ceptors. Nakajima et al. [36] reported that the number of IFN receptors on peripheral blood mononuclear cells in patients with chronic hepatitis B decreased to about 50% of the baseline with a 5-fold increase in 2′,5′-oligoadenylate synthetase activity when the patients were treated with IFN for 2 or 4 weeks. These results suggest that the downregulation of the IFN receptor is not always associated with a decrease in the action of IFN. We chronologically examined the relationship between the antiproliferative effect and the expression of the IFNAR-2 subunit in HAK-1B cells up to 240 h after the addition of PEG-IFN-α2b. The expression of the IFNAR-2 subunit was significantly downregulated at 3 h compared with the control and then significantly upregulated at 48 h. Expression decreased in a time-dependent manner after 72 h, and the viable cell number continuously decreased over time [17]. Similarly, IFNAR-2 expression in the tumor was lower in mice that received PEG-IFN-α2b than in mice that received IFN-α2b or in control mice as a result of the long-term continuous action of PEG-IFN-α2b, but, in fact, the tumor size was smaller in mice that received PEG-IFN-α2b than in mice that received IFN-α2b or in control mice. The results suggest that, at least for the HCC cell line, HAK-1B, the IFNAR-2 subunit is downregulated, but an efficient antiproliferative effect is induced with continuous contact with PEG-IFN-α2b in vitro and in vivo.

Growth-Inhibitory Effects and Mechanism of Action of Combined IFN-α and 5-FU Treatment in Human Liver Cancer Cells in vitro

Alterations in cell cycle progression via upregulation of p27^{kip1} [37] or cyclin A [22], induction of apoptosis by downregulation of Bcl-xl [38], modulation of the immune response via the TRAIL/TRAIL-R pathway [39] and Fas/Fas ligand pathway [40], and alteration of 5-FU metabolism (e.g., increase of 5-fluoro-2′-deoxyuridine-5′-monophosphate and decrease of thymidylate synthase) have been reported as the mechanism of synergistic antitumor action of the combined IFN-α and 5-FU treatment in HCC. We examined the growth-inhibitory effects of the combined IFN-α and 5-FU treatment in 6 HCC cell lines in vitro by using isobologram analysis and found that the cell lines could be divided into two groups: the S group (3 cell lines) showing synergistic effects and the A group (3 cell lines) showing additive effects. In addition, mRNA and protein expressions of type I IFN receptor subunits, IFNAR-1 and IFNAR-2, were specifically upregulated by 5-FU in all cell lines of the S group except IFNAR-2 in one cell line, but not in those of the A group. IFN-α modulated the protein expression levels of six enzymes (thymidylate synthase, dihydropyrimidine dehydrogenase, orotate phosphoribosyl transferase, thymidine phosphorylase, uridine phosphorylase, and thymidine kinase) regulating sensitivity to 5-FU, but none of them were altered in the same way in cells in the S or A group. We concluded that the 5-FU-induced modulation of type I IFN receptor expression, at least in part, contributes to the induction of synergistic effects of combined IFN-α and 5-FU therapy.

Conclusions

We show that (1) almost all liver cancer cell lines express type I IFN receptor, (2) each IFN-α preparation or subtype presents very different antiproliferative activities in different human liver cancer cell lines, (3) a common mechanism of in vitro growth suppression by type I IFN is cell cycle arrest with or without caspase-dependent apoptosis induction, and (4) the mechanism of in vivo growth inhibition by type I IFN is the induction of apoptosis with or without the inhibition of angiogenesis. These lines of evidence suggest that the direct antiproliferative action of type I IFN may be involved in the suppressive mechanisms of type I IFN on hepatocellular carcinogenesis. In addition, we would expect pegylated IFN-α preparations to produce more potent effects in the prevention and treatment of HCC than do nonpegylated IFN preparations.

Disclosure Statement

References

1 Pestka S, Langer JA, Zoon KC, Samuel CE: Interferons and their actions. Annu Rev Biochem 1987;56:727–777.
2 Diaz MO, Bohlander S, Allen G: Nomenclature of human interferon genes. J Interferon Res 1993;13:243–244.
3 Rani MR, Ransohoff RM: Alternative and accessory pathways in the regulation of IFN-β-mediated gene expression. J Interferon Cytokine Res 2005;25:788–798.
4 Gutterman JU: Cytokine therapeutics: lessons from interferon α. Proc Natl Acad Sci USA 1994;91:1198–1205.
5 Shiratori Y, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, Teratani T, Tohgo G, Toda N, Ohashi M, Ogura K, Niwa Y, Kawabe T, Omata M: Characteristic difference of hepatocellular carcinoma between hepatitis B- and C- viral infection in Japan. Hepatology 1995;22:1027–1033.
6 Kashiwagi K, Furusyo N, Kubo N, Nakashima H, Nomura H, Kashiwagi S, Hayashi J: A prospective comparison of the effect of interferon-alpha and interferon-beta treatment in patients with chronic hepatitis C on the incidence of hepatocellular carcinoma development. J Infect Chemother 2003;9:333–340.
7 Mazzella G, Accogli E, Sottili S, Festi D, Orsini M, Salzetta A, Novelli V, Cipolla A, Fabbri C, Pezzoli A, Roda E: Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. J Hepatol 1996;24:141–147.
8 Nishiguchi S, Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, Shiomi S, Seki S, Kobayashi K, Otani S: Randomised trial of effects of interferon-α on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 1995;346:1051–1055.
9 Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, Shiomi S, Tamori A, Oka H, Igawa S, Kuroki T, Kinoshita H: Effects of long-term postoperative interferon-α therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. Ann Intern Med 2001;134:963–967.
10 Ikeda K, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Chayama K, Murashima N, Kumada H: Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor – a prospective randomized study of hepatitis C virus-related liver cancer. Hepatology 2000;32:228–232.
11 Obi S, Yoshida H, Toune R, Unuma T, Kanda M, Sato S, Tateishi R, Teratani T, Shiina S, Omata M: Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. Cancer 2006;106:1990–1997.

12 Sakon M, Nagano H, Dono K, Nakamori S, Umeshita K, Yamada A, Kawata S, Imai Y, Iijima S, Monden M: Combined intraarterial 5-fluorouracil and subcutaneous interferon-α therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer 2002;94:435–442.
13 Takayama A, Yano H, Ogasawara S, Higaki K, Kojiro M: Expression of Hu-IFN-αR2 chain of type I interferon receptor in human hepatocellular carcinoma and non-cancerous tissues. Int J Mol Med 2000;6:621–627.
14 Ogasawara S, Yano H, Momosaki S, Akiba J, Nishida N, Kojiro S, Moriya F, Ishizaki H, Kuratomi K, Kojiro M: Growth inhibitory effects of IFN-β on human liver cancer cells in vitro and in vivo. J Interferon Cytokine Res 2007;27:507–516.
15 Hisaka T, Yano H, Ogasawara S, Momosaki S, Nishida N, Takemoto Y, Kojiro S, Katafuchi Y, Kojiro M: Interferon-αCon1 suppresses proliferation of liver cancer cell lines in vitro and in vivo. J Hepatol 2004;41:782–789.
16 Yano H, Iemura A, Haramaki M, Ogasawara S, Takayama A, Akiba J, Kojiro M: Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines. Hepatology 1999;29:1708–1717.
17 Yano H, Ogasawara S, Momosaki S, Akiba J, Kojiro S, Fukahori S, Ishizaki H, Kuratomi K, Basaki Y, Oie S, Kuwano M, Kojiro M: Growth inhibitory effects of pegylated IFN α-2b on human liver cancer cells in vitro and in vivo. Liver Int 2006;26:964–975.
18 Yano H, Yanai Y, Momosaki S, Ogasawara S, Akiba J, Kojiro S, Moriya F, Fukahori S, Kuruimoto M, Kojiro M: Growth inhibitory effects of interferon-α subtypes vary according to human liver cancer cell lines. J Gastroenterol Hepatol 2006;21:1720–1725.
19 Yano H, Ogasawara S, Momosaki S, Akiba J, Nishida N, Kojiro S, Ishizaki H, Kojiro M: Expression and activation of apoptosis-related molecules involved in interferon-α-mediated apoptosis in human liver cancer cells. Int J Oncol 2005;26:1645–1652.
20 Oie S, Ono M, Yano H, Maruyama Y, Terada T, Yamada Y, Ueno T, Kojiro M, Hirano K, Kuwano M: The upregulation of type I interferon receptor gene plays a key role in hepatocellular carcinoma cells in the synergistic antiproliferative effect by 5-fluorouracil and interferon-α. Int J Oncol 2006;29:1469–1478.
21 Oie S, Ono M, Fukushima H, Hosoi F, Yano H, Maruyama Y, Kojiro M, Terada T, Hirano K, Kuwano M, Yamada Y: Alteration of dihydropyrimidine dehydrogenase expression by IFN-α affects the antiproliferative effects of 5-fluorouracil in human hepatocellular carcinoma cells. Mol Cancer Ther 2007;6:2310–2318.

