thrombosis due to endotoxin administration. EX-LPD emulsion has a sustained-releasing effect on endotoxin [24]. Endotoxin induces the secretion of cytokines, including interleukin-1 β and tumor necrosis

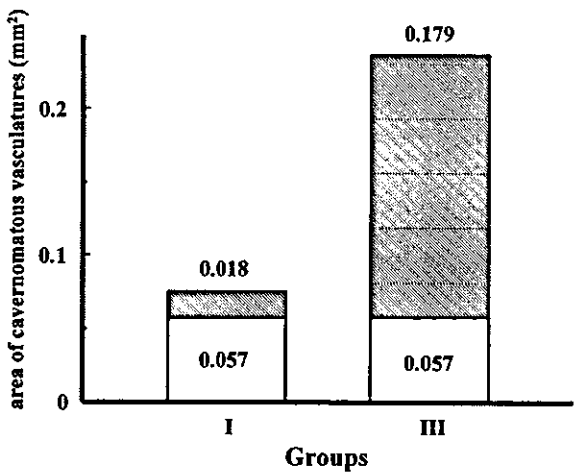

Fig. 7. Anti-α-SMA staining and changes in the total area of the cavernomatous vasculatures per portal tract. α-SMA: ■, positive; □, negative.

factor, which both promote an abnormal activation of coagulation on endothelial cells in portal veins. Both thrombin and activated platelets can synergize with endotoxin to stimulate monokine release [25,26]. Although several animal models of EHO have been reported in the literature [23,27], all are developed by a ligation of the portal vein. The portal vein ligation is not a physiological state and may disturb the secondary development of collaterals. Compared with these models, our model has one advantage that the physiological substance is used as an inducer of thrombus formation. Collaterals are also likely to develop in our model.

In this study, the developmental process of the cavernomatous vasculatures in the portal tracts was evaluated. Secondary to thrombus formation in the portal veins, both the number and total area of cavernomatous vasculatures increased within 48 h. The development in the total area occurred first, followed by a subsequent increase in their number. At 72 h, both increased 2- to 3-folds more than in the control group and these increases were statistically significant. This phenomenon is considered to be a rapid reaction against the abrupt cessation of the portal venous flow and stagnation. At 7 days, the cavernomatous vasculatures tended to decrease in both number and area, which attributed to the reopening of the portal flow due to the absorption of the organized thrombi. In nine patients with acute thrombosis, De Gaetano et al. [28] observed, by the hemodynamic consequences of cavernomatous transformation using Doppler sonography, that cavernomatous transformation developed within 6–20 days. Our study seems to confirm such clinical evidence. After the thrombus formation, microscopic cavernomatous vasculatures tend to develop within a few days subsequent to the evolution of macroscopic cavernomatous transformation within a few weeks.

As for the nature of these increased cavernomatous vasculatures around the thrombotic portal veins, tiny portal venous branches might develop as collaterals. In fact, Terada et al. demonstrated such budding of new vessels from the portal vein by serial section observations [29]. However, using routine histological techniques, it was impossible to clearly discriminate between portal venous branches and lymph vessels.

α-SMA is the isoform of actin and is routinely used as a reliable marker for smooth muscle cell differentiation. Fetal liver showed α-SMA positive cells along the blood vessels of the portal tract, terminal hepatic venule and at the perisinusoidal spaces [30]. In addition, in the portal tract of normal livers, α-SMA positive cells were noted in the vascular wall [31]. Masuda et al. [32] reported that, in the portal tracts of the human liver, lymph vessels measuring < 100 μm in diameter can be distinguished from the portal venous branches by the immunohistochemical detection of α-SMA. The authors confirmed their method using rabbit livers in our preliminary experiment; α-SMA positive vasculatures were portal venous branches and negative ones were lymph vessels.

The immunostaining of the α-SMA not only well characterized the vessel wall in the portal tract, but was also applicable to the sequential observations during the increase of the cavernomatous vasculatures in this study. A majority of the increased cavernomatous vasculatures was found to be positive for α-SMA and therefore the venous branches are considered to be related to collateral formation. In other words, peri-portal capillary venules [22] developed as cavernomatous vasculatures within 48 h to compensate for the blocked portal venous flow by thrombosis.

Oikawa et al. [30] examined the lymph vessels and the portal venous branches in liver wedge biopsies of IPH liver. In their study, the number of lymph vessels increased more in the IPH samples than in control. This phenomenon in IPH patients contrasts sharply with the results obtained from our model of portal venous thrombosis. These differences may be attributable to the specificity in the mechanism of reduction in the portal blood flow between two different situations. For instance, the hepatopetal collateral formations are distinctive features in EHO but they could not be observed in IPH.

The peribiliary vascular plexus plays an important role in bile flow physiology. The peribiliary vascular plexus, a network of small vessels surrounding the intrahepatic bile ducts, is supplied by the hepatic arterial branches and drains into the sinusoids or the portal venous branches. In some cases of portal hypertension associated with ductular proliferations, the peribiliary vascular plexus are increased in number and diameter, suggesting that the peribiliary vascular plexus serves as intrahepatic collaterals [33]. However, the proliferation of peribiliary vascular plexus was not an outstanding feature in our study. It is likely that the increase in peribiliary vascular plexus may not reflect the abrupt cessation of the portal venous flow and stagnation, but chronic changes in regional blood flow related to the intrahepatic bile ducts in various hepatobiliary diseases.

In conclusion, the results of this study demonstrated that endotoxin played an important role in the pathogenesis of portal venous thrombosis. A single application of EX-LPD emulsion induced portal venous thrombosis within 48 h and the resulting thrombus existed for over 7 days. Most of the developed cavernomatous vasculatures consist of portal venous branches as collaterals. However, we could not demonstrate the typical thrombosis located only around the hepatic hilum similar to that observed in EHO and this aspect should therefore be the focus of future investigations.