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＜原　著＞

C型肝炎に対するIFN治療後10年経過観察例についての検討

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要　旨：IFN治療後10年経過観察した148例を対象にretrospective検討した結果，CRではHCV RNAは全例10年間持続陰性であった．CRやICRでは組織学的，生化学的にも明らかな改善を認め生命予後も良かったがPR，NRでは有意に累積発癌率は高く累積生存率は低かった．NRやPRでもIFN再投与を繰り返すことによって組織学的な改善や発癌率の抑制が期待でき生命予後の改善に繋がると考えられた．

索引用語：C型慢性肝炎　長期的予後　インターフェロン　肝組織像　肝細胞癌

はじめに

1992年にC型慢性肝炎に対するIFN治療が保険適応になってから10年が経過し，投与方法や判定基準について一定のコンセンサスは得られているもののgenotype 1b，高ウイルス量では依然として満足のいく結果ではない．これまでC型慢性肝炎に対するIFN治療後の長期予後を検討した報告はいくつかあるが[1~11] 10年以上経過観察した報告は2つしかない[4~5]．今回，われわれはIFN治療が始まって10年が経ったひとつの区切りとしてIFN治療の効果とその後の組織学的な変化，発癌率，生命予後との関係などについてretrospectiveに検討した．

対象と方法

対象は当院で1987年から1992年の間にIFN単独で治療を行ったC型肝炎148例（男77例，女71例，年齢25～72歳，平均57±9.4歳）を対象としてretrospectiveに検討を行った．観察期間は脱落例，死亡例を含め0.3～10年（平均6.8±3.3年）である．HBs抗原陽性，自己免疫性肝炎，原発性胆汁性肝硬変，アルコール性肝硬変の合併例は除外した．投与方法はrecombinant IFN-α2b 1回量10 MUを4週間連日投与後，週3回20週間投与し総投与量880 MU（18例），同量を2週間連日投与後，週3回22週間投与し総投与量800 MU（5例），1回量9 MUを4週間連日投与後，週3回20週間投与し総投与量792 MU（7例），同量を2週間投与後，週3回22週間投与し総投与量720 MU（3例），1回量6 MUを4週間連日投与後，週3回20週間投与し総投与量528 MU（31例），同量を2週間連日投与後，週3回22週間投与し総投与量480 MU（5例），natural IFN-α 1回量3 MUを4週間連日投与後，週3回20週間投与し総投与量264 MU（71例），同量を週3回24週間投与し総投与量216 MU（8例）である．効果判定は以下によった．IFN投与後6カ月間ALT正常かつ投与終了後6カ月後のHCV RNAが陰性であるcomplete responder（CR）（32例，男18例女14例．平均年齢54±12歳），IFN投与後6カ月間ALT正常かつ投与終了6カ月後のHCV RNAが陽性であるincomplete responder（ICR）（12例，男10例女2例．平均年齢56±8歳），HCV RNA陽性かつ投与後6カ月以内にALTが正常上限値の2倍以下に改善したpartial responder（PR）（19例，男13例女6例．平均年齢58±6歳），上記以外のnon responder（NR）（85例，男36例女49例．平均年齢58±9歳）．genotype 1bの占める割合はそれぞれ60%，46%，89%，90%であった（Table 1）．肝組織は新犬山分類[12]に基づいて評価し，IFN投与前組織は，生検を行っていない2例を除きF1 13例，F2 41例，F3 67例，F4 25例であった．うち74例では2回以上肝生検を行い組織学的変化を検討した．統計学的検定には治療前後の血液生化学検査の変化はWilcoxon test（両側検定），発癌率，生存率はKaplan-Meier法で求めグループ間の比較はlog rank testを用いた．検定の結果は平均値±標準偏差（m±SD）で表示しp<0.05を有意差ありとした．

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Table 1 Characteristics of chronic hepatitis C patients treated with interferon

	CR	ICR	PR	NR
n	32	12	19	85
Age	54±12	56±8	58±6	58±9
M/F	18/14	10/2	13/6	36/49
HCV genotype				
1b(%)	60	46	89	90
Stage before IFN	2.5±0.6	2.1±0.7	2.8±1.0	2.9±0.9

CR：complete responder, ICR：incomplete responder, PR：partial responder, NR：nonresponder. Values are expressed as mean±S.D.

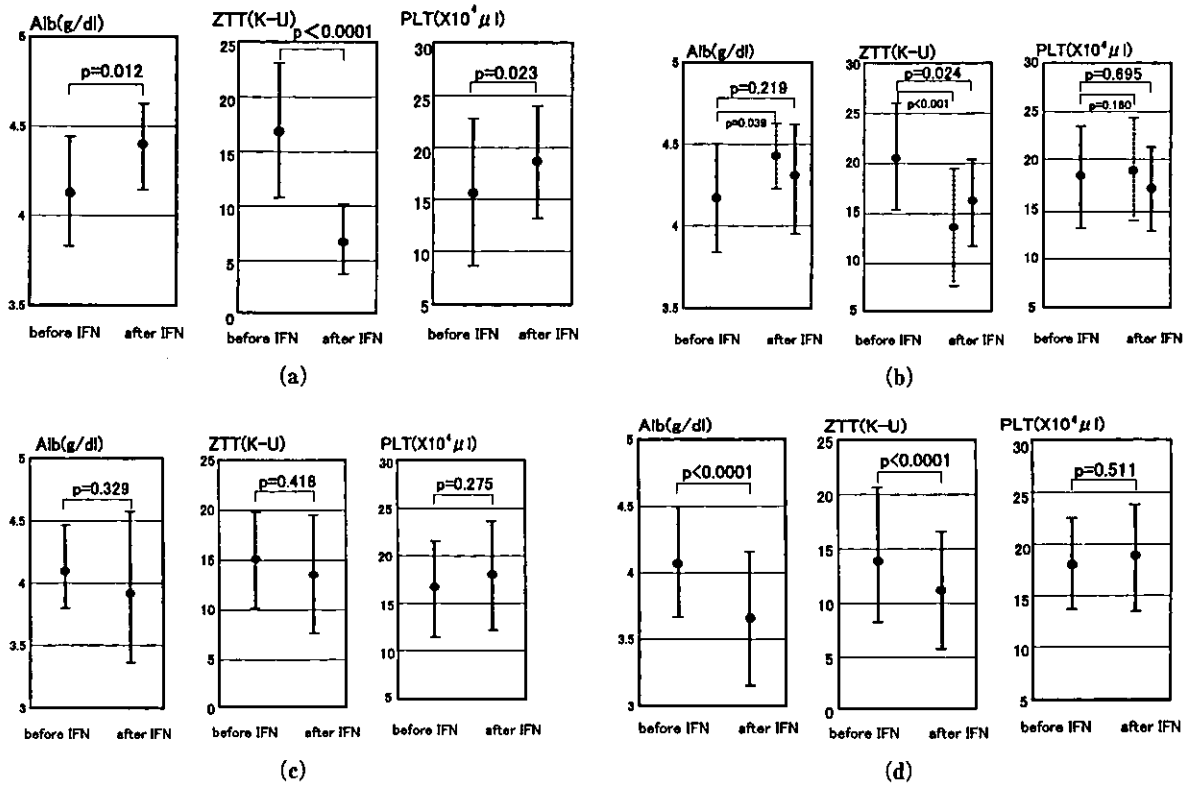


Fig. 1 Comparison of laboratory data between before and after interferon treatment. (a) Complete responder. Test interval is 2.5～10 years (ave. 8.0±2.7 years). (b) Incomplete responder. Test interval is 5.1～10 years (ave. 8.7±2.0 years). Dotted lines show mean values during normalization of ALT after IFN treatment. Test interval is 1.1～10 years (ave. 5.8±3.5 years). (c) Partial responder. Test interval is 2.1～10 years (ave. 5.9±3.0 years). (d) Nonresponder. Test interval is 0.6～10 years (ave. 7.0±3.2 years).

結　果

1. CR例の検討

観察期間中，HCV RNAが再陽性化した例はなかった．1例はγGTP，他の3例はγGTPとASTが軽度異常で持続しているが前者は糖尿病と脂肪肝，後者は常習飲酒家である．発癌や死亡例はなくIFN投与前後で比較したalbumin，ZTT，血小板数は有意に改善し(Fig. 1-a)，組織的にもstageの年変化率は−0.19±0.34(生検間隔0.2～8.7年，平均3.9±2.4年)と改善した．

2．前組織F4例の予後

前組織F4であった25例にIFN投与を行いCR 1例，PR 4例，NR 20例であった．10例に再投与した結果，NRであった7例からCR 2例，PR 1例，NR 4例，PRであった3例からCR 1例，PR 1例，NR 1例となった．12例が発癌した．8例（初回PR 3例，NR 5例）がIFN投与から2.8～8.3年（平均6.1±1.8年）で4例が発癌，4例が肝不全により死亡した．

3．治療前後の血液生化学検査の変化（Fig. 1）

albumin，ZTT，血小板数をIFN投与前後で比較するとCRでは2.5～10年（平均8.0±2.7年）の観察期間でそれぞれ4.2±0.3 g/dlから4.4±0.3 g/dl（p=0.012），16.8±6.0 K-Uから6.9±2.9 K-U（p<0.0001），15.8±6.9×10⁴/μlから18.8±5.4×10⁴/μl（p=0.023）へといずれも有意に改善した（Fig. 1-a）．ICRでは5.1～10年（平均8.7±2.0年）で4.2±0.3 g/dlから4.3±0.4 g/dl（p=0.219），20.7±5.0 K-Uから16.3±4.5 K-U（p=0.024），18.0±5.4×10⁴/μlから17.1±4.4×10⁴/μl（p=0.695）とZTTのみ改善がみられたが，ALT正常の期間中で比較すると1.1～10年（平均5.8±3.5年）の観察期間後それぞれ4.4±0.2 g/dl（p=0.039），14.2±5.2 K-U（p<0.001），18.8±5.6×10⁴/μl（p=0.160）となりalbuminとZTTの改善がみられた（Fig. 1-b）．PRでは2.1～10年（平均5.9±3.0年）で4.1±0.3 g/dlから3.9±0.7 g/dl（p=0.329），15.0±4.8 K-Uから13.4±5.7 K-U（p=0.418），16.9±5.3×10⁴/μlから17.9±5.9×10⁴/μl（p=0.275）といずれも有意差はなかった（Fig. 1-c）．NRでは0.6～10年（平均7.0±3.2年）で4.1±0.4 g/dlから3.7±0.5 g/dl（p<0.0001），14.2±7.0 K-Uから11.6±5.5 K-U（p<0.0001），17.8±4.7×10⁴/μlから18.2±5.7×10⁴/μl（p=0.511）へとalbuminの悪化とZTTの改善がみられた（Fig. 1-d）．