22 Kojiro S, Yano H, Ogasawara S, Momosaki S, Takemoto Y, Nishida N, Kojiro M: Antiproliferative effects of 5-fluorouracil and interferon-alpha in combination on a hepatocellular carcinoma cell line in vitro and in vivo. J Gastroenterol Hepatol 2006;21:129–137.
23 Lutfalla G, Holland SJ, Cinato E, Monneron D, Reboul J, Rogers NC, Smith JM, Stark GR, Gardiner K, Mogensen KE, et al: Mutant U5A cells are complemented by an interferon-αβ receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster. EMBO J 1995;14:5100–5108.
24 Domanski P, Nadeau OW, Platanias LC, Fish E, Kellum M, Pitha P, Colamonici OR: Differential use of the βI subunit of the type I interferon (IFN) receptor determines signaling specificity for IFNα2 and IFNβ. J Biol Chem 1998;273:3144–3147.
25 Kondo M, Nagano H, Sakon M, Yamamoto H, Morimoto O, Arai I, Miyamoto A, Eguchi H, Dono K, Nakamori S, Umeshita K, Wakasa K, Ohmoto Y, Monden M: Expression of interferon α/β receptor in human hepatocellular carcinoma. Int J Oncol 2000;17:83–88.
26 Ota H, Nagano H, Doki Y, Sekimoto M, Kondo M, Wada H, Nakamura M, Noda T, Damdinsuren B, Marubashi S, Miyamoto A, Takeda Y, Dono K, Umeshita K, Nakamori S, Wakasa K, Sakon M, Monden M: Expression of type I interferon receptor as a predictor of clinical response to interferon-α therapy of gastrointestinal cancers. Oncol Rep 2006;16:249–255.
27 Ota H, Nagano H, Sakon M, Eguchi H, Kondo M, Yamamoto T, Nakamura M, Damdinsuren B, Wada H, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Wakasa K, Monden M: Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-α and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. Br J Cancer 2005;93:557–564.
28 Fish EN, Banerjee K, Stebbing N: Human leukocyte interferon subtypes have different antiproliferative and antiviral activities on human cells. Biochem Biophys Res Commun 1983;112:537–546.
29 Foster GR, Rodrigues O, Ghouze F, Schulte-Frohlinde E, Testa D, Liao MJ, Stark GR, Leadbeater L, Thomas HC: Different relative activities of human cell-derived interferon-alpha subtypes: IFN-alpha 8 has very high antiviral potency. J Interferon Cytokine Res 1996;16:1027–1033.
30 Blatt LM, Davis JM, Klein SB, Taylor MW: The biologic activity and molecular characterization of a novel synthetic interferon-alpha species, consensus interferon. J Interferon Cytokine Res 1996;16:489–499.

31 Ruzicka FJ, Jach ME, Borden EC: Binding of recombinant-produced interferon β ser to human lymphoblastoid cells. Evidence for two binding domains. J Biol Chem 1987;262:16142–16149.

32 Russell-Harde D, Wagner TC, Perez HD, Croze E: Formation of a uniquely stable type I interferon receptor complex by interferon β is dependent upon particular interactions between interferon β and its receptor and independent of tyrosine phosphorylation. Biochem Biophys Res Commun 1999;255:539–544.

33 Oshima K, Yanase N, Ibukiyama C, Yamashina A, Kayagaki N, Yagita H, Mizuguchi J: Involvement of TRAIL/TRAIL-R interaction in IFN-α-induced apoptosis of Daudi B lymphoma cells. Cytokine 2001;14:193–201.

34 Yano H, Iemura A, Fukuda K, Mizoguchi A, Haramaki M, Kojiro M: Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. Hepatology 1993;18:320–327.

35 Zoon KC, Zur Nedden D, Hu R, Arnheiter H: Analysis of the steady state binding, internalization, and degradation of human interferon-α2. J Biol Chem 1986;261:4993–4996.

36 Nakajima S, Kuroki T, Shintani M, Kurai O, Takeda T, Nishiguchi S, Shiomi S, Seki S, Kobayashi K: Changes in interferon receptors on peripheral blood mononuclear cells from patients with chronic hepatitis B being treated with interferon. Hepatology 1990;12:1261–1265.

37 Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, Dono K, Nakamori S, Umeshita K, Sakon M, Monden M: Augmentation of antitumor activity of 5-fluorouracil by interferon α is associated with up-regulation of p27^{kip1} in human hepatocellular carcinoma cells. Clin Cancer Res 2000;6:2881–2890.

38 Kondo M, Nagano H, Wada H, Damdinsuren B, Yamamoto H, Hiraoka N, Eguchi H, Miyamoto A, Yamamoto T, Ota H, Nakamura M, Marubashi S, Dono K, Umeshita K, Nakamori S, Sakon M, Monden M: Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. Clin Cancer Res 2005;11:1277–1286.

39 Yamamoto T, Nagano H, Sakon M, Wada H, Eguchi H, Kondo M, Damdinsuren B, Ota H, Nakamura M, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Yagita H, Monden M: Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against hepatocellular carcinoma. Clin Cancer Res 2004;10:7884–7895.

40 Nakamura M, Nagano H, Sakon M, Yamamoto T, Ota H, Wada H, Damdinsuren B, Noda T, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Nakamori S, Dono K, Monden M: Role of the FAS/FASL pathway in combination therapy with interferon-α and fluorouracil against hepatocellular carcinoma in vitro. J Hepatol 2007;46:77–88.

Review

肝癌の発生・進展とインターフェロンによる制御

矢野　博久

Development and progression of hepatocellular carcinoma and chemoprevention of HCC by interferon

Hirohisa Yano

Abstract

　Most early hepatocellular carcinomas (HCCs) are well differentiated, with an ill-defined nodular appearance. When a well-differentiated HCC reaches a size of about 1.5 cm in diameter, less-differentiated cancerous tissues with greater proliferative activity evolve within it. Clonally-related HCC cell lines (HAK-1A and HAK-1B) established from a single HCC nodule with a variety in the histological grade suggest that less-differentiated cancerous tissues develop from clonal dedifferentiation of well-differentiated HCC tissues. Subsequently, moderately to poorly differentiated HCC tissues gradually replace the initial surrounding HCC.

　HCC frequently occurs multicentrically whether synchronously or metachronously, defying complete cure by conventional therapies; therefore, chemoprevention of HCC is very important. Interferon (IFN)-α has been used for the treatment of chronic viral liver diseases to eradicate virus. Recently, IFN-α has been shown to possess highly suppressive effects on hepatocellular carcinogenesis. We found that type I IFN preparations inhibit the growth of 13 liver cancer cell lines at various degrees in vitro and in vivo, and the clinical dose of IFN preparations was effective in vivo. The data suggest that IFNs may inhibit the growth of clinically undetectable HCC cells and prevent or delay the development of HCC in patients with chronic viral liver diseases.

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緒　言

　本稿では，肝細胞癌（肝癌）の発生と進展に関する病理形態学的研究，肝癌の脱分化機構やインターフェロン（IFN）の肝癌細胞に対する増殖抑制作用に関する培養肝癌細胞を用いた分子・実験病理学的研究について我々の施設で集積してきたデータの一部を紹介する．

I．肝癌の発生：早期肝癌の特徴

　肝癌の多くは，ウイルス性慢性肝炎・肝硬変を背景に発生する．腫瘍径2cm前後までの小さな肝癌は，肉眼的に単純結節型と境界不明瞭型に大別される[1]．単純結節型は明瞭な結節を形成し，その約60％に被膜形成と中分化型肝癌組織が認められ古典的な肝癌の像を呈する[2]．一方，境界不明瞭型は単純結節型よりやや腫瘍径が小さいものが多く（1cm前後），癌結節が不明瞭で被膜形成は認められず，脈管侵襲も転移も示さず，高分化型肝癌組織のみから構成される[2]．「原発性肝癌取扱い規約，第4版」では小結節境界不明瞭型を「早期肝細胞癌」と定義している[1]．この境界不明瞭型肝癌は，細胞異型・構造異型の比較的乏しいため，境界病変（前癌病変）といわれている異型腺腫様過形成（high grade dysplastic nodule）との鑑別が問題となることがあるが，その際には結節内部の門脈域への癌細胞の浸潤像の有無が鑑別の指標となる．浸潤があれば高分化癌，なければ異型腺腫様過形成と診断される[2]．

II．肝癌の進展：組織多彩性の出現と
　　 高分化型肝癌細胞の脱分化

　腫瘍径が1cm前後の境界不明瞭型肝癌が高分化型肝癌組織のみから構成されるのに対し，腫瘍径2～3cmの癌結節は約40％に分化度の異なる癌組織が混在し組織多彩性が認められる[3]．この場合，分化度の低い癌組織が結節の内側に，高分化な癌組織が外側に位置している．腫瘍径の増大とともに外側に位置している高分化型肝組織は面積を減じ，3cmを超えるようになると中分化あるいは低分化型癌組織で置換される．このような形態推移の定型的なものは"nodule-in-nodule"像として肉眼的，画像的に認められる[3]．我々は，中心部が低分化型肝癌で，周囲が脂肪化を伴う，あるいは伴わない高分化型肝癌からなり"nodule-in-nodule"像を呈する肝癌結節から高分化型肝癌細胞株（HAK-1A）と低分化型肝癌細胞株（HAK-1B）の分離樹立に成功した（Fig. 1）[4]．HAK-1Bは，HAK-1Aに比べ形態的に低分化で，生物学的悪性度も高い．しかし，これら2つの細胞株には，p53遺伝子の242番目のコドンに共通の変異を認めることから，2つの細胞株が同一起源であり，おそらく，低分化型癌細胞が高分化型癌細胞の脱分化に由来することが示唆される．このように肝癌は，高分化型癌として発生するが，高分化型肝癌細胞の脱分化により，より低分化で悪性度の高い細胞が発生することが肝癌の進展に深く関係する可能性が示唆される[3]（Fig. 2）．

III．肝癌の多中心性発生と
　　 IFNによる発生・再発抑制

　筆者らの施設で実施された切除肝癌の検討では，肝硬変を背景に肝癌を発症した場合，約35％の症例で同時性多中心性発生が認められ，また，2cm以下の肝癌を発症し切除治療を受けた症例で，術後5年までに異時性多中心性発生を認めた症例は約60％に上ることが判明した[5]．多中心性発生は肝癌の1つの特徴であるが，このような患者は，背景病変にC型慢性肝炎や肝硬変を有するものが多い．このような患者にIFNを投与することにより，肝機能の改善や肝発癌率の低下が誘導されることが報告されている[6]-[8]．また，同様に肝癌の切除術後の再発防止に対するIFN投与の有用性も報告されている[9]．しかしながら，このようなIFNの発癌抑制の機序はいまだ十分に解明されていない．IFNには細胞増殖抑制作用があることから，肝癌細胞に対して直接的に作用して，発癌抑制や抗癌作用を示している可能性も考えられる．そこで，我々は，培養肝癌細胞を使用しIFNの肝癌細胞に対する直接的作用の検討を行った．

IV．IFN-α製剤及びIFN-β製剤の
　　 肝癌細胞株に対するin vitroの
　　 増殖抑制作用と増殖抑制機序

　現在我が国において臨床的に使用されているI型IFN製剤には，天然型には，リンパ芽球由来の天然型IFN-α（オーアイエフ®）や線維芽細胞由来の天然型IFN-β（フエロン®）がある．遺伝子組換え型のIFNとしては，IFN-
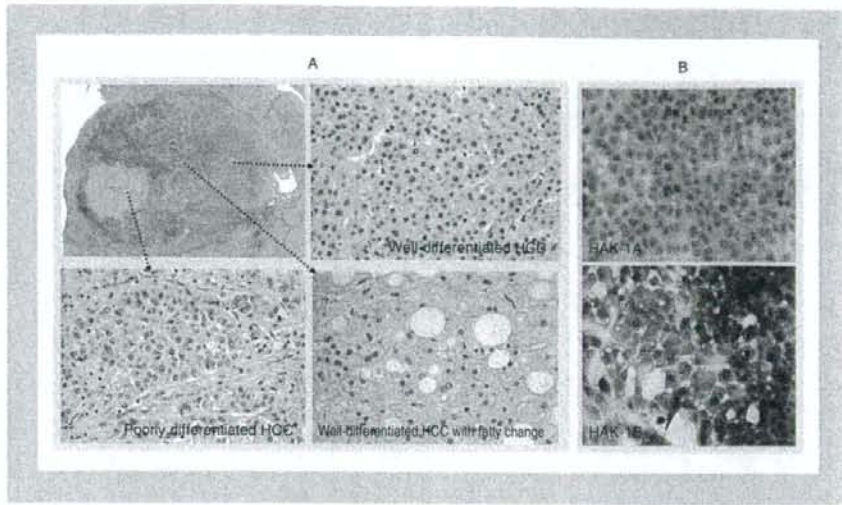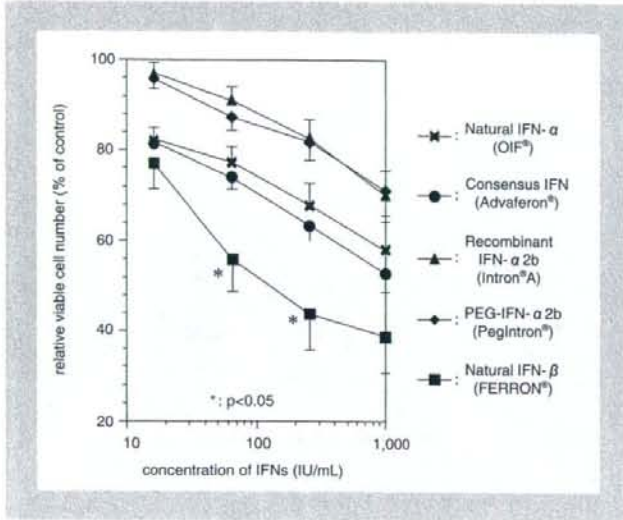
Figure 1

A: upper left, low-power photomicrograph showing the distinct three-layered pattern of the tumor and suggesting the presence of different histological features in each layer (Azan staining); upper right, cells in the outer layer show well-differentiated hepatocellular carcinoma (HCC) (H&E stain, ×100); lower left, cells in the inner layer show poorly differentiated HCC (H&E stain, ×100); lower right, cells in the intermediate layer show well-differentiated HCC with fatty change (H&E stain, ×100).

B: op, HAK-1A cells showing cobble stone-like arrangement of monomorphic cells; bottom, HAK-1B cells showing relatively large pleomorphic cells.
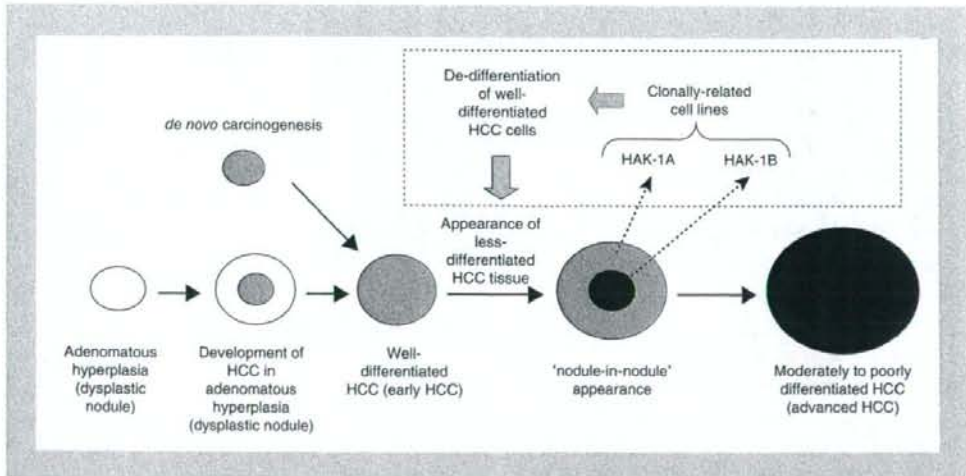
Figure 2

Development and progression of hepatocellular carcinoma (HCC). HCC is well differentiated and vaguely nodular in the early stage, and develops by dedifferentiation in a multistep fashion. Dedifferentiation of well-differentiated HCC cells produces less-differentiated and biologically aggressive HCC cells.

Figure 3

Antiproliferative effects of type I interferon (IFN) preparations, including natural IFN-α, consensus IFN, recombinant IFN-α2b, PEG-IFN-α2b, and natural IFN-β. Thirteen liver cancer cell lines were cultured with or without culture medium containing 10-1,024 IU/mL of one of the 5 type I IFN preparations, and relative viable cell numbers (% of control) were examined. Average of relative viable cell numbers was assessed for each type I preparation and plotted. Antiproliferative effect was strongest in IFN-β, followed by consensus IFN, natural IFN-α, and IFN-α2b or PEG-IFN-α2b.

α2b（イントロン®A）や，特殊なものとして，IFN-α の 13種類のサブタイプ遺伝子のそれぞれのアミノ酸配列について，各位置で最も出現頻度が高いアミノ酸を選択することによりアミノ酸配列を決定し，人工的に作製したコンセンサスIFN（rIFN-α Con1，アドバフェロン®）がある。最近開発されたIFNとしては，遺伝子組換え型 IFN-α にメトキシポリエチレングリコール（PEG）を結合させることにより生物学的半減期を延長させ，少ない投与回数で高い効果が期待可能なPEG-IFN-α2b（ペグイントロン®）やPEG-IFN-α2a（ペガシス®）がある。これらのIFNのなかからPEG-IFN-α2aを除く4種類のIFN-α製剤と，1種類のIFN-β製剤を用いて13種類の肝癌細胞株に対する増殖抑制作用について比較検討を行った。1,024 IU/mLの各種IFN添加培地で96時間培養後に，IFN非添加培養（コントロール）と比べ生細胞数の割合が50%以下まで低下した細胞株の数は，ヒト天然型 IFN-α では5株[10]，コンセンサスIFNでは7株[11]，IFN-α2bとPEG-IFN-α2bではいずれも2株[12]，天然型 IFN-β では10株であった[13]。IFN製剤別の増殖抑制作用を13株の平均値で比較すると，天然型IFN-β，コンセンサスIFN，天然型IFN-α，PEG-IFN-α2b・IFN-α2bの順に強い作用を認めた（**Fig. 3**）。特に，天然型IFN-β では，

経時的に増殖抑制作用が増大し，接触96時間後では，低濃度でも比較的強い増殖抑制効果が見られた。

各種IFNを肝癌細胞の培地に添加し，48～72時間培養し細胞形態を観察すると細胞質の縮小や核の濃縮，核の断片化など，アポトーシスに特徴的な細胞像の出現が認められた。アポトーシス誘導は，使用したIFNの種類，濃度，そして細胞株により差を認めるものの，最低でも 13株中10株で認められた[10]-[13]。IFN-α 誘導性アポトーシスでは，caspase-9，-8，-7，-3の活性化とともに cytochrome cやSmac/DIABLOのミトコンドリアから細胞質への放出が見られ，ミトコンドリア系のアポトーシス誘導経路の関与が示唆されるが[14]，TRAILや TRAIL-R1，-R2などの発現亢進も見られており（未発表データ），デスリガンド-デスレセプターを介した経路の関与も考えられ，今後更なる検討が必要である。アポトーシス誘導以外の増殖抑制の機序としてすべての細胞株で細胞周期の進行停止誘導が認められ，S期での停止誘導が11株，G2/M期での停止誘導が1株，G1期での停止誘導が1株で認められた[10] [12]。

V. IFN-α 製剤及びIFN-β 製剤の肝癌細胞株に対するin vivoの増殖抑制作用と増殖抑制機序

肝細胞癌細胞株HAK-1B[4]をヌードマウスの皮下に接種し、約1週間後5〜10mmの腫瘍径の腫瘍が形成された時点から、各種IFN製剤を投与しin vivoにおける増殖抑制作用の検討を行った。天然型IFN-αは、C型慢性肝炎患者の治療に使用される投与量にほぼ相当する量（臨床量）(4,000 IU/mouse, 2.0×10^5 IU/kg)、その10倍量あるいは100倍量を14日間連日マウスの皮下に接種し、腫瘍の経時的な推定体積や、15日目に摘出された腫瘍の組織像を比較検討した。コンセンサスIFN-αは、臨床量の約1.4倍量 (0.01μg/mouse, 0.5μg/kg)、その10倍量、100倍量を同様に投与し、天然型IFN-βは、臨床量の約半量 (1,000 IU/mouse, 5.0×10^4 IU/kg)、その10倍量、100倍量を腹腔内に投与し同様に検討した。その結果、14日目の腫瘍体積は、最小量のIFN投与によりIFNを投与