4．治療前後のstageの改善率と年変化率

IFN治療前後で2回以上生検した74例におけるstageの変化は，改善18例（24.3%），不変45例（60.8%），悪化11例（14.9%）であった．（最終生検時stage－IFN投与前stage）/生検間隔（年）で算出した治療前後のstageの年変化率は，CRは－0.25±0.37（n=9，生検間隔0.2～7年，平均3.4±2.1年），ICRは－0.07±0.17（n=9，生検間隔1.3～8.7年，平均5.0±2.6年），PRは－0.024±0.35（n=11，生検間隔0.3～9.1年，平均3.9±2.6年），NRは0.04±0.24（n=45，生検間隔1～10年，平均4.3±2.6年）であった．

5．発癌（Fig. 2）

(1) 経過観察中に32例が発癌した．Kaplan-Meier法による累積発癌率は5年14.4%，10年31%であった（Fig. 2-a）．

(2) CR，ICR，PR+NRの3群で累積発癌率を比較するとCRからの発癌はなく，ICRの1例（前組織F3）から4.3年後に発癌がみられた（年発癌率0.8%/人/年）．残りはPR 6例とNR 25例から発癌し累積発癌率は5年19.8%，10年44.2%で（年発癌率3.1%/人/年）でCR，ICRに比して有意に高率であった（p=0.0025，Fig. 2-b）．

(3) 男女それぞれのCR，ICR，PR+NR群の累積発癌率を比較すると男女ともCRからの発癌はなく，男1例がICRから発癌した以外，全例PR+NRからの発癌であった（p=0.0023，Fig. 2-c）．しかしPR+NRのみを男女間で比較すると有意差はなかった（p=0.571）．

(4) 60歳以下と60歳以上でのCR，ICR，PR+NR群の累積発癌率を比較すると60歳以下のICRから1例が発癌した以外は両群ともにPR+NR群から発癌した（Fig.2-d）．PR+NRのみで比較すると60歳以下より60歳以上で有意に発癌率が高かった（p=0.012）．さらに55歳，50歳で分けてもそれぞれp=0.021，p=0.016と以下群より以上群で高かったが45歳で分けるとp=0.111と有意差はなかった．

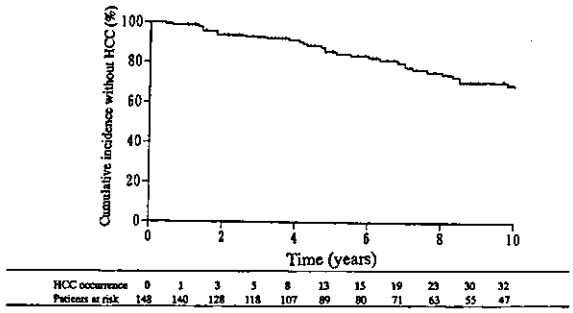
(5) F stage別に比較するとIFN投与前組織F1からの発癌はなくF2から4例，F3から17例，F4から11例とF2，F3，F4の順に発癌率が高くなった（p<0.001，Fig. 2-e）．

6．生存率（Fig. 3）

(1) 脱落例を除き13例が死亡し，Kaplan-Meier法による累積生存率は5年93.7%，10年86.1%であった（Fig. 3-a）．

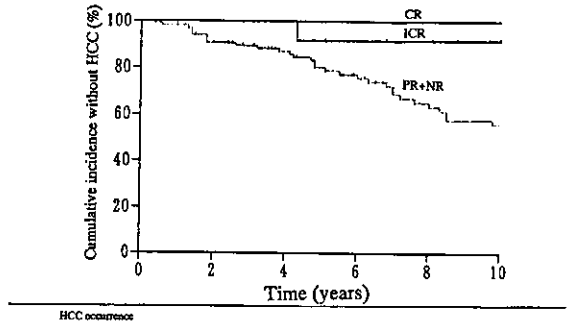
(2) CR，ICR，PR+NRの3群で累積生存率を比較するとCR，ICRは5年，10年100%，NR+PRは5年89%，10年78.1%で有意にNR+PRの方が低率であった（p=0.001，Fig. 3-b）．

(3) 男女それぞれのCR，ICR，PR+NR群の累積生存率を比較するとCR，ICRからの死亡はなく，男女ともPR+NR群の死亡例が経過とともに増加し



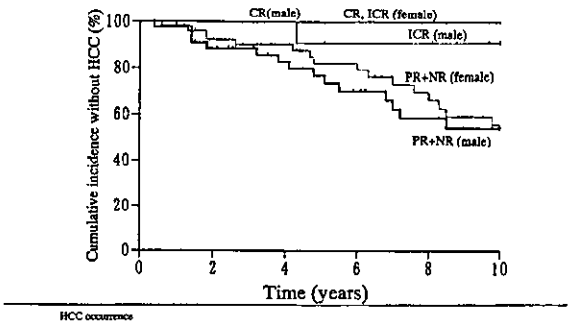
HCC occurrence	0	1	3	5	8	13	15	19	23	30	32
Patients at risk	148	140	128	118	107	89	80	71	63	55	47

(a)



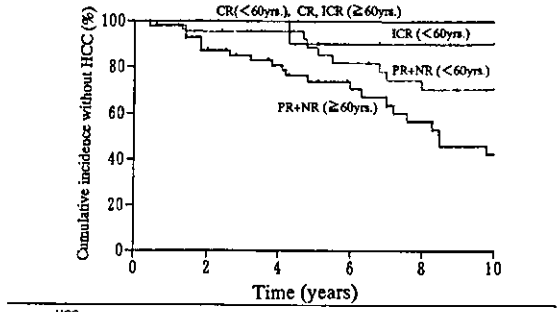
HCC occurrence											
CR	0	0	0	0	0	0	0	0	0	0	0
ICR	0	0	0	0	1	1	1	1	1	1	1
PR+NR	0	1	3	5	8	12	14	18	22	29	31
Patients at risk											
CR	31	31	31	28	25	23	20	20	17	16	13
ICR	12	12	12	12	12	11	9	8	8	6	5
PR+NR	105	101	85	78	70	55	51	43	38	33	29

(b)



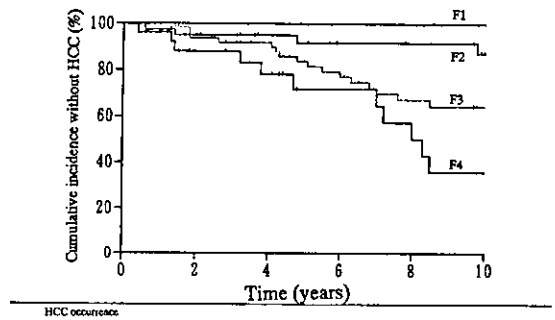
HCC occurrence											
Male											
CR	0	0	0	0	0	0	0	0	0	0	0
ICR	0	0	0	0	1	1	1	1	1	1	1
PR+NR	0	1	2	3	5	7	9	11	12	15	15
Female											
CR, ICR	0	0	0	0	0	0	0	0	0	0	0
PR+NR	0	0	1	2	2	5	5	7	10	14	16
Patients at risk											
Male											
CR	18	18	18	17	15	13	10	10	7	7	7
ICR	11	11	11	11	11	10	8	8	6	6	4
PR+NR	48	44	38	30	30	26	22	19	17	15	13
Female											
CR	13	13	13	10	10	10	10	10	10	9	6
ICR	2	2	2	2	2	2	2	1	1	1	1
PR+NR	55	55	46	43	39	28	28	23	20	17	16

(c)



HCC occurrence											
<60yrs.											
CR	0	0	0	0	0	0	0	0	0	0	0
ICR	0	0	0	0	0	1	1	1	1	1	1
PR+NR	0	1	2	2	4	6	7	8	9	9	
≧60yrs.											
CR, ICR	0	0	0	0	0	0	0	0	0	0	0
PR+NR	0	0	1	3	6	8	8	11	14	20	22
Patients at risk											
<60yrs.											
CR	19	19	17	14	12	10	10	8	8	6	6
ICR	10	10	10	10	10	9	8	7	7	5	4
PR+NR	49	46	38	37	32	28	26	23	21	19	18
≧60yrs.											
CR	12	12	12	10	10	10	9	9	8	7	7
ICR	3	3	3	3	3	3	2	2	2	2	1
PR+NR	55	54	46	40	37	26	24	19	16	13	11

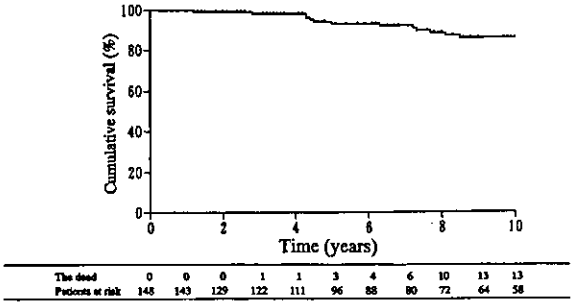
(d)



HCC occurrence											
F1	0	0	0	0	0	0	0	0	0	0	0
F2	0	0	0	0	0	1	1	1	1	2	4
F3	0	0	1	2	3	6	8	12	15	17	17
F4	0	1	2	3	5	6	6	6	7	11	11
Patients at risk											
F1	14	14	14	14	13	11	10	8	8	5	4
F2	41	39	37	34	30	27	22	22	21	21	17
F3	66	66	57	51	46	40	37	30	25	23	20
F4	26	24	19	18	16	10	10	10	8	5	5

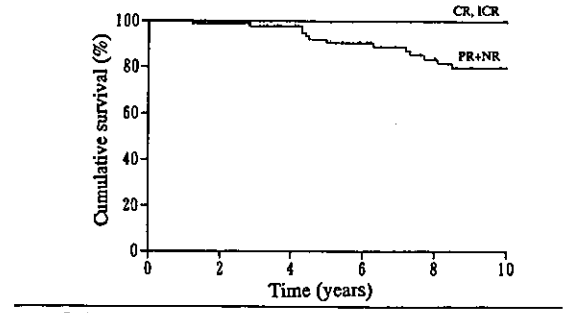
(e)

Fig. 2 Comparison of cumulative incidence without hepatocellular carcinoma. (a) All patients. Cumulative incidence at 5 years is 14.4% and at 10 years is 31%. (b) CR, ICR and PR+NR groups. CR : complete responder. ICR : incomplete responder. PR : partial responder. NR : nonresponder. (c) CR, ICR and PR+NR groups in male and female. (d) CR, ICR and PR+NR groups under and over 60 years of age. (e) F1, F2, F3 and F4 groups. Differences among the three groups (b), $p=0.0025$, among the six groups (c), $p=0.0023$, (d), $p=0.012$, among the four groups (e), $p<0.001$; log rank test



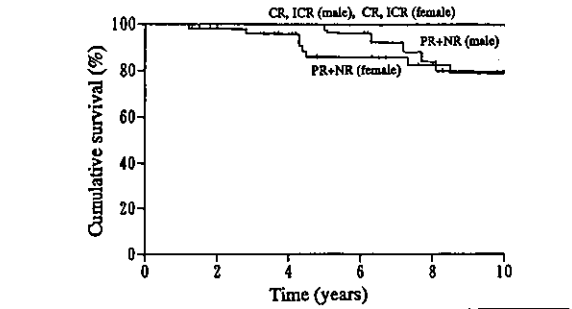
	0	2	4	6	8	10
The dead	0	0	1	3	6	13
Patients at risk	148	129	111	88	72	58