しなかったマウス（コントロール）に比べ、天然型IFNで30%前後（未発表データ）、コンセンサスIFN-αで40%前後[11]、天然型IFN-βで15%前後[13]減少した。このように、臨床量前後のIFN製剤の投与は、in vivoにおいて肝癌細胞の増殖を抑制した。IFNを投与されたマウスの腫瘍組織では、IFNの濃度依存性に肝癌細胞のアポトーシス数の増加を認め、コンセンサスIFN-αを投与されたマウスの腫瘍では、腫瘍内血管の減少も認められた。

PEG-IFN-α2bは、前述のごとくPEG化により吸収・排泄速度が低下し、通常のIFNに比べ生物学的半減期が数倍延長する結果、長時間IFN-α2bの血液濃度が維持されるという特徴を有する。臨床量の1/3量 (640 IU/mouse, 3.2×10^4 IU/kg)、その10倍量、100倍量、1,000倍量を1週間に2回、合計4回皮下に投与し腫瘍の経時的な推定体積や、15日目に摘出された腫瘍の重量や組織像を比較した。その結果、臨床量の1/3量の投与でコントロールに比べ約50%前後の腫瘍の体積及び重量の減少が認められた[12] (Fig. 4A)。増殖抑制機序としては、アポトー
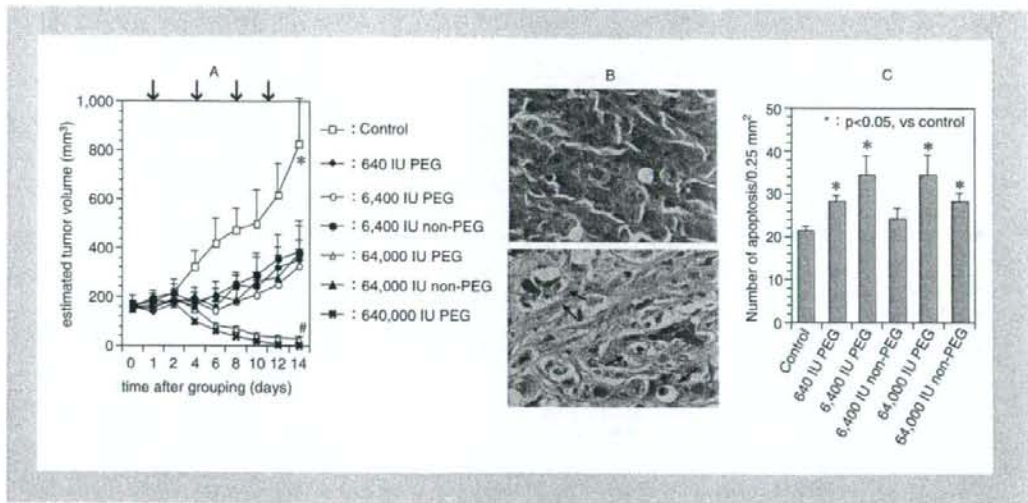

Figure 4

A: Time-course change in estimated tumor volumes of subcutaneously transplanted human hepatocellular carcinoma (HCC) tumors in nude mice. The mice received a subcutaneous injection of 640, 6,400, 64,000, or 640,000 IU of PEG-IFN-α2b, or 6400 or 64,000 IU of IFN-α2b, or medium alone (control). The arrows show the days of injection. * : p<0.001, vs the other groups, # : p<0.0001, vs IFN-α2b (64,000 IU).

B: Photomicrograph of subcutaneous human HCC tumor in nude mice. Top, a control mouse that received culture medium alone; bottom, a mouse that received a subcutaneous injection of 6,400 IU PEG-IFN-α2b, showing some apoptotic tumor cells (arrows).

C: The numbers of apoptotic tumor cells in subcutaneous human HCC tumors in nude mice that received 6,400 IU or 64,000 IU of IFN-α2b, or 640, 6,400, or 64,000 IU of PEG-IFN-α2b, or medium alone (control).

シスの誘導を認めたが，血管新生抑制は確認できなかった（**Fig. 4B**）．PEG-IFN-α2bと同じ活性（IU）のIFN-α2bを同様の方法で投与し，抗腫瘍作用をPEG-IFN-α2bを投与した場合と比較すると，PEG-IFN-α2bを投与した方が，腫瘍のアポトーシス誘導は高度であり，有意により強い抗腫瘍作用を認めた[12]（**Fig. 4C**）．*In vitro* では，PEG-IFN-α2bはIFN-α2bと同程度に増殖抑制効果が最も低いIFN-α製剤であったが，PEG化により長時間血中IFN-α2bの濃度が維持されたことにより，肝癌細胞に持続的に作用し，非PEG化IFN-α2bや他のIFN-α製剤より強い増殖抑制作用を発揮したと推察される．

結　語

肝癌の発生・進展に関して，肝癌の多くは，ウイルス性慢性肝炎・肝硬変を背景に高分化型肝癌として発生するが，腫瘍径の増大とともに増殖活性の高いより低分化な癌組織が高分化型癌細胞の脱分化により発生・増殖し，その結果，進行肝癌は，中・低分化型癌組織のみで占められるようになる．肝癌は，再発や多中心性発生を示す頻度も高いが，IFN製剤の投与はこれらを有意に抑制する．今回紹介したIFN製剤が肝癌細胞に対しアポトーシスなどを誘導し直接的に抗腫瘍作用を発揮するという実験結果は，IFN製剤による肝発癌抑制機序の1つとして，ごく初期のまだ不顕性な段階の肝癌細胞に対する直接的な増殖抑制作用が発癌抑制に寄与している可能性を支持している．

References

1）日本肝癌研究会 編：臨床・病理　原発性肝癌取扱い規約．第4版．東京．金原出版．2000

2）Nakashima O, Sugihara S, Kage M, Kojiro M: Pathomorphologic characteristics of small hepatocellular carcinoma: a special reference to small hepatocellular carcinoma with indistinct margins. Hepatology **22**: 101-105, 1995

3）Kojiro M: Morphologic evolution of hepatocellular carcinoma: from early to advanced. *in* Pathology of Hepatocellular carcinoma. UK, Blackwell Publishing Ltd, 51-61, 2006

4）Yano H, Iemura A, Fukuda K, Mizoguchi A, Haramaki M, Kojiro M: Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. Hepatology **18**: 320-327, 1993

5）Kojiro M: Multicentric occurrence of hepatocellular carcinoma. *in* Pathology of Hepatocellular carcinoma. UK, Blackwell Publishing Ltd, 97-104, 2006

6）Kashiwagi K, Furusyo N, Kubo N, Nakashima H, Nomura H, Kashiwagi S, Hayashi J: A prospective comparison of the effect of interferon-alpha and interferon-beta treatment in patients with chronic hepatitis C on the incidence of hepatocellular carcinoma development. J Infect Chemother **9**: 333-340, 2003

7）Mazzella G, Accogli E, Sottili S, Festi D, Orsini M, Salzetta A, Novelli V, Cipolla A, Fabbri C, Pezzoli A, Roda E: Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. J Hepatol **24**: 141-147, 1996

8）Nishiguchi S, Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, Shiomi S, Seki S, Kobayashi K, Otani S: Randomised trial of effects of interferon-α on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet **346**: 1051-1055, 1995

9）Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, Shiomi S, Tamori A, Oka H, Igawa S, Kuroki T, Kinoshita H: Effects of long-term postoperative interferon-α therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. Ann Intern Med **134**: 963-967, 2001

10）Yano H, Iemura A, Haramaki M, Ogasawara S, Takayama A, Akiba J, Kojiro M: Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines. Hepatology **29**: 1708-1717, 1999

11）Hisaka T, Yano H, Ogasawara S, Momosaki S, Nishida N, Takemoto Y, Kojiro S, Katafuchi Y, Kojiro M: Interferon-α Con1 suppresses proliferation of liver cancer cell lines *in vitro* and *in vivo*. J Hepatol **41**: 782-789, 2004

12）Yano H, Ogasawara S, Momosaki S, Akiba J, Kojiro S, Fukahori S, Ishizaki H, Kuratomi K, Basaki Y, Oie S, Kuwano M, Kojiro M: Growth inhibitory effects of pegylated IFN α-2b on human liver cancer cells *in vitro* and *in vivo*. Liver Int **26**: 964-975, 2006

13）Ogasawara S, Yano H, Momosaki S, Akiba J, Nishida N, Kojiro S, Moriya F, Ishizaki H, Kuratomi K, Kojiro M: Growth inhibitory effects of IFN-β on human liver cancer cells *in vitro* and *in vivo*. J Interferon Cytokine Res **27**: 507-516, 2007

14）Yano H, Ogasawara S, Momosaki S, Akiba J, Nishida N, Kojiro S, Ishizaki H, Kojiro M: Expression and activation of apoptosis-related molecules involved in interferon-α-mediated apoptosis in human liver cancer cells. Int J Oncol **26**: 1645-1652, 2005

Growth inhibitory effects of pegylated IFN-α2b and 5-fluorouracil in combination on renal cell carcinoma cell lines *in vitro* and *in vivo*

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Abstract. We investigated the effects of pegylated IFN-α2b (PEG-IFN-α2b) alone and PEG-IFN-α2b plus 5-fluorouracil (5-FU) *in vitro* on the proliferation of renal cell carcinoma (RCC) cell lines. After the transplantation of RCC cells into nude mice, we administered IFN (PEG-IFN-α2b or IFN-α2b) alone, 5-FU alone, or IFN (PEG-IFN-α2b or IFN-α2b) plus 5-FU; and investigated tumor volume, tumor weight, the numbers of apoptotic cells and artery-like blood vessels, relative mRNA expression levels of enzymes which relate to 5-FU metabolism, angiogenesis factor, and type I interferon receptor. RCC cells *in vitro* were generally and relatively resistant to the anti-proliferative effects of PEG-IFN-α2b, but the addition of 5-FU augmented IFN-induced anti-proliferative effects with the induction of apoptosis. PEG-IFN-α2b *in vivo* presented stronger anti-tumor effects than IFN-α2b, and its combination with 5-FU augmented the effects. The significant anti-tumor effect of the combination treatment was the increase in apoptotic cell number, but there were no significant differences in the suppression of angiogenesis, expression of IFN receptor, and the actions of metabolic enzymes of 5-FU. In conclusion, PEG-IFN-α2b presents stronger anti-tumor effects than non-pegylated IFN, and the effects are augmented in the combination with 5-FU. Our findings suggest the clinical usefulness of PEG-IFN-α2b in the treatment of RCC.

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Introduction

Renal cell carcinoma (RCC) is highly resistant to conventional chemotherapy. The objective response rate is 6-9% for vinblastine and 5-8% for 5-fluorouracil (5-FU) (1). The response rates of treatment regimens using interleukin-2 are 6-31% (2), and the therapeutic response rates of interferon (IFN)-α are 4-33% in patients with metastatic RCC (3). The response rates of immunochemical therapies that utilize chemotherapeutic agents with IFN-α or interleukin-2 range between 8 and 39% (4). Immunochemical therapy is the best treatment for advanced RCC, but potential synergetic effects of the medicines as well as their mechanisms remain to be elucidated.

Wadler and Wienik (5) for the first time proposed a combination therapy of IFN-α and 5-FU in 1988 in their study using colon cancer cell lines. Later, this combination therapy was applied to various types of human malignancies including RCC and hepatocellular carcinoma (HCC). 5-FU has two major anti-tumor mechanisms: one involves its active metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), inhibiting the activity of thymidylate synthase (TS) and consequently DNA synthesis; the other is related to the incorporation of 5-FU metabolite into RNA and DNA, thereby disrupting normal RNA processing and function. The sensitivity of cancer cells to 5-FU is often influenced by the enzymes affecting 5-FU metabolism, including TS, dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), thymidine phosphorylase (TP), uridine phosphorylase (UP) and thymidine kinase (TK).

PEG-IFN-α2b, a new interferon, is a covalent conjugate of recombinant IFN-α2b with monomethoxy polyethylene glycol (PEG) in a 1:1 molar ratio that produces a 31,000-Da molecule (6). PEG conjugation increases the size of the molecule, therefore, the absorption of the pegylated molecule is slower, its serum half-life is longer, and its rate of clearance from the plasma is lower than that of the unmodified molecule. PEG-IFN-α2b thereby increases patient exposure

to IFN-α2b and requires less frequent administration (6). Clinical trials in chronic hepatitis C patients have suggested that PEG-IFN-α preparations produce more potent therapeutic effects than IFN-α preparations (6-10). Yano *et al* (11) examined the *in vitro* and *in vivo* anti-tumor effects of PEG- and non-PEG-IFN-α2b on human liver cancer cells, and they reported that the anti-tumor effect of PEG-IFN-α2b was significantly more potent than that of non-PEG-IFN-α2b. In addition, Motzer *et al* (12) conducted a phase I study of PEG-IFN-α2b on advanced renal cancer patients, and reported that partial response was obtained in 5 (19%) patients. Yet, there have been few basic studies evaluating the efficacy of PEG-IFN-α2b on RCC *in vitro* and *in vivo*.

Our current study examined the *in vitro* and *in vivo* anti-tumor effects of PEG-IFN-αb, IFN-α2b, 5-FU, and the combination of one of the two IFNs and 5-FU, on RCC cell lines, using PEG-IFN-α2b concentrations close to the clinical dosage. We also examined the effects of the therapies on apoptotic cells, artery-like blood vessels, the enzymes affecting 5-FU metabolism, vascular endothelial growth factor (VEGF), and type 1 IFN receptor subunits in human RCC tumors which were developed in nude mice.

Materials and methods

Cell lines and cell culture. This study used 8 human RCC cell lines. KRC/Y (13) was established in our laboratory. KUR11 and KURM were donated by Professor K. Itoh of the Department of Immunology at our University. Caki-1, Caki-2, and ACHN were purchased from American Type Culture Collection. VMRC-RCW was purchased from Japan Health Sciences Foundation. OS-RC-2 was purchased from Riken Cell Bank (Tsukuba, Japan).

Culture medium for KRC/Y consisted of Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with heat-inactivated (56°C, 30 min) 5% fetal bovine serum (FBS, Bioserum, Vic, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL/Life Technologies Inc., Gaithersburg, MD). Culture medium for Caki-1, Caki-2, VMRC-RCW and ACHN consisted of modified Eagle's medium (Gibco); the medium for KUR11, KURM and OS-RC-2 consisted of RPMI-1640; and each medium was supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in an atmosphere of 5% CO_2 in air at 37°C. 5-FU was purchased from Kyowa Hakko K.K. (Tokyo, Japan).

IFN and reagents. PEG-IFN-α2b (PEG Intron®) and IFN-α2b (Intron® A) were provided by Schering-Plough K.K. (Osaka, Japan). The specific activity of PEG-IFN-α2b was 6.4×10^7 IU/mg protein and that of IFN-α2b was 2.6×10^8 IU/mg protein.

Rat antibody against mouse endothelial cells (anti-CD34, clone MEC14.7) was purchased from Serotec Co., Oxford, UK; and mouse monoclonal antibody against human α-smooth muscle actin (SMA) that cross-reacts with mouse α-SMA (clone 1A4), from Immunon (Pittsburgh, PA).

Effects of PEG-IFN-α2b on the proliferation of RCC cell lines in vitro. The effects of PEG-IFN-α2b or 5-FU on cell proliferation were examined in colorimetric assays by using 3-(4,5-dimethylthiazol-2yl-yl-)-2,5-diphenyl tetrazolium bromide (MTT) cell growth assay kits (Chemicon International Inc.) as described elsewhere (14). Briefly, the RCC cells (1.5-5×10^3 cells per well) were seeded on 96-well plates (Nunc Inc., Roskilde, Denmark), cultured for 24 h, and the culture medium was changed to a new medium with or without PEG-IFN-α2b (8, 32, 128, 512 or 2,048 IU/ml). After culturing for 24, 48, 72 or 96 h, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength to 570 nm and the reference wavelength to 630 nm. To keep the optical density within linear range, all experiments were performed when the cells were in the logarithmic growth phase. The effects of IFN-α2b on the growth of VMRC-RCW cells were also examined in the same manner.

Effects of combination therapy of PEG-IFN-α2b and 5-FU on the proliferation of RCC cell lines in vitro. RCC cells (VMRC-RCW, 3,000 cells/well) were seeded on 96-well plates (Nunc Inc.), cultured for 24 h, and then the culture medium was changed to a new medium containing PEG-IFN-α2b alone (0, 160, 317, 625, 1,250, 2,500, 5,000 or 10,000 IU/ml); 5-FU alone (0, 0.6, 1.25, 2.5, 5 or 10 μM); or both 5-FU (0, 0.6, 1.25, 2.5, 5, 10 μM) and PEG-IFN-α2b (0, 160, 317, 625, 1,250, 2,500, 5,000 or 10,000 IU/ml). After 96 h of culture, the number of viable cells was examined by MTT assay as described above.

The synergy of cooperative cytotoxicity was determined by the median-effect principle as described by Chou and Talalay (15). Data from each sample were analyzed by using CalcuSyn ver. 2 (Biosoft, Cambridge, UK).

Morphological observation. For morphological observation by light microscopy, 8 RCC cell lines were seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with or without PEG-IFN-α2b (1,024, 4,098 or 8,192 IU/ml) for 72 h, fixed for 30 min in Carnoy's solution, and stained with hematoxylin and eosin (H&E).

In another experiment, one RCC cell line (VMRC-RCW, 8,000 cells/chamber) was seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with PEG-IFN-α2b alone (0, 160, 317, 625, 1,250, 2,500, 5,000 IU/ml); 5-FU alone (0, 0.6, 1.25, 2.5, 5.0 μM); PEG-IFN-α2b (0, 160, 317, 625, 1,250, 2,500, 5,000 IU/ml) plus 5-FU (0, 0.6, 1.25, 2.5, 5.0 μM), or PBS, for 72 h, fixed for 30 min in Carnoy's solution and H&E stained.