(a)



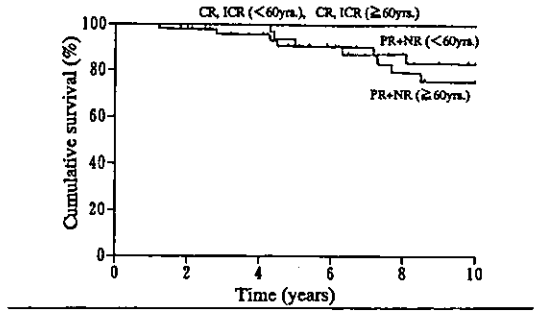
	0	2	4	6	8	10
The dead						
CR, ICR	0	0	0	0	0	0
PR+NR	0	0	1	3	10	13
Patients at risk						
CR	31	31	28	23	17	13
ICR	12	12	12	9	6	5
PR+NR	105	88	74	59	43	40

(b)



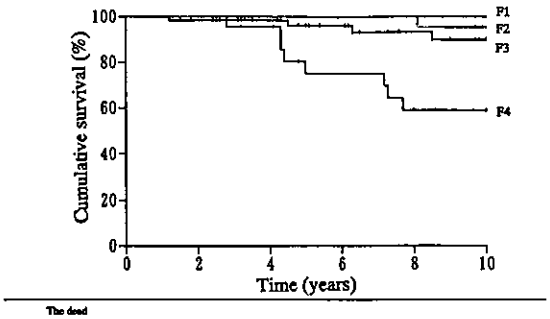
	0	2	4	6	8	10
The dead						
Male						
CR, ICR	0	0	0	0	0	0
PR+NR	0	0	0	1	2	4
Female						
CR, ICR	0	0	0	0	0	0
PR+NR	0	0	1	3	4	6
Patients at risk						
Male						
CR	18	18	17	15	13	10
ICR	11	11	11	11	8	8
PR+NR	48	40	36	32	30	27
Female						
CR	13	13	11	10	10	10
ICR	2	2	2	2	2	2
PR+NR	56	47	45	41	32	31

(c)



	0	2	4	6	8	10
The dead						
< 60yrs.						
CR, ICR	0	0	0	0	0	0
PR+NR	0	0	0	1	3	5
≧ 60yrs.						
CR, ICR	0	0	0	0	0	0
PR+NR	0	0	1	3	7	8
Patients at risk						
< 60yrs.						
CR	19	19	17	12	8	6
ICR	10	10	10	8	7	4
PR+NR	49	40	35	30	25	21
≧ 60yrs.						
CR	12	12	11	11	9	7
ICR	3	3	3	3	2	1
PR+NR	55	47	42	31	25	19

(d)



	0	2	4	6	8	10
The dead						
F1	0	0	0	0	0	0
F2	0	0	0	0	0	1
F3	0	0	0	1	2	4
F4	0	0	1	2	4	8
Patients at risk						
F1	14	14	13	11	8	5
F2	41	37	30	23	22	18
F3	66	58	48	41	32	27
F4	26	21	19	13	10	8

(e)

Fig. 3　Cumulative survival. (a) All patients. Cumulative incidence at 5 years is 93.7% and at 10 years is 86.1 %. (b) CR, ICR and PR+NR groups. CR：complete responder. ICR：incomplete responder. PR：partial responder. NR：nonresponder. (c) CR, ICR and PR+NR groups in male and female. (d) CR, ICR and PR+NR groups under and over 60 years of age. (e) F1, F2, F3 and F4 groups. Differences among the three groups(b), $p=0.001$, among the six groups(c), $p=0.047$, (d), $p=0.021$, among the four groups(e), $p<0.001$；log rank test

た(p=0.047, Fig. 3-c). しかしPR+NR群のみで男女間を比較しても有意差はなかった(p=0.451).

(4) 60歳以下と60歳以上でのCR, ICR, PR+NR群の累積生存率を比較すると両群ともにPR+NR群のみ死亡がみられた(p=0.021, Fig. 3-d). しかしPR+NR群のみで60歳以下と60歳以上を比較しても有意差はなかった(p=0.165). さらに55歳, 50歳, 45歳で分けてもそれぞれp=0.908, p=0.369, p=0.227と有意差はなかった.

(5) F stage別に比較するとF1からの死亡例はなくF2, F3, F4の順に生存率が低くなったが, 特にF4の低下が顕著であった(p<0.001, Fig. 3-e).

7. 再投与例(Fig. 4)

初回IFN投与でNRであった85例中39例, PRであった19例中6例, ICRであった12例中5例の計50例に初回投与終了後0.1～8.2年(平均2.2±2.0年)で再投与を行った. そのうち9例には再投与終了後0.1～9年(平均3.3±3.4年)で3回目の投与を, 3例には3回投与終了後0.1～6.1年(平均3.2±2.5年)で4回目の投与を行った. 計62回の投与方法はrecombinant IFN-α2b 1回量10 MUを4週間連日投与後, 週3回20週間投与し総投与量880 MU(31回), 同量を2週間連日投与後, 週3回22週間投与し総投与量800 MU(8回), 1回量9 MUを4週間連日投与後, 週3回20週間投与し総投与量792 MU(7回), 同量を2週間連日投与週3回22週間投与し総投与量720 MU(5回), 1回量6 MUを4週間連日投与後, 週3回20週間投与し総投与量528 MU(6回), 同量を2週間連日投与後, 週3回22週間投与し総投

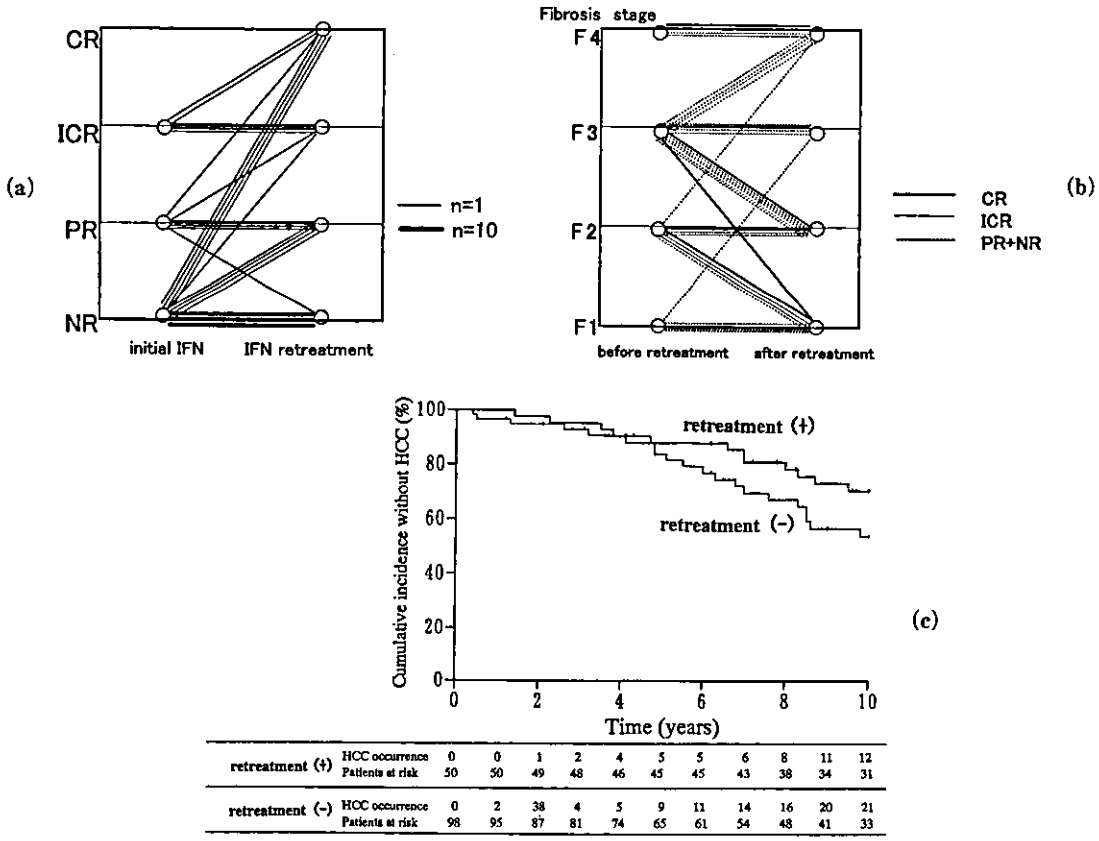


Fig. 4 (a) Response to interferon of initial treatment and retreatment. Time interval between the end of initial treatment and retreatment is 0.1～8.2 years(ave.2.2±2.0 years). (b) Fibrosis changes after retreatment with interferon in 31 patients. 11 patients(36%) improved, 14 patients(45%) unchanged and 6 patients(19%) worsened. Biopsy interval is 2.3～9.6 years(ave.6.1±2.1 years). (c) Comparison of cumulative incidence without hepatocellular carcinoma between groups with and without retreatment(p=0.118 ; log rank test).

与量480 MU(2回)，natural IFN-α 1回量3 MUを4週間連日投与後，週3回20週間投与し総投与量264 MU(3回)である．その結果CR 7例，ICR 5例，PR 7例，NR 31例となった(Fig. 4-a)．また再投与前後で肝生検を行った31例のstageの変化は改善11例(36%)，不変14例(45%)，悪化6例(19%)でありPR，NRでもそれぞれ3/7例(42.8%)，2/31例(6.5%)にstageの改善を認めた(Fig. 4-b)．再投与群と非再投与群の間に平均年齢，IFN投与前のF4の占める割合，初回IFN投与のPR，NR例の割合に差はなく累積発癌率を比較すると有意差はみられなかったが再投与群の方が低い傾向にあった(p=0.118，Fig. 4-c)．

考　察

これまでC型慢性肝炎に対するIFN治療後の組織学的変化や発癌，生命予後に関する報告はいくつかあるが組織学的な推移を観察した期間は平均約5年までである[1~11]．10年以上経過観察したLauら[4]とAjelloら[5]の報告が最長であるが症例数はそれぞれ10例，31例と少なく組織学的な推移を検討したのは前者のみである．われわれはIFN投与後10年経過観察した148例を対象に組織学的変化や発癌，生命予後に関してretrospectiveに検討した．

IFN治療後の組織学的な改善に関して荒瀬ら[1]はCR 31例を検討した結果，stageの改善率は1~3年で17.4%，3~7年で62.5%とstageの改善には長期間かかるとしている．このことからstageの改善の評価には観察期間も考慮する必要がある．Toccaceliら[11]はCR 112例の検討ではIFN投与後36~76カ月の観察期間で44%に組織学的な改善が，Shiratoriら[8]はsustained responder 183例1~10年(平均3.7年)の観察期間で59%にfibrosisの改善をみた．最も長期間観察したLauら[4]の報告ではIFN投与終了6カ月後にHCV RNAが陰性であった5例では5~11年後もHCV RNA陰性で組織のactivity，fibrosisともに全例が改善し，正常あるいはmildな非特異的炎症性変化を残すのみとなった．以上よりわれわれの検討を含めIFN投与終了6カ月後にHCV RNAが陰性のCRならその後，少なくとも10年はHCV RNA陰性が持続し組織学的な改善が期待できる．

Shindoら[10]はICR 26例では2年後，stageの有意な改善はみられなかったとしているが，われわれICR 13例の検討では生検間隔1.3~8.7年(平均5.3±2.6年)で線維化の年変化率は−0.07±0.16と改善していた．ALT正常のまま2回目の生検をした6例では生検間隔が1.3~3.1年の4例は不変，2.5年と5.1年の2例は改善していたのでALT持続正常例ではstageの改善には2~3年要すると考えられる．またALTが再上昇した後に2回目の生検をした5例でも生検間隔4.3~8.7年(平均7.4年)−ALT正常から再上昇までの期間1.5~5.8年(平均2.8年)，再上昇(ALTが正常の2倍以上で持続が4例，2倍以下で持続が1例)から生検までの期間0.3~5.6年(平均2.4年)−で改善1例，不変3，悪化1例と比較的長期間，同じstageに保たれていた．

IFNと発癌の関係に関して，われわれの肝硬変を含めた検討では累積発癌率はCRでは10年0%，ICRでは5年，10年8.3%，PR+NRでは5年19.8%，10年44.2%とこれまでの報告と同様，NRからの発癌率が高かった．Kasaharaら[13]のC型慢性肝炎を対象としたretrospectiveな検討ではCRからの発癌は3年1.6%，5年4.3%，7年4.3%．TRではそれぞれ3.4%，4.7%，4.7%，NRでは6.3%，21.4%，26.1%とNRの5年発癌率はわれわれとほぼ同じであったがCRからも発癌を認めている．またALT異常のbiochemical non-responderはALT正常のbiochemical sustained responderよりも発癌のリスクは8倍高かった．Mahmoodら[14]はNRとPR 83例の検討で，ALT>80 IU/lが2年以上持続すると有意に発癌率が高いと述べている．以上からNRではできる限りALTを低く保つことが発癌の抑制につながるといえる．しかしCRでもIFN投与後，最長8年6カ月後に発癌した報告があり[15]定期的なエコー検査を怠ることはできない．Nishiguchi[16]やValla ら[17]はrandomized controlled studyでLCにIFNを投与し有意な発癌抑制効果を認めているので肝硬変でも肝予備能が良い例には試みる価値がある．

累積生存率に関して，われわれの検討ではCR，ICRでは10年まで100%，NR+PRでは5年89%，10年78.1%と有意に後者のほうが低かった(p=0.001：log-rank test)．しかしNRとPRの間ではIFN投与後の観察期間が長いためか有意差はなかった．Tanakaら[18]はbiochemical transient responderよりもbiochemical sustained responderのほうが累積生存率は良く，これら両群の死亡率はNRやIFN非投与群に比べて1/5であったと報告している．Ajelloら[5]も10年の経過観察でIFN投与終了6カ月後のHCV RNA陰性またはALT正常であったres-

ponder 10例では10年後の死亡例，発癌例はなかったがNR 21例では死亡4例，発癌5例と自然経過を変える程の効果はなかったとしている．

C型肝炎患者の死因のほとんどが肝硬変による肝不全か肝癌である．われわれ10年間の検討でも肝疾患以外の死亡は1例のみであった．肝癌の80%以上は肝硬変から発生するためC型肝炎の予後を改善するにはいかに線維化を抑制し肝硬変へ移行するのを食い止めるかである．IFNには抗線維化作用のあることが示されている[19,20]．線維化の評価はF stageでは0～4の5段階であるが，線維部分をコンピューターで画像処理し標本全体に占める割合を算出するmorphometryを用いるとわずかな線維幅の変化を比較することができる．われわれはこの方法でNR 76例の6～123カ月(平均34.6カ月)後の改善率をmorphometryで検討した結果，F stageの評価では23.7%であったがmorphometryで検討すると65.8%であった[21]．Guerretら[7]も同様な方法によりNR 24例の5年間の観察で，慢性肝炎ではIFN投与期間が1年以上でも以下でも，また投与量に関係なく全例改善し，肝硬変でも1年以下の短期投与では43%に，1年以上の長期投与では全例に改善がみられたとしている．われわれの検討でNRでもZTTの改善がみられたのはこのことを反映しているかもしれない．さらにNRに対してIFNを再投与することでCRやICRになる例があるのみならず，再びNRになってもIFNを繰り返し投与することで線維化が抑制され，延いては発癌や肝不全に至るのを減少させる可能性がある．Reichardら[22]は繰り返しIFNを投与してNRでも組織の改善を認めている．われわれも初回IFN投与でNRまたはPRであった45例に1～3回再投与を行った結果CR 5例，ICR 2例となりPRやNRでもそれぞれ3/7例(42.8%)，2/31例(6.5%)にstageの改善を認めた．肝硬変25例に対してIFNを投与したところCRは1例であったが，NR 7例に再投与したところ2例がCRになった．そして累積発癌率も再投与群の方が低い傾向にあった．

以上，われわれの10年以上経過例についての検討結果はこれまでの長期観察例の延長線上にあるといえる．そしてIFN投与終了6カ月後にHCV RNAが陰性のCRならばその後も10年はHCV RNA陰性が持続し，組織学的な改善も持続すると考えられた．たとえNRでもIFNの再投与や他の肝庇護剤でALT値をできるだけ低く抑えることが予後の改善に繋がるものと考えられた．

結　語

IFN治療後10年経過観察した例を対象にretrospectiveに検討を行った結果，

1) IFN終了時および終了6カ月目にHCV RNAが陰性ならばその後も陰性が持続する．

2) CRでは組織的，生化学的にも明らかな改善を認め生命予後も良かったがPR，NRでは累積発癌率や累積生存率はCRより有意に悪かった．

3) NRやPRでもIFNの再投与を繰り返すことで組織的な改善や発癌率の抑制が期待でき予後の改善にも繋がる．

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Ten-year follow-up after interferon therapy in hepatitis C patients

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We retrospectively conducted ten-year follow-up study in 148 patients with hepatitis C patients treated with interferon(IFN). Complete responder(CR), defined as clearance of HCV RNA and normalization of serum ALT levels at six months after IFN therapy, had remained negative serum HCV RNA levels throughout observation period and albumin, ZTT and platelet counts significantly improved, Cumulative incidence of hepatocellular carcinoma(HCC)was higher and cumulative survival rate was lower in partial responder(PR), defined as no clearance of HCV RNA and decrease in serum ALT levels compared with prestudy levels to a level less than twice the upper normal limit at six months after IFN therapy, and non-responder(NR), defined as no clearance of HCV RNA and or decrease in serum ALT levels compared with the prestudy levels after IFN therapy, than CR. When IFN was repeatedly administered, histological improvement and decrease of cumulative incidence of development of HCC could be expected even in PR and NR after initial IFN therapy, resulting in better life expectancy.

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慢性HBV感染患者のHBVプレコア／コアプロモーター変異の検討

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I．緒　　言

B型肝炎ウイルス（HBV）はRNA intermediateの逆転写を介して非相称的に増殖し[1]，これがHBVの変異を生じさせる素地となっている。もっとも代表的なHBV変異はHBe抗原産生を阻止するprecore stop codon mutation（$G_{1896}A$）[2]とHBe抗原産生をdown-regulateするcore promoter領域の重複mutation（$A_{1762}T$, $G_{1764}A$）である[3]。プレコアとコアプロモーター変異はヨーロッパとアジアのHBe抗原陰性慢性B型肝炎患者の50～80％に報告されている[4]。

最近，HBVプレコア／コアプロモーター変異と慢性HBV感染の肝疾患進行および血清HBV DNA値との間に関連があることが報告された[5),6)]。今回，われわれはこれらの報告を確認する目的で，慢性HBV感染患者のHBVプレコア／コアプロモーター変異を検討した。

II．対象と方法

対象患者は国立病院呉医療センター消化器科で治療を受けている慢性HBV感染患者58例（男性38例，女性20例）で，平均年齢は50.6±14.1歳であった。肝生検（46例），画像検査（腹部超音波検査，CT検査）（12例）で行った臨床診断は慢性肝炎27例，肝硬変18例，肝細胞癌13例であった。

血清HBe抗原／抗体はRIA法，血清HBV DNA値はPCR法，HBVプレコア／コアプロモーター変異はプレコア／コアプロモーター領域における野生型と変異型を遺伝子増幅法で検出するHBV DNA検出キット（プレコア／コアプロモーター）を用いて測定した。58例中11例で，HBVゲノタイプをPCR法で測定した（国立病院長崎医療センター臨床研究部で測定）。

統計学的解析はχ^2検定で有意差を検定した。

III．結　　果

測定された11例のHBVゲノタイプは全例ゲノタイプCであった。平均年齢が50.6歳であったので，50歳未満と50歳以上に年齢を分け，HBVプレコア／コアプロモーター変異を検討した。HBVプレコア領域の変異は50歳未満で野生株8/27（29.6％），混合型10/27（37.0％），変異株9/27（33.3％），50歳以上で野生株9/31（29.0％），混合型5/31（16.1％），変異株17/31（54.8％）であり，加齢に伴って変異株の占める割合が高くなった（p＝0.1402）。HBVコアプロモーター領域の変異は50歳未満で野生株7/27（25.9％），混合型3/27（11.1％），変異株17/27（63.0％），50歳以上で野生株3/28（10.7％），混合型2/28（7.1％），変異株23/28（82.2％）であり，HBVプレコア領域と同様に，加齢とともに混合株の占める割合が高くなった（p＝0.2615）（図1）。HBVプレコア領域の変異はHBe抗原／抗体（－／＋）で，野生株8/39（20.5％），混合型10/39（25.6％），変異株21/39（53.8％），HBe抗原／抗体（＋／－）で，野生株10/17（58.8％），混合型4/17（23.5％），変異株（17.6％）であり，HBe抗原／抗体（－／＋）で野生株の占める割合が有意に高かった（p＝0.0109）。HBVコアプロモーター領域の変異はHBe抗原／抗体（－／＋）で，野生株7/36（19.4％），混合型2/36（5.6％），変異株27/36（75.0％），HBe抗原／抗体（＋／－）で野生株4/17（23.5％），混合型3/17（17.6％），変異株10/17（58.8％）であり，HBe抗原／抗体（－／＋）で変異株の占める割合が高かった（p＝0.3142）（図2）。HBV DNA値を5.0 LC/mL未満と5.0 LC/mL以上に分け，HBVプレコア／コアプロモーター変異を検討した。HBVプレコア領域の変異はHBV DNA値5.0 LC/mL未満で野生株6/21（28.6％），混合型6/21（28.6％），変異株9/21（42.9％）で，HBV DNA値5.0 LC/mL以上で野生株11/37（29.7％），混合型9/37（24.3％），変異株17/37（45.9％）であり，HBV DNA値で両群間に差は認められなかった（p＝0.9380）。HBVコアプロモーター領域の変異はHBV DNA値5.0 LC/mL未満で野生株6/20（30.0％），混合型1/20（5.0％），変異株13/20（65.0％），HBV DNA値5.0 LC/mL以上で野生株15/35（14.3％），混