Effects of PEG-IFN-α2b and IFN-α2b on RCC cell proliferation in nude mice. Cultured VMRC-RCW cells (1.0×10^7 cells/mouse) were subcutaneously (s.c.) injected into the backs of 4-week-old female BALB/c athymic nude mice (n=62) (Clea Japan, Inc, Osaka, Japan). One week later when the largest diameter of the tumor reached ~10 mm (day 0), the mice were divided into 7 groups (n=8 or 9 each) in a manner to equalize the mean tumor diameter of each group. Each mouse received a subcutaneous injection of 0.1 ml of medium alone (control group), medium containing 640, 6,400, 64,000 or 640,000 IU of PEG-IFN-α2b, or medium containing 640 or 6,400 IU of IFN-α2b, twice a week for 2 consecutive weeks (on day 1, 4,
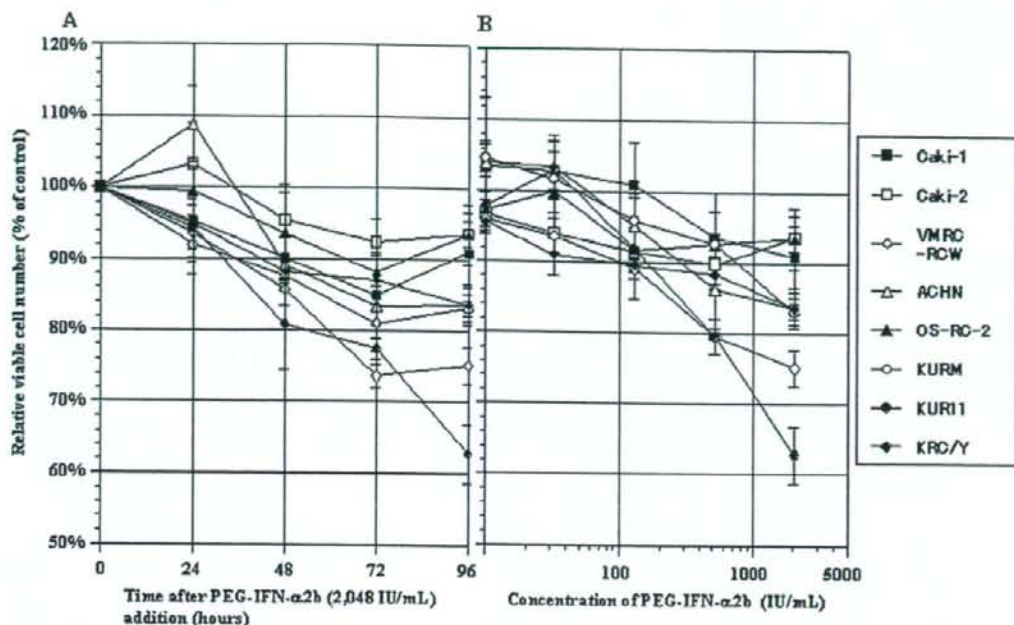
Figure 1. Anti-proliferative effects of PEG-IFN-α2b. (A) Chronological changes in relative viable cell number (% of the control) after adding 2,048 IU/ml of PEG-IFN-α2b. Growth was significantly suppressed over time in 2 cell lines (KUR11 and KRC/Y). (B) Ninety-six hours after adding 8, 32, 128, 512 or 2,048 IU/ml of PEG-IFN-α2b. Cell proliferation was suppressed in a dose-dependent manner in 6 cell lines (all but Caki-2 and OS-RC-2). Eight samples were used in each experiment. The experiment was repeated at least three times for each cell line. The values represent the average ± SE.

8 and 11). The clinical daily dose of IFN-α2b for human RCC is 600x10⁴ IU/body (1.2x10⁵ IU/kg), and this is approximately four times the lowest dose (3.2x10⁴ IU/kg) used in this experiment. Tumor size was measured in two directions by using calipers on the first and second days of s.c. injection (day 1 and 2) and then once every 2 days until day 14, and tumor volume (mm³) was estimated by using the equation: Length x (Width)² x 0.5. Mouse body weight was measured on day 0, 8 and 14. On day 15, all mice were sacrificed and the tumors were removed.

The animals received human care according to criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Effects of combination therapy of PEG-IFN-α2b and 5-FU on RCC cell proliferation in nude mice. VMRC-RCW cells (7.5x10⁶ cells/mouse) were subcutaneously injected into 4-week-old female BALB/c athymic nude mice (n=58). The mice were divided into 7 groups (n=8 or 9 each) on day 7 when tumor size reached ~10 mm in diameter, and each group was assigned to one of the 7 treatments: i) PEG-IFN-α2b alone (6,400 IU); ii) IFN-α2b alone (6,400 IU); iii) low dose 5-FU alone (160 μg); iv) high dose 5-FU alone (320 μg); v) combination therapy of PEG-IFN-α2b (6,400 IU) and low

dose 5-FU; vi) combination therapy of IFN-α2b (6,400 IU) and low dose 5-FU; and (vii) control.

5-FU was administered intra-abdominally every day for 2 consecutive weeks. The dose of 5-FU (160 μg/mouse, 8 mg/kg) is comparable to the clinical dose.

Tumor size measurement and IFN administration were performed in the same manner as described above. On day 15, all mice were sacrificed and each tumor was removed. After the tumor weight was measured, half of the obtained tumors were used for histological examination and the other half were used for quantitative real-time RT-PCR.

The number of cells showing characteristics of apoptosis such as cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation was counted in ten 0.25 mm² areas within an H&E-stained specimen, and the average number per area was obtained. The TUNEL technique (ApopTag® Peroxidase In Situ Apoptosis Detection Kits, Chemicon International, CA, USA) was also used to detect apoptotic cells. The average number of TUNEL-positive cells per area was obtained as described above.

Immunohistochemistry. Double immunohistochemical staining was performed by using anti-mouse endothelial cell (anti-CD34) antibody, anti-human α smooth muscle actin (α-SMA) antibody and histofine simple stain mouse Max-Po (Rat) kits (Nichirei, Tokyo, Japan) as described elsewhere (16). We
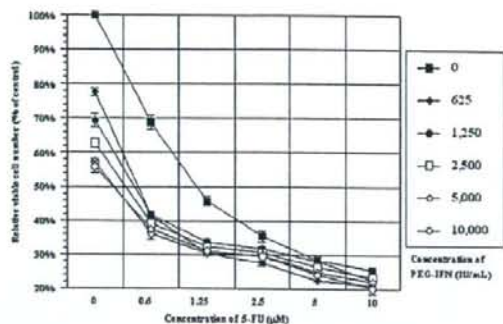
Figure 2. Anti-proliferative effects of the combination therapy of PEG-IFN-α2b and 5-FU on VMRC-RCW cells in a 96-h culture. The relative viable cell number decreased dose-dependently. Two samples were used in each experiment. The experiment was repeated three times. The values represent the average ± SE. PEG-IFN, PEG-IFN-α2b.

calculated the number of artery-like blood vessels in the entire area of each section and obtained for each the mean number per mm^2.

cDNA preparation and quantitative real-time RT-PCR. Total RNA was extracted using RNA-Bee™ (Tel-Test, Inc., TX) and reverse transcribed using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with an ABI PRISM 7300 (Applied Biosystems, Foster City, CA). We examined 6 enzymes related to 5-FU metabolism, i.e., TS, TP, DPD, OPRT, UP and

TK. The sequences of the primers and probes for the 6 enzymes are listed elsewhere (17). The sequences for VEGF were 5'-CCATGAACTTTCTGCTGTCTTGG-3' as the forward primer, 5'-CTGCGCTGATAGACATCCATGA-3' as the reverse primer, and 5'-TGCTCTACCTCCACCATGC CAAGT-3' as the probe. The sequences of the primers and probes for VEGFR-1, IFNAR-1, IFNAR-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems.

Statistical analysis. We used two-factorial ANOVA for the comparisons of tumor volume, tumor weight, number of apoptotic cells, number of artery-like blood vessels, and relative levels of mRNAs related to 5-FU metabolism.

Results

Effects of PEG-IFN-α2b on the proliferation of RCC cell lines in vitro. After adding 2,048 IU/ml of PEG-IFN-α2b, the relative viable cell number of the cultured 8 cell lines was suppressed in a time-dependent manner until 72 h, but at 96 h, suppression was noted in only 2 cell lines (KRC/Y and KUR11). On the other hand, with different doses of PEG-IFN-α2b, the relative viable cell number at 96 h was suppressed in the 6 cell lines, i.e., VMRC-RCW, KRC/Y, KURM, KUR11, ACHN and Caki-1. In the 8 cell lines, IC$_{50}$ was not reached for either time- and dose-dependent suppressions, but the most sensitive case was KUR11 with the dose of 2,048 IU/ml at 96 h, i.e., the relative viable cell number was 62.7% of the control (Fig. 1).

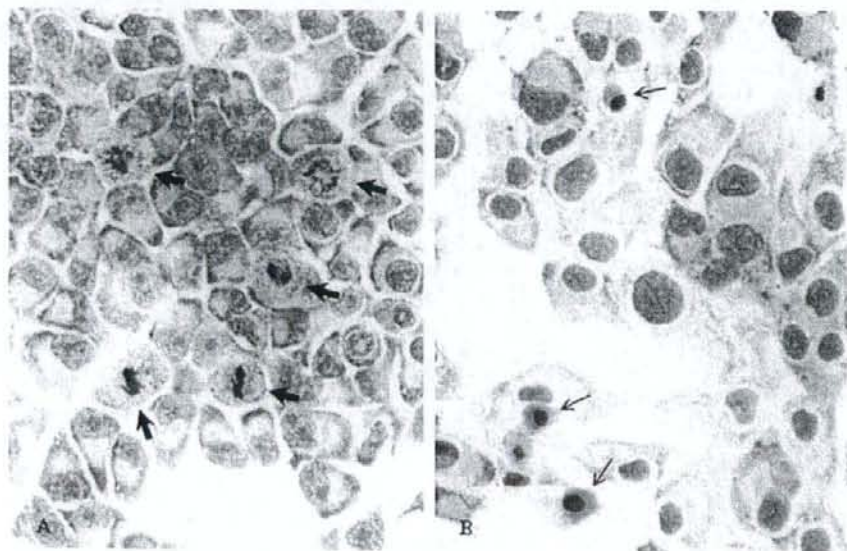In the VMRC-RCW cell line, the anti-tumor effects of PEG-IFN-α2b and IFN-α2b were not markedly different.

Figure 3. Photomicrograph of VMRC-RCW cells cultured for 72 h on a Lab-Tek Chamber Slide. (A) Without PEG-IFN-α2b in culture medium. Mitotic figures (thick arrows) were noted. (B) With 2,500 IU/m of PEG-IFN-α2b and 2.5 μM of 5-FU in culture medium. Apoptotic cells (thin arrows) characterized by cytoplasmic shrinkage and chromatic condensation were noted (H&E staining, x200).

Table I. Effects of PEG-IFN-α2b and IFN-α2b on RCC cell proliferation in nude mice.

Treatment group	Number	Tumor weight (g, mean ± SE)	Body weight (g, mean ± SE on day 15)
Control (culture medium)	9	1.835±0.132	17.122±0.362
IFN-α2b (640 IU)	9	1.735±0.177	16.089±0.599
IFN-α2b (6,400 IU)	9	1.455±0.140	16.667±0.420
PEG-IFN-α2b (640 IU)	9	1.267±0.072ᵃ·ᶜ	16.156±0.308
PEG-IFN-α2b (6,400 IU)	9	1.160±0.075ᵇ	15.244±0.313
PEG-IFN-α2b (64,000 IU)	9	0.920±0.126ᵇ	16.922±0.601
PEG-IFN-α2b (640,000 IU)	8	0.444±0.077ᵇ	17.638±0.717

Cultured VMRC-RCW cells were subcutaneously transplanted in each nude mouse (1.0x10⁷/mouse). Seven days later, when the largest diameter of the tumor reached ~10 mm, mice were treated twice per week with s.c. injection of PEG-IFN-α2b, IFN-α2b, or culture medium. All mice were sacrificed on day 15. ᵃp<0.01 and ᵇp<0.001 vs. control; ᶜp<0.05 vs. the same concentration of IFN.
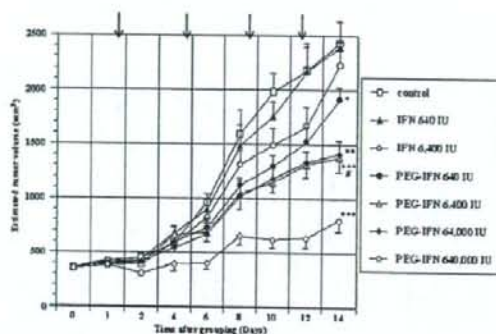

Figure 4. Chronological changes in the estimated volume of subcutaneously transplanted RCC tumors (VMRC-RCW cells, 1.0x10⁷) in nude mice according to the treatment dose. Seven days after the transplantation, when the largest tumor diameter reached ~10 mm (day 0), mice were divided into 7 groups (n=8 or 9, each). The arrows show the days of treatment. *p<0.05, **p<0.01 and ***p<0.001 vs. control; #p<0.01 vs. the same dose of IFN-α2b (6,400 IU). The values represent the average ± SE. PEG-IFN, PEG-IFN-α2b.
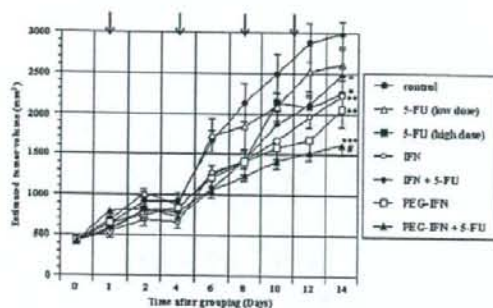

Figure 5. Chronological changes in the estimated volume of subcutaneously transplanted RCC tumors (VMRC-RCW cells, 7.5x10⁶) in nude mice according to the treatment method. Seven days after the transplantation, when the largest tumor diameter reached ~10 mm (day 0), mice were divided into 7 groups (n=8 or 9, each), i.e., PEG-IFN-α2b (6,400 IU) alone; IFN-α2b (6,400 IU) alone; combination of 5-FU and PEG-IFN-α2b (6,400 IU) or IFN-α2b (6,400 IU); 5-FU alone (low or high dose); and culture medium alone (control). The arrows show the days of treatment. *p<0.05, **p<0.01 and ***p<0.001 vs. control; #p<0.001 vs. IFN-α2b and 5-FU. The values represent the average ± SE. PEG-IFN, PEG-IFN-α2b.

Effects of the combination treatment of PEG-IFN-α2b and 5-FU on the growth of the VMRC-RCW cell line in vitro. Without 5-FU, the relative viable cell number did not decrease to 50% or lower of the control even when the highest dose of PEG-IFN-α2b (5,000 IU/ml) was added to the culture. When 5-FU (0.6 μM) was used in combination, the relative viable cell number was suppressed to 41.6% even when PEG-IFN-α2b was at the lowest dose (625 IU/ml, Fig. 2). The anti-proliferative effect of these two agents was additive, not synergistic.

Morphological examination in vitro. The 8 cell lines presented such apoptotic features as cytoplasmic shrinkage and chromatin condensation in a varying degree and in a dose-dependent manner at 72 h after adding PEG-IFN-α2b. For the combination treatment of PEG-IFN-α2b and 5-FU, more apoptotic cells were observed than in the PEG-IFN-α2b

alone-treated cells, and the apoptotic cells increased dose-dependently to PEG-IFN-α2b plus 5-FU (Fig. 3).

Effects of PEG-IFN-α2b on RCC cell proliferation in nude mice. Chronological changes in estimated tumor volume after IFN administration to nude mice are summarized in Fig. 4. Dose-dependent suppression of tumor volume was observed in mice receiving PEG-IFN-α2b. The estimated tumor volume on day 14 in the mice receiving 6,400 IU of PEG-IFN-α2b became 61.9% of the mice receiving the same dose of IFN-α2b (p<0.01) and 56.8% of the control (p<0.001). The tumor weight on day 15 in the mice receiving 6,400 IU of PEG-IFN-α2b became 63.2% of the control (p<0.001, Table I).

Significant differences in the estimated tumor volume were observed between each PEG-IFN-α2b group (640, 6,400,

Table II. Effects of combination therapy of PEG-IFN-α2b and 5-FU on RCC cell proliferation in nude mice.

Treatment group	Number	Tumor weight (g, mean ± SE)	Body weight (g, mean ± SE on day 15)
Control (culture medium)	8	2.255±0.102	17.188±0.578
5-FU (low dose)	9	2.430±0.185	16.778±0.595
5-FU (high dose)	7	1.603±0.107[c]	15.686±0.814
IFN-α2b alone	8	1.812±0.084[b]	16.363±0.692
IFN-α2b + 5-FU	8	1.917±0.170	16.344±0.426
PEG-IFN-α2b	8	1.771±0.172[a]	15.963±0.459
PEG-IFN-α2b + 5-FU	9	1.742±0.194[a]	15.767±0.621

Cultured VMRC-RCW cells were subcutaneously transplanted in each nude mouse ($7.5x10^6$/mouse). Seven days later, when the largest diameter of the tumor reached ~10 mm, mice were treated with s.c. injection of IFNs and/or intraperitoneal injection of 5-fluorouracil (5-FU) daily. All mice were sacrificed on day 15. The concentration of both PEG-IFN-α2b and IFN-α2b was 6,400 IU/ml. [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ vs. control.
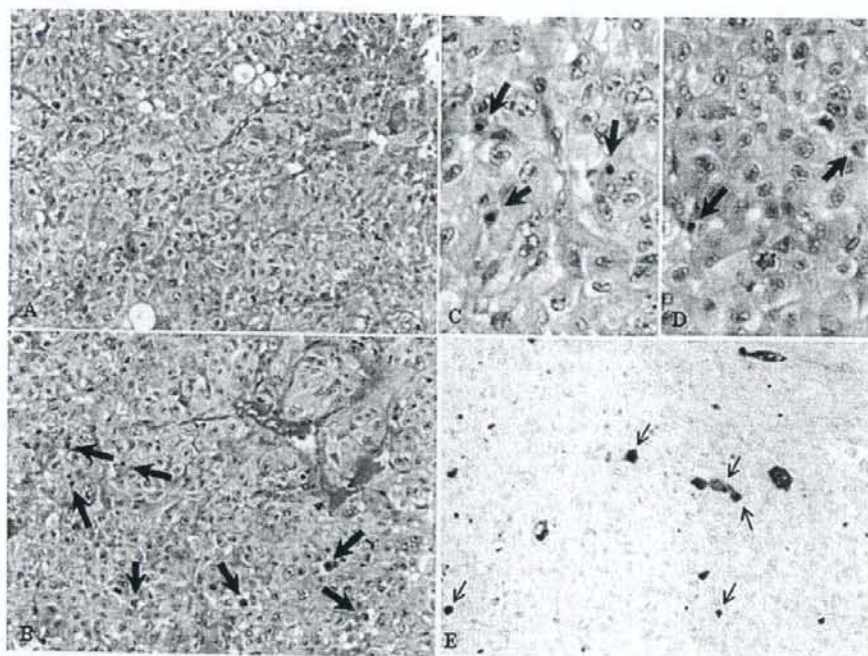

Figure 6. Photomicrograph of a subcutaneous human RCC tumor in nude mice, which developed after the injection of VMRC-RCW cells. (A) A control mouse that received culture medium alone. The tumor showed a thick trabecular arrangement of tumor cells and thin fibrous connective tissues and capillary vessels in the stroma. (B) A mouse that received PEG-IFN-α2b and 5-FU. There were many apoptotic tumor-cells (thick arrows, H&E staining, x200). (C and D) Higher magnifications of B (x400). Apoptotic tumor-cells characterized by shrinkage and eosinophilic change in the cytoplasm and chromatin condensation are shown (thick arrows, H&E staining). (E) TUNEL-positive apoptotic cells showing brown nuclei (thin arrows, TUNEL staining, x200).

64,000, 640,000 IU) and the control ($p<0.05$ to $p<0.001$, Fig. 3). There was no significant difference between 640 or 6,400 IU of the IFN-α2b group and the control. There were no significant differences in body weight of the mice among the groups.

Effects of the combination therapy of PEG-IFN-α2b and 5-FU on RCC cell proliferation in nude mice. Chronological changes in estimated tumor volume are shown in Fig. 5. The tumor volume on day 14 for the combination therapy of PEG-IFN-α2b and 5-FU was 54.2% of the control ($p<0.0001$).

Table III. Relative mRNA expression levels of the enzymes related with 5-FU metabolism, VEGF, VEGFR-1 and type I IFN receptor subunits.

Treatment group	DPD	TP	TK	TS	UP	OPRT	VEGF	VEGFR-1	IFNAR-1	IFNAR-2
5-FU (low dose)	129	101	68	182	59	48	116	135	122	94
5-FU (high dose)	72	50	52	40ᵃ	86	41	30	93	59	90
IFN-α2b	110	71	82	119	100	103	97	134	108	174
IFN-α2b + 5-FU	95	80	85	74	53	106	56	111	63	44ᵃᵈ
PEG-IFN-α2b	648ᵇᵈ	420ᵃ	313	297ᵇᵈ	76	124	366ᵃᵈ	277	159	217
PEG-IFN-α2b + 5-FU	159ᶜ	143ᶜ	162	129	129	86	251ᵃᵉ	138	91	141

mRNA levels were examined by quantitative real-time RT-PCR and normalized with GAPDH. The values of relative mRNA expression level represent the average of the ratio to the level of control in each group. ᵃp<0.05 and ᵇp<0.01 vs. control; ᶜp<0.05 vs. PEG-IFN-α2b; ᵈp<0.05 vs. IFN-α2b; and ᵉp<0.01 vs. IFN-α2b plus 5-FU. DPD, dihydropyrimidine dehydrogenase; TP, thymidine phosphorylase; TK, thymidine kinase; TS, thymidylate synthase; UP, uridine phosphorylase; OPRT, orotate phosphoribosyl transferase; VEGF, vascular endothelial growth factor; VEGFR-1, VEGF receptor 1; IFNAR-1, type I interferon receptor subunit 1; and IFNAR-2, type I interferon receptor subunit 2.
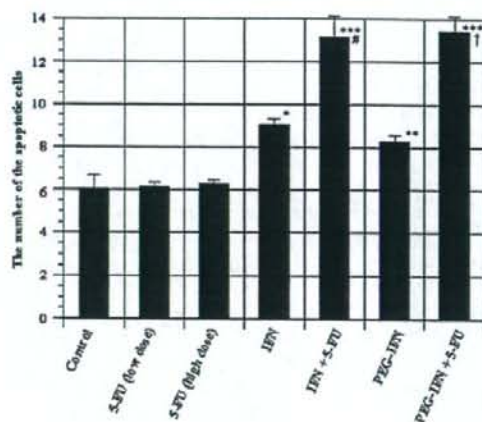

Figure 7. Number of apoptotic cells in the tumors. The number was counted in ten 0.25 mm² areas in each section, and the average number per area in each group was obtained. *p<0.05, **p<0.01 and ***p<0.001 vs. control; #p<0.05 vs. IFN-α2b; †p<0.001 vs PEG-IFN-α2b. PEG-IFN, PEG-IFN-α2b.

The tumor weights of the mice on day 15 were significantly different between the control and the 5-FU high dose group, each IFN alone group, and the combination group of PEG-IFN-α2b and 5-FU. The two types of IFNs and/or 5-FU did not affect the body weight of the mice (Table II).

Histological examination of the RCC tumor specimens stained with H&E revealed that the number of apoptotic cells was significantly higher in the mice treated with 6,400 IU of PEG-IFN-α2b (p<0.01) or 6,400 IU of IFN-α2b (p<0.05) in comparison to the control (Fig. 6A-D). The incidence of apoptosis in TUNEL-stained sections showed the same tendencies as those obtained in the H&E-stained sections (Fig. 6E). The number of apoptotic cells significantly increased in the mouse tumors treated with the combination

therapy in comparison to the control (for each IFN, p<0.0001). The number also significantly increased with the combination treatment of PEG-IFN-α2b and 5-FU in comparison to PEG-IFN-α2b alone (p<0.0001), and with the combination of IFN-α2b and 5-FU in comparison to IFN-α2b alone (p<0.05, Fig. 7).

The results of quantitative real-time RT-PCR are shown in Table III. The VEGF mRNA levels increased significantly in the PEG-IFN-α2b alone group (p<0.05 vs. control, p<0.05 vs. IFN-α2b) and in the combination (PEG-IFN-α2b plus 5-FU) group (p<0.05 vs. control, p<0.01 vs. IFN-α2b plus 5-FU). There were also significant increases in the expression levels of DPD (p<0.01), TP (p<0.05), and TS (p<0.05) in the PEG-IFN-α2b alone group in comparison to the control. On the other hand, significant decreases were observed in the expression levels of DPD (p<0.05) and TP (p<0.05) in the combination (PEG-IFN-α2b plus 5-FU) group in comparison to the PEG-IFN-α2b alone group. In addition, the TS mRNA levels in the PEG-IFN-α2b group increased in comparison to the IFN-α2b group (p<0.05). The relative mRNA levels of IFN-α2b receptors in the combination group were lower than the levels of the IFN alone group.

The number of artery-like blood vessels increased slightly in comparison to the control in the groups receiving IFN-α2b alone, PEG-IFN-α2b alone, or the combination therapies; and there were no significant differences among the 7 groups (Fig. 8).

Discussion

Shang et al (18) examined 5 RCC cell lines and reported that the greatest decrease in the viable cell number after adding 1,600 IU/ml of Sumiferon to the cultures was 42% (58% of the control). On the other hand, Vyas et al (19) comparatively examined the anti-tumor effects of PEG-IFN-α2b and IFN-α2b by using an RCC cell line, ACHN, and reported that the addition of 1,033 IU/ml of PEG-IFN-α2b suppressed the viable cell number to 50% of the control. Our current
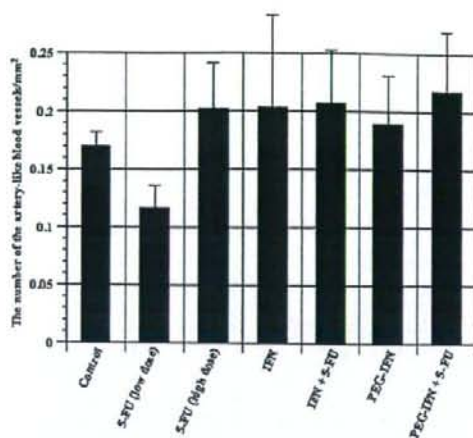
Figure 8. Number of artery-like blood vessels in the tumors. The number was counted in the whole area of each section, and mean number per mm² was obtained. Each figure shows the average ± SE. PEG-IFN: PEG-IFN-α2b.

experiment used concentration levels close to those of Shang *et al*, and similar anti-tumor effects were obtained. However, the level of suppression in our study did not reach 50% of the control even though the concentrations were higher than those of Vyas *et al*. The reasons for these disparate findings are not clear, however, they may be related to the different cell density in the experiments, different measurement methods, and possible changes in cell characteristics due to cultures. Vyas *et al* (19) also reported that the anti-tumor effects of PEG-IFN-α2b and IFN-α2b were not markedly different as we demonstrated in the present study.

Some medical institutions administer the combination therapy of IFN-α and 5-FU in the treatment for advanced RCC. Sella *et al* (20) reported that the combination of IFN-α and chemotherapy (5-FU and mitomycin C) resulted in a significant clinical effect on RCC patients. Our results support the anti-tumor effects reported by Sella *et al* regarding the combination of IFN-α and chemotherapy. Moreover, in the present study, the combination of PEG-IFN-α2b and 5-FU exhibited enhanced anti-tumor effects in comparison to the combination of IFN-α2b and 5-FU *in vivo*.

The induction of apoptosis is a mechanism of the anti-tumor effects of IFN-α2b, and Vyas *et al* (19) reported a dose-dependent increase in apoptotic cell number for PEG-IFN-α2b in cell cultures. Shang *et al* (18) found that apoptosis induction by IFN-α2b was not significant even at a dose of 5,000 IU/ml. On the other hand, 5-FU induced apoptosis in a dose-dependent manner, and 50 and 100 IU/ml of IFN-α2b was able to promote 5-FU-induced apoptosis in RCC cells. In our current study, the number of apoptotic cells *in vitro* increased proportionally to the dose of PEG-IFN-α2b and to the doses of PEG-IFN-α2b plus 5-FU in the combination treatment. The apoptotic cell number in the tumors also increased dose-dependently in the IFN-α2b alone group and PEG-IFN-α2b alone group, and the number in each group further increased with the combination of 5-FU. This indicates that PEG-IFN-

α2b and IFN-α2b induced apoptosis and the combination with 5-FU induced apoptosis more extensively. Comparing the two combination treatments, i.e., PEG-IFN-α2b plus 5-FU vs. IFN-α2b plus 5-FU, the estimated tumor volume was significantly smaller in the PEG-IFN-α2b plus 5-FU group, but the number of apoptotic cells did not differ markedly between the groups. Shang *et al* (18) revealed that IFN-α2b caused cell cycle arrest at G1 in ACHN cells and at G2/M in Caki-1 cells in their flow cytometric analyses, and this suggests that cell cycle arrest could be the reason why there was no remarkable difference in the number of apoptotic cells in our results.

Anti-angiogenesis activity is a biological effect of IFN-α2b, and it has been shown that IFN-α2b inhibits angiogenesis by down-regulating angiogenesis factors. For example, Dinney *et al* (21) systematically administered IFN-α in a nude mouse model of bladder tumor and reported a decrease in *in vivo* blood vessel density in the tumors, which then resulted in the shrinkage of the tumor size. On the other hand, Kojiro *et al* (16) reported that there was no significant relation between the tumor shrinkage effects and angiogenesis factors or artery-like blood vessels when IFN-α and 5-FU were administered in combination to nude mice receiving transplantation of HCC cells. In our current study, the mRNA expression of VEGF and the number of artery-like blood vessels in the tumors were not suppressed in the PEG-IFN-α2b alone group and the PEG-IFN-α2b plus 5-FU group, but the estimated tumor volume of the PEG-IFN-α2b plus 5-FU group was the most suppressed among the groups. The reason for these contrary findings is unclear. Angiogenesis plays an important role in the proliferation and metastasis of solid tumors such as renal cancer, therefore the relation between angiogenesis factors and anti-tumor effects should be investigated in future studies by using different IFN preparations and other RCC cell lines.

It has been reported that IFN directly suppresses tumor proliferation and at the same time augments the suppressive effects of 5-FU on tumor growth, including the induction of apoptosis (15,22). In regards to the mechanism of this augmentation, several researchers reported that IFN-α acts on the metabolic pathway of 5-FU (23,24). Low levels of TS and DPD and high levels of OPRT, TP, UP and TK render cancer cells sensitive to 5-FU. In our results, the enzymes related to 5-FU metabolism, except OPRT, slightly