Eiichi Takezaki, Toshitaka Tsuda, Shigeki Mizuno, Eiji Miyoshi, Tatsuma Fukuhara, Satoe Yokoyama, Taiji Okamoto: Significance of HBV precore/core promoter variants in patients with chronic HBV infection. Department of Gastroenterology, National Kure Medical Center.
国立病院呉医療センター消化器科

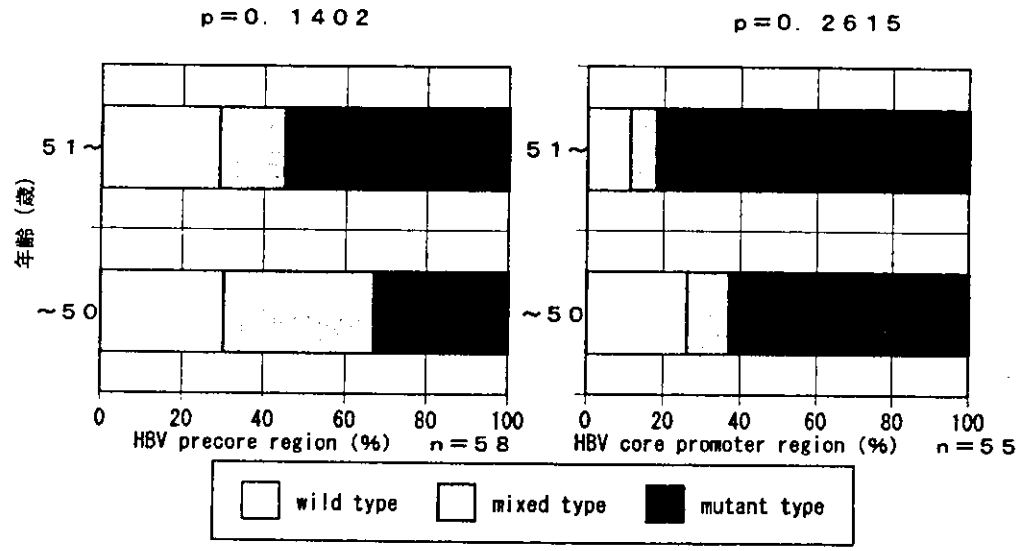

図1　年齢別のHBVプレコア／コアプロモーター変異の頻度

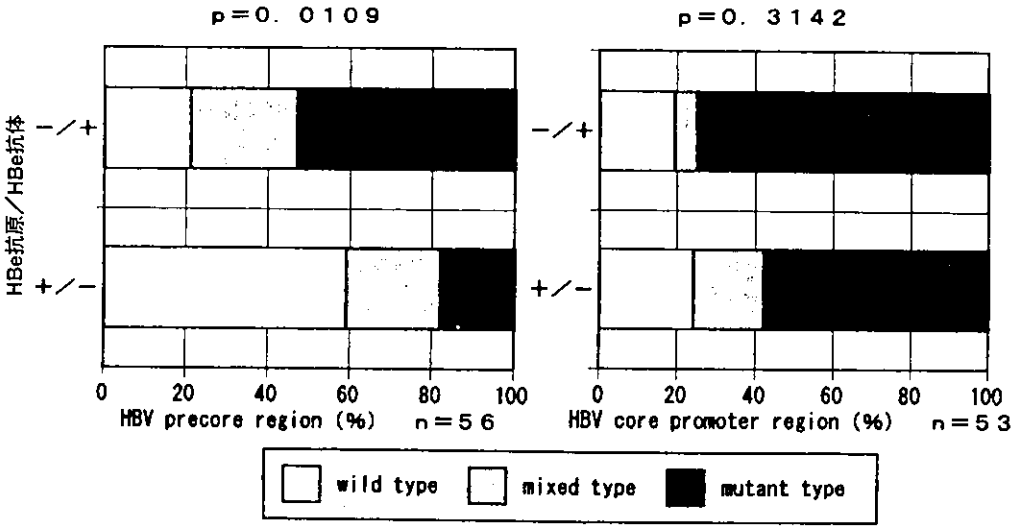

図2　HBe抗原・抗体状態でみたHBVプレコア／コアプロモーター変異の頻度

合型4/35（11.4%），変異株26/35（74.3%）であり，HBV DNA値5.0LC/mL以上で野生株の占める割合が高かった（p=0.3159）（図3）。HBVプレコア／コアプロモーター変異を慢性HBV感染患者の肝疾患の進行の程度で分けて検討した。HBVプレコア領域の変異は慢性肝炎で野生型12/28（42.9%），混合型6/28（21.4%），変異株10/28（35.7%），肝硬変で野生株1/17（5.9%），混合型6/17（35.3%），変異株10/17（58.8%），肝細胞癌で野生株4/13（30.8%），混合型3/13（23.1%），変異株6/13（46.2%）であり，慢性肝炎に比べて，肝硬変，肝細胞癌で変異株の占める割合が高かった（p=0.1333）。HBVコアプロモーター領域の変異は慢性肝炎で野生株6/27（22.2%），混合型4/27（14.8%），変異株17/27（63.0%），肝硬変で野生株4/17（23.5%），混合型0/17（0.0%），変異株13/17（76.5%），肝細胞癌で野生株1/11（9.1%），混合型1/11（9.1%），変異株9/11（81.8%）であり，慢性肝疾患の進行に伴って変異株の占める割合が高かった（p=0.4274）（図4）。

Ⅳ．考　　察

HBVゲノタイプBとCは日本を含めたアジアの代表的なHBVゲノタイプである[7]。HBVゲノタイプCはゲノタイプBに比べて，HBe抗原seroconversion率が低く，程度の高い肝疾患と関連することが示唆され[8]，また，HBVゲノタイプCはゲノタイプBに比べて，

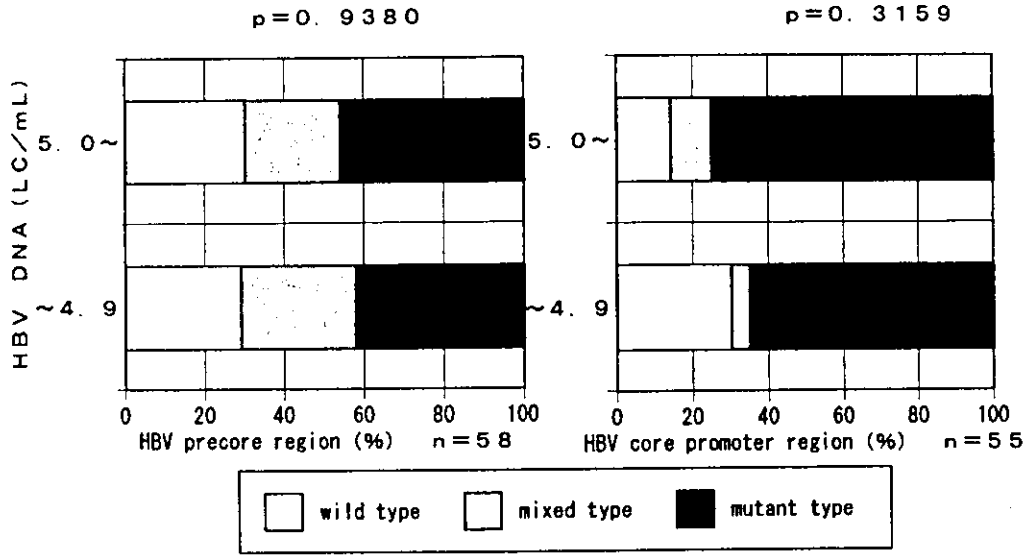

図3　HBV DNA値でみたHBVプレコア　コアプロモーター変異の頻度

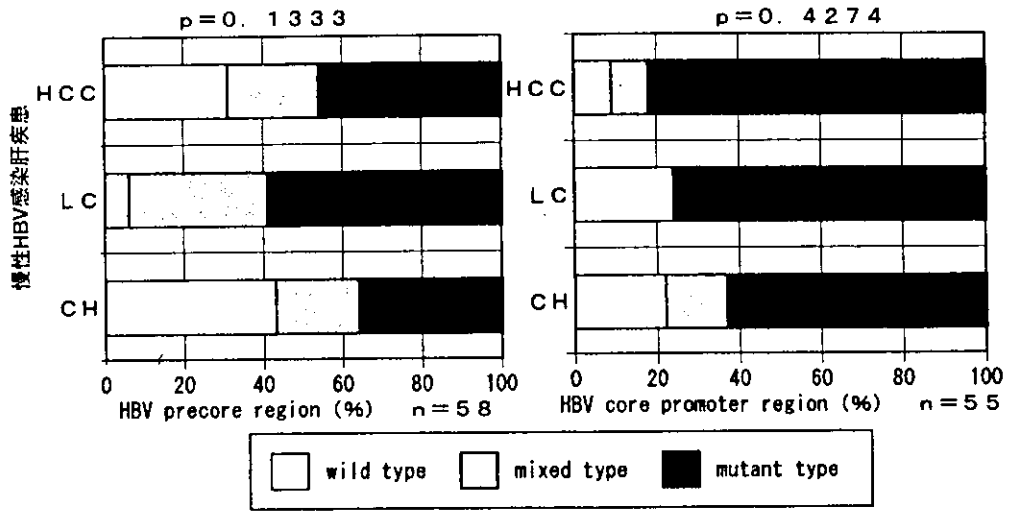

図4　慢性肝疾患の進行でみたHBVプレコア　コアプロモーター変異の頻度
（CH；慢性肝炎，LC；肝硬変，HCC；肝細胞癌）

コアプロモーター変異（T1762/A1764変異）の頻度が高いことが示されている[9]。

今回，われわれは国立病院呉医療センターで加療中の患者を対象にして，HBVプレコア　コアプロモーター変異を検討した。HBVゲノタイプは58例中11例で測定されているが，全例ゲノタイプCであった。肝細胞癌とHBVコアプロモーター変異との関連を示したKaoらはHBVコアプロモーター変異の存在が肝細胞癌と有意に相関し，これはHBVゲノタイプBあるいはCに関係なく，若年者肝細胞癌患者のHBVコアプロモーター変異の頻度が，年齢が一致する非活動性キャリアーに比べて有意に高く，高齢者肝細胞癌患者との間に差がなかったことを報告している[5]。これが事実とすると，若年者でHBVコアプロモーター変異を有する患者は肝細胞癌発生の危険性が高い群として，注意深い観察が必要であることを意味している。われわれの対象患者は平均年齢が50.6歳であり，肝細胞癌13例の中に若年者肝細胞癌患者が含まれていなかったが，HBVコアプロモーター変異の頻度は加齢と平行して増加する傾向を示した。したがって，肝細胞癌患者に有意に高い頻度のHBVコアプロモーターは認めることができなかった。

一方，Chuらはアメリカの HBVキャリアーを対象に，HBVコアプロモーター変異の存在が重篤な慢性肝

疾患と関連している可能性があり，HBe 抗原陰性患者では，血清 HBV DNA 高値と関連していることを示している[6]。われわれの HBe 抗原／抗体（−/+）患者は（+/−）患者に比べて，HBV プレコア変異の頻度が有意に高く，コアプロモーター変異の頻度も高い傾向を示したが，血清 HBV DNA 値に関しては，HBV プレコア変異の存在の関与は認められず，コアプロモーター変異を有する患者で高値となる傾向が認められた。

以上を考え合わせると，HBV プレコア／コアプロモーター変異が加齢に伴って頻度が増加し，変異が肝疾患の進行に加担していることが示唆されるが，今回の対象患者の症例数および年齢分布に限界があり，結論を導くまでには至らなかった。今後，特に若年者肝細胞癌患者を含めて，多数例を対象にした検討が必要であると考えられる。

V. 結　　語

慢性 HBV 感染患者 58 例を対象にして，HBV プレコア／コアプロモーター変異を検討した。HBV プレコア／コアプロモーター変異が加齢と関連していることが示唆されたが，コアプロモーター変異が慢性肝疾患の進行に加担している可能性があり，若年者での HBV コアプロモーター変異と肝細胞癌発生の間の関連を検討する必要性が認められた。

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（受付　2004－2－18）

Antiangiogenic Property of Pigment Epithelium-Derived Factor in Hepatocellular Carcinoma

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Pigment epithelium-derived factor (PEDF) is one of the most powerful endogenous antiangiogenic reagents discovered to date. Its antiangiogenic potential in neoplastic disease remains unclear. In this study, we investigated antiangiogenic property of PEDF in hepatocellular carcinoma (HCC), a typical hypervascular tumor. In HCC cell lines, constitutive messenger RNA and protein expression of PEDF varied. Genomic DNA encoding the PEDF gene was the same in the cell lines examined by Southern blotting. In chemically induced hypoxic conditions, secreted PEDF protein was suppressed in contrast to elevation of vascular endothelial growth factor protein. When PEDF was overexpressed by gene transfer, proliferation and migration of endothelial cells were inhibited in conditioned media derived from all HCC cell lines. However, the serum concentration of PEDF, as measured by enzyme-linked immunosorbent assay, was decreased in patients with cirrhosis or HCC complicated by cirrhosis compared to healthy volunteers and patients with chronic hepatitis. According to the endothelial cell proliferation assay, the serum PEDF of patients with HCC had antiangiogenic activity. Moreover, intratumoral injection of a PEDF-expressing plasmid in athymic mouse models caused significant inhibition of preestablished tumor growth. In conclusion, PEDF plays a role in the angiogenic properties of HCC. Reduction of serum PEDF concentration associated with the development of chronic liver diseases may contribute to the progression of HCC. In addition, gene therapy using PEDF may provide an efficient treatment for HCC. (HEPATOLOGY 2004;40:252–259.)

Neovascularization is essential for the growth of solid malignant tumors larger than 1 to 2 mm in diameter.[1,2] Cancer cells constantly require high oxygen and nutrient concentrations because of their rapid cell division. Therefore, these cells are always exposed to a certain degree of vascular starvation, and blood vessels in the area attempt to sprout from preexisting vessels. This phenomenon is called *angiogenesis*.[2,3] Many secretory agents involved in angiogenesis, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and angiopoietin-1, have been discovered and are well studied in various types of cancer.[4–6] Conversely, several endogenous antiangiogenic factors also have been identified.

Pigment epithelium-derived factor (PEDF) was first discovered in 1989 by Tombran-Tink as a neurotrophic serpin that was secreted by retinal pigment epithelial cells.[7,8] Recently, PEDF was implicated in inhibition of angiogenesis in a dose-dependent manner both *in vitro* and *in vivo*.[9–11] The antiangiogenic efficiency of PEDF is more potent than that of other endogenous angiogenic inhibitors, including angiostatin, thrombospondin-1, and endostatin.[12] PEDF is found throughout the body and is particularly highly expressed in the normal liver.[13,14] In this regard, in hepatocellular carcinoma (HCC), it is speculated that PEDF expression is disadvantageous for tumor progression, but paradoxically, HCC is known to be one of the most hypervascular cancers. PEDF expression has not been well investigated in neoplastic diseases, including HCC. Furthermore, because the clinical nature of premalignant conditions associated with HCC are clearly elucidated, including chronic hepatitis (CH) or liver cirrhosis (LC) resulting from hepatitis B or C virus infection,[15] patterns of PEDF expression in these liver diseases also are of interest.

Abbreviations: VEGF, vascular endothelial growth factor; PEDF, pigment epithelium-derived factor; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis; RPMI, Roswell Park Memorial Institute; HUVEC, human umbilical vascular endothelial cells; CM, conditioned media; ELISA, enzyme-linked immunosorben assay; CM-P, CM from pcDNA3-PEDF-transfected cells; RT-PCR, reverse-transcriptase polymerase chain reaction; mRNA, messenger RNA.

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In the present study, we investigated the antiangiogenic properties of PEDF both *in vitro* and *in vivo* using HCC cell lines and human serum samples from patients with premalignant liver diseases. In addition, we studied the effects of PEDF gene overexpression on angiogenesis *in vitro* and on progression of tumors implanted subcutaneously in nude mice *in vivo*. These issues are extremely relevant to the field of antiangiogenic gene therapy.

Materials and Methods

Cell Culture. Human HCC cell lines were maintained in Roswell Park Memorial Institute (RPMI) supplemented with 10% bovine calf serum (Huh-7, HepG2, or PLC/PRF/5). Human umbilical vascular endothelial cells (HUVECs) were purchased from Sankyo Junyaku (Tokyo, Japan) and were grown in EBM2 medium.

Southern Blotting. DNA samples were digested with *EcoRI*. Ten micrograms of each digested DNA sample were fractionated on a 1% agarose gel, were blotted onto a nylon membrane (Hybond N+; Amersham, Little Chalfont, UK), and were hybridized with a [^{32}P]-labeled PEDF cDNA.

Northern Blotting. Total RNA was isolated using the guanidinium isothiocyanate method. Total RNA (10 μg) was fractionated on a 1% formaldehyde agarose gel, was transferred to a nylon membrane, and was hybridized with [^{32}P]-labeled PEDF or VEGF cDNA probes.

Western Blotting. Conditioned media (CM) or serum containing 10 μg protein was subjected to 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transblotted onto nitrocellulose membrane. Blots were blocked with a solution of 5% nonfat dry milk/Tris-buffered saline containing 0.1% Tween 20 for 1 hour and then incubated overnight at 4°C in the presence of mouse anti-PEDF monoclonal antibody (Chemicon International Inc., Temecula, CA) or rabbit antihexahistidine antibody (ICN, Costa Mesa, CA). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and were incubated with horseradish peroxidase-conjugated antimouse immunoglobulin G. After washing with Tris-buffered saline containing 0.1% Tween 20, immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

Enzyme-Linked Immunosorbent Assay. VEGF and PEDF concentrations were assayed using the Quantikine HS Human VEGF Immunoassay Kit (R & D Systems, Minneapolis, MN) and the ChemiKine PEDF sandwich enzyme-linked immunosorbent assay (ELISA) kit (Chemicon International Inc.), according to the instructions provided by the manufacturer.

Serum from Healthy Volunteers and Patients With Liver Diseases. We collected sera from healthy volunteers (n = 8), patients with CH (n = 8), patients with LC (n = 8), and patients with HCC complicated with LC (n = 8). HCC patients had undergone selective hepatic angiography or computed tomography, and they showed hypervascularity. Informed consent was obtained from each patient before entering this study according to the guidelines of the Ethics Committee of Nagasaki University.

Immunoprecipitation. Immunoprecipitation was performed using 25 μg of mouse anti-PEDF antibody or normal mouse immunoglobulin G for 500 μL serum with 20 μL packed Protein G/A (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Transfection of Cells. The human hexahistidine-tagged PEDF cDNA was cloned into pcDNA3 (Invitrogen, Carlsbad, CA) from pCEP4-PEDF (kindly provided by Dr. Noel P. Bouck, Northwestern University) to construct pcDNA3-PEDF. Transfection was performed using 10 μg pEGFP (Clontech, Palo Alto, CA), pcDNA3, or pcDNA3-PEDF by the lipofectin (Life Technologies Inc., Gaithersburg, MD).

Preparation of Conditioned Media Derived From HCC Cell Lines. Huh-7, HepG2, or PLC/PRF/5 cells (approximately 2 $\times$ 10^6) were plated on 100-mm cell culture dishes. After 24 hours, transfection was performed and medium was replaced with 10 mL serum-free RPMI. After a further 48 hours of incubation, CM was collected from nontransfected cells, from pcDNA3-PEDF transfected cells (CM-P), or from pCDNA3 transfected cells transfected cells.

Proliferation and Migration Assay of HUVECs. HUVECs were plated onto 96-well culture plates at approximately 5 $\times$ 10^3 cells/well and were incubated for 24 hours. Medium was then replaced with 100 μL of RPMI, CM derived from nontransfected, or CM derived from transfected HCC cells. After 48 hours, proliferation of HUVECs was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). However, migration was measured using 8.0-μm 24-well Transwells (Corning, Acton, MA), as described previously with some modifications.[16] Briefly, 600 μL of each CM sample was placed in the lower chamber. Subconfluent 16-hour cultures of HUVEC in the growth factor-free medium were harvested, washed, and resuspended in serum-free RPMI. HUVECs (approximately 5 $\times$ 10^4) in 100 μL serum-free RPMI were added to the upper chamber. After 24 hours of incubation, all nonmigrating cells were removed from the upper surface of the membrane with a cotton swab; cells that had migrated to the lower surface were fixed with absolute meth-

anol and were stained with Giemsa. The numbers of migrated cells were counted using a light microscope under a high-power field (magnification, ×200). All experiments were performed in triplicate.

Murine Hepatocellular Carcinoma Tumor Model. Four-week-old male BALB/c nu/nu athymic mice were purchased from Charles River (Yokohama, Japan). Animal experiments were performed in accordance with institutional guidelines, and the study was approved by the Ethics Committee of Nagasaki University. Huh-7 cells (3×10^6) were implanted subcutaneously into the left thigh. Tumor volume was calculated as follows; tumor volume = length (mm) × width^2 (mm) × 1/2. When the tumor volume reached 60 to 110 mm^3, pcDNA3-PEDF or pcDNA3 (75 μg plasmid/100μL of TE [Tris ethylenediamine tetra acetic acid] buffer) with 20 μL of lipofectin was injected into the tumor once weekly for 3 weeks. As a control, 100 μL of vehicle (TE buffer) with lipofectin was injected. Each group consisted of five mice. Tumor volume was measured every 3 or 4 days. Two other mice were killed at day 24 or 32 in each group; tumors were removed and analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR).

RT-PCR. RNA was used after contaminating DNA was completely removed by DNase I treatment. RT-PCR was performed using the instructions provided by the supplier of the OneStep RT-PCR Kit (Qiagen, Valencia, CA), using primers specific for PEDF, VEGF and glyceraldehyde 3-phosphate dehydrogenase. RT-PCR amplification of glyceraldehyde 3-phosphate dehydrogenase was used as a control to assess the integrity of RNA. Ten-microliter samples of the amplification reactions were loaded on 1.2% TAE (Tris Acetic Acid + TE) agarose gels, and the products were visualized by ethidium bromide staining.

Statistical Analysis. All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using Student's t test. All reported P values are two-tailed, and those less than .01 were considered statistically significant.

Results

Expression and Oxygen Regulation Analysis of PEDF in HCC Cell Lines. We first investigated PEDF expression in HCC cell lines. Northern blot showed that PEDF messenger RNA (mRNA) expression was abundant in HepG2 cells and was detected at considerable levels in Huh-7 cells, whereas it was undetectable in PLC/PRF/5 cells (Fig. 1B). Secreted PEDF protein in CM assessed by Western blotting exhibited a pattern similar to that of mRNA expression (Fig. 1C). In contrast, Southern blot showed that

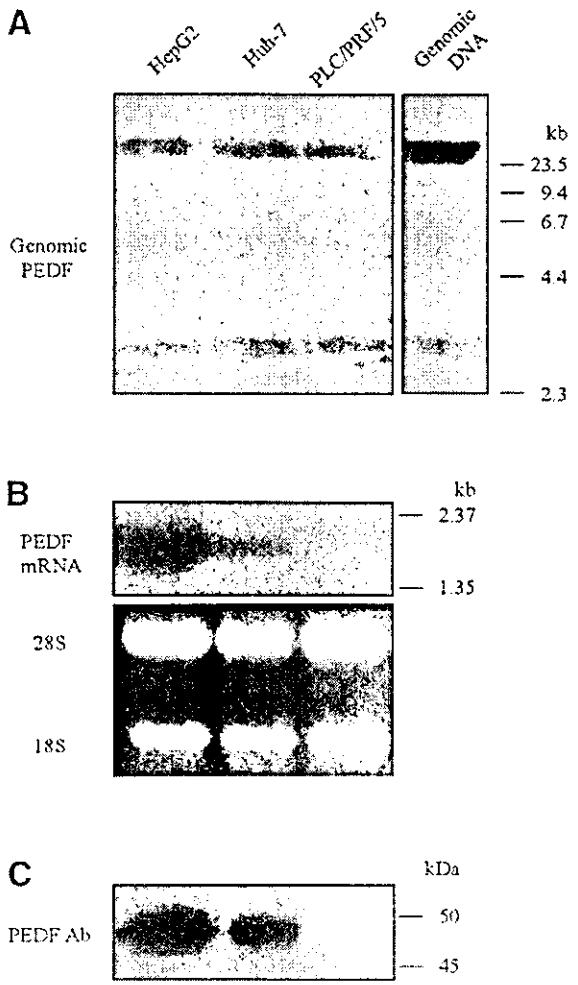

Fig. 1. Expression of pigment epithelium-derived factor (PEDF) in cell lines. (A) Southern blot analysis of genomic DNA derived from hepatocellular carcinoma cell lines and human genomic DNA. (B) PEDF messenger RNA (mRNA) as determined by Northern blot analysis. The 1.5-kb band indicates PEDF gene mRNA. The lower panel shows 28S and 18S ribosomal RNA as internal controls. (C) Expression of PEDF protein in conditioned media derived from each cell line analyzed by Western blotting using anti-PEDF antibody.

genomic DNA encoding the PEDF gene did not differ among the three cell lines compared with normal human genome (Fig. 1A). For chemical induction of hypoxic conditions,[17–19] HepG2 or PLC/PRF/5 cells were incubated for 12 hours with 400 μmol/L of cobalt chloride or 260 μmol/L of desferrioxamine. After exposure to these compounds, PEDF protein was suppressed in CM derived from HepG2 cells despite no alteration of PEDF mRNA expression (Fig. 2A, B), whereas the VEGF protein level was increased (Fig. 2C). In PLC/PRF/5 cells, both PEDF mRNA and protein remained undetectable, and VEGF protein was increased, similar to that in HepG2 (Fig. 2A–C).

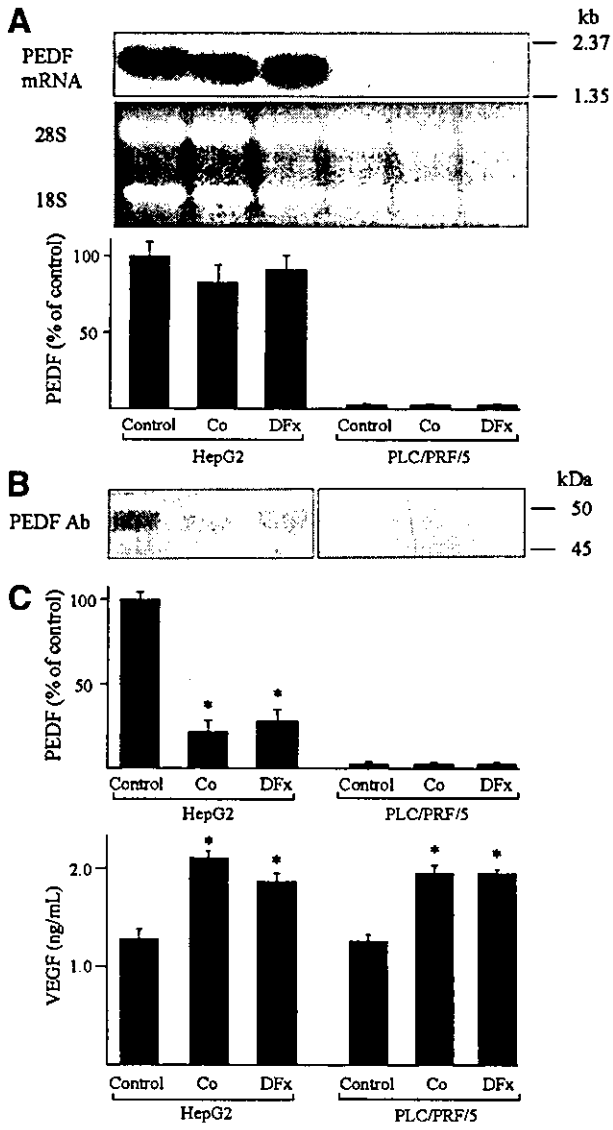

Fig. 2. Effect of chemically induced hypoxia on the expression of pigment epithelium-derived factor (PEDF) or vascular endothelial growth factor (VEGF) in hepatocellular carcinoma cell lines. HepG2 or PLC/PRF/5 cells were incubated for 12 hours with 400 μmol/L cobalt chloride (Co) or 260 μmol/L desferrioxamine (DFx). (A) Northern blot analysis. PEDF messenger RNA (mRNA) expression was quantified by densitometry and was normalized against the density of 28S ribosomal RNA. The PEDF mRNA level is indicated as a percentage of the respective control. (B) PEDF protein analyzed by Western blotting in each conditioned media (CM). The quantification of PEDF protein measured by densitometry is indicated as a percentage of the respective control. (C) VEGF concentrations in the CM derived from HepG2 or PLC/PRF/5 cells measured by enzyme-linked immunosorbent assay. Data are mean ± SD of three separate experiments. *$P < .01$ versus control.

PEDF Expression in Serum and Inhibition of HUVEC Proliferation. ELISA revealed that PEDF protein in both human serum from patients with LC and HCC complicated with LC were lower than that in healthy volunteers and patients with CH (Fig. 3A). Serum PEDF concentrations in patients with LC and in those with HCC complicating LC were almost similar. Next, we investigated the effect of serum PEDF of HCC patients on HUVEC proliferation. PEDF was extracted from the serum of HCC patients by immunoprecipitation, and the effect of this process on HUVEC proliferation was evaluated. Western blotting revealed that PEDF was almost completely removed from the serum by immunoprecipitation using anti-PEDF antibody (Fig. 3B). HUVEC proliferation was significantly higher in PEDF-free serum (1.26 times) than in PEDF-containing serum ($P < .01$; Fig. 3C). In contrast, the serum that was immunoprecipitated with normal mouse immunoglobulin G as a control contained the same amount of PEDF and resulted in the same HUVEC proliferation rate as the PEDF-containing serum. These results suggest that serum PEDF from HCC patients could inhibit angiogenesis.

***Induction of a PEDF Expression Vector in HCC Cell Lines and Its Antiangiogenic Effect* In Vitro.** We constructed a mammalian expression vector for the PEDF gene tagged with hexahistidine (pcDNA3-PEDF). HCC cell lines were transiently transfected with pcDNA3 or pcDNA3-PEDF. To determine the efficiency of transfection, the expression of green fluorescent protein was observed 24 hours after pEGFP transfection in each cell lines. In these experiments, 3%, 7%, and 21% of HepG2, Huh-7, and PLC/PRF/5 cells, respectively, were transfected with the vector. CM derived from 48 hours of incubation of each of the pcDNA3-PEDF-transfected HCC cells (CM-P-G2, CM-P-7, CM-P-PLC) contained more PEDF protein than that derived from nontransfected or pcDNA3-transfected cells. Western blotting with antihexahistidine antibody clearly showed that the increased PEDF protein in CM-P mainly was the result of pcDNA3-PEDF expression (Fig. 4A). Proliferation of each HCC cell line was not influenced by pcDNA3 or pcDNA3-PEDF transfection (Fig. 4B). To determine the proliferation of HUVEC, these cells were incubated in RPMI or CM derived from the transfected or nontransfected cells for 48 hours. We also investigated the migration of HUVECs after 24 hours incubation in RPMI or CM derived from transfected or nontransfected cells as described in Materials and Methods. Quantitative analysis showed that both proliferation and migration of HUVECs were significantly suppressed in CM-P compared with CM collected from nontransfected cells or CM collected from pCDNA3-transfected cells from all three cell lines (Fig. 4C). The average suppressive effect of CM-P on proliferation and migration was 30% and

36.3%, respectively, compared with CM collected from nontransfected cells.

Effect of PEDF Gene Induction on Progression of Preestablished Huh-7 Tumors in an Athymic Mouse Model. Huh-7 cells were subcutaneously implanted and tumors were established in athymic mice because Huh-7 cells were more efficiently transplantable than other cell lines. After reaching an adequate size, the tumor was directly injected with pcDNA3-PEDF, and the effect of treatment on tumor size was determined. Injection of pcDNA3-PEDF resulted in a significant reduction of tumor volume in compared with vehicle- or pcDNA3-injected mice at day 24 after the start of treatment ($P < .01$; Fig. 5B). When the expression levels of PEDF and VEGF mRNAs were analyzed by RT-PCR, PEDF mRNA was increased in pcDNA3-PEDF-injected mice 3 days after pcDNA3-PEDF-injection (Fig. 5C; day 24). However, pcDNA3-PEDF-injected tumors seemed to escape the growth suppression effect at the last time point (Fig. 5B). PEDF overexpression was not seen at day 11 after the third injection (Fig. 5C; day 32). Thus, the period of PEDF expression by pcDNA3-PEDF injection was limited for 24 days. However, expression of VEGF mRNA was not altered at days 3 and 11 after the third injection (Fig. 5C). Therefore, the loss of PEDF expression in the tumor, rather than the increased VEGF expression, seems a better explanation for the escape of growth suppression noted at day 32.

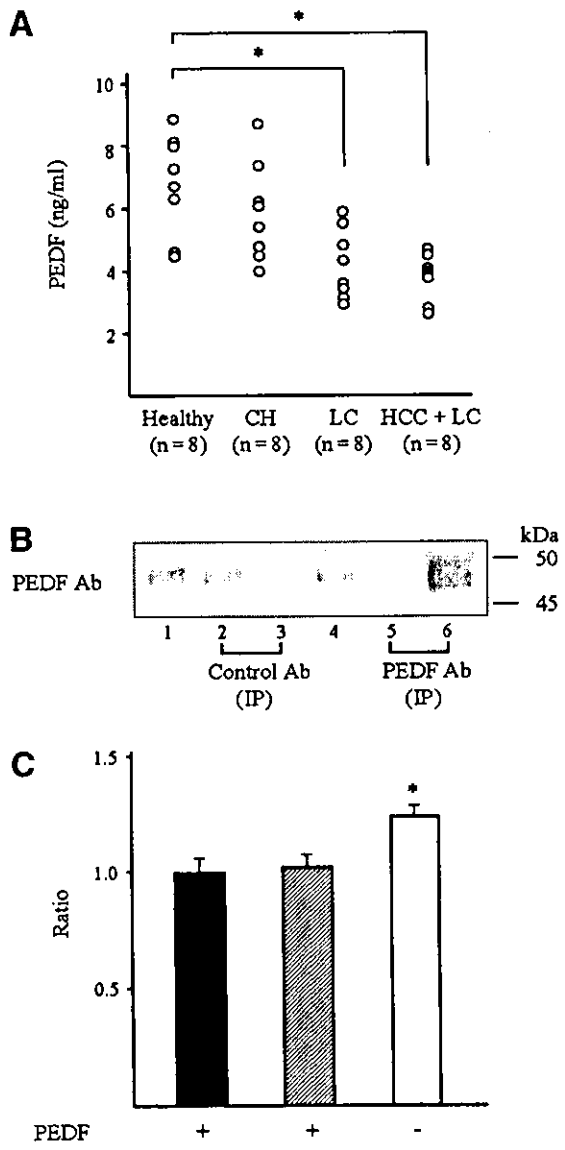

Fig. 3. Expression of pigment epithelium-derived factor (PEDF) in human samples and its inhibitory effect on human umbilical vascular endothelial cell (HUVEC) proliferation. (A) PEDF protein expression in serum of patients with liver diseases. Serum samples were analyzed by enzyme-linked immunosorbent assay. $*P < .01$ versus healthy volunteers. (B) Removal of human PEDF from the serum. Serum samples of hepatocellular carcinoma (HCC) patients were immunoprecipitated using anti-PEDF antibody or anti-mouse immunoglobulin G (IgG) antibody as control. An equal amount of flow-through fraction was analyzed by Western blotting using anti-PEDF antibody. Original serum (**lanes 1 and 4**), flow-through fraction of immunoprecipitation (**lanes 2 and 5**), and elution of immunoprecipitation (**lanes 3 and 6**). (C) Effect of PEDF in serum of HCC patients on HUVEC proliferation. HUVECs were incubated in the conditioned media containing 5% of original serum (**solid bar**), immunoprecipitated serum using normal mouse IgG (**hatched bar**) or anti-PEDF antibody (**open bar**). After 48-hour incubation, HUVEC numbers were determined and expressed as the ratio to their numbers in the original serum (PEDF-containing serum). Data are mean ± SD of all eight HCC patients. $*P < .01$ versus original serum and immunoprecipitated serum using normal mouse IgG. CH, chronic hepatitis; LC, liver cirrhosis; HCC + LC, hepatocellular carcinoma complicated with liver cirrhosis; IP, immunoprecipitation.

Discussion

Human PEDF is expressed in various tissues in the body[13,14] and is involved in retinal angiogenesis, however, there have been only a few specific studies of the antiangiogenic properties of PEDF in neoplastic diseases.[20-24] In the present study, we demonstrated that the mRNA and protein expression of PEDF varied in three HCC cell lines. PEDF mRNA was not detected in PLC/PRF/5, which is consistent with PEDF suppression being advantageous for tumor progression. Southern blotting showed a similar pattern of genomic PEDF in these cell lines. Therefore, PEDF expression seems to be regulated at the transcriptional level or in association with RNA stability. However, PEDF expression was suppressed at the protein level by chemically induced hypoxic conditions in the constitutive PEDF-expressing cell line, HepG2. Although PEDF mRNA was abundantly expressed in

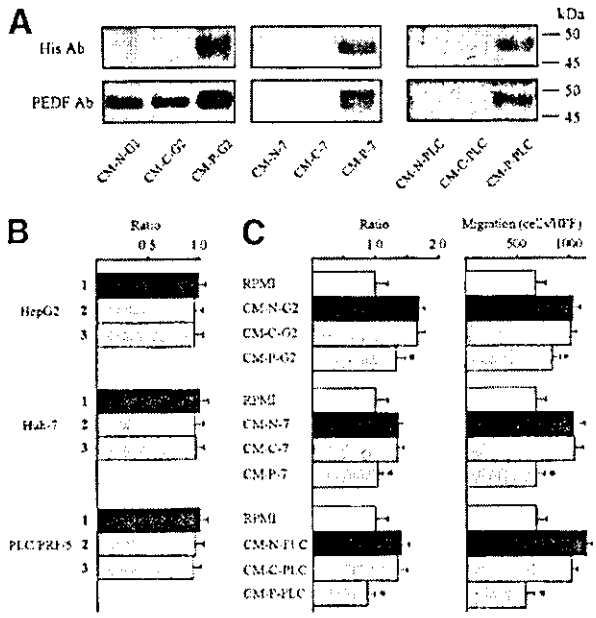

Fig. 4. Inhibition of proliferation and migration of human umbilical vascular endothelial cells (HUVECs) by pigment epithelium-derived factor (PEDF) gene induction. (A) Expression of PEDF protein in conditioned media (CM) derived from each transfected or nontransfected cell line. CM was collected after 48-hour incubation as described in Materials and Methods. Expression of PEDF protein was analyzed by Western blotting using antihexahistidine antibody or anti-PEDF antibody. (B) Growth of transfected or nontransfected HCC cells. HCC cell lines were incubated for 48 hours after transfection, and numbers of viable cells were determined. Data are expressed as the ratio to nontransfected cells (mean ± SD of three separate experiments). **1**, nontransfected; **2**, pcDNA3 transfected; **3**, pcDNA3-PEDF transfected. (C) Endothelial cell proliferation and migration. HUVECs were incubated in CM derived from each cell line. The numbers of proliferated cells after 48-hour incubation were estimated and expressed as the ratio to the number of cells incubated in RPMI. The numbers of migrated cells after 24 hours of incubation was determined under a light microscope with high-power field (magnification, ×200), as described in Materials and Methods. Data represent mean ± SD of three separate experiments. $*P < .01$ versus CM-N or CM-C. His Ab, ; Ab, ; CM-N, CM collected from nontransfected cells; CM-C, CM collected from pCDNA3 transfected cells; CM-P, CM collected from pcDNA3-PEDF transfected cells; CM-G2, CM collected from HepG2; CM-7, CM collected from Huh-7; CM-PLC, CM collected from PLC/PRF/5; RPMI, Roswell Park Memorial Institute; HPF, higher-power field.

HepG2, PEDF protein expression was suppressed in chemically induced hypoxic conditions in contrast to the elevation of VEGF protein expression. Constitutive overexpression of PEDF in HepG2 cells seems to be contradictive because the local environment should shift toward angiogenic conditions in cancer cells for rapid tumor growth. Thus, HepG2 cells may produce enough angiogenic reagents in excess of the level of antiangiogenic reagents such as PEDF. Indeed, VEGF, which is known as a major angiogenic factor, is expressed in adequate amounts in HepG2 cells.[23,24] Moreover, it is possible that PEDF is suppressed in HepG2 cells, as demonstrated in our hypoxic study when the cells were grown *in vivo* and were exposed to more hypoxic conditions than in culture media *in vitro*. Because we did not evaluate angiogenic and antiangiogenic reagents other than VEGF and PEDF and there are no adequate methods for estimating the local angiogenic or antiangiogenic activity directly and separately, precise evaluation of the angiogenic phenotype of specific tumors may be difficult. However, it can be concluded that PEDF must be involved as an antiangiogenic factor in HCC.

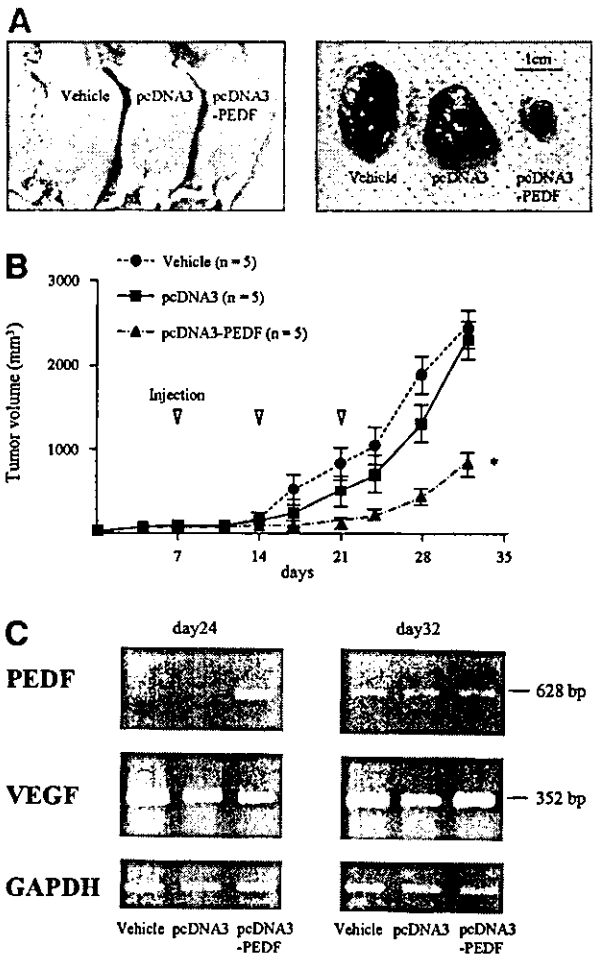

Fig. 5. Inhibition of pre-established tumor growth by injection of pcDNA3-pigment epithelium-derived factor (PEDF) plasmid in athymic mice. Vehicle, pcDNA3, or pcDNA3-PEDF was injected intratumorally into pre-established tumors of Huh-7 cells. Mice were killed on day 24 or day 32 and subcutaneous tumors were extracted. (A) Representative photographs of harvested tumors. (B) Serial changes in tumor volume in the three different groups. Data are mean ± SD of tumor volume. $*P < .01$ versus vehicle or pcDNA3. (C) Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis for PEDF and vascular endothelial growth factor (VEGF) messenger RNA (mRNA) expression, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. Total RNA was extracted from tumor tissues at days 24 and 32. RT-PCR was performed with primers specific for PEDF, VEGF, and GAPDH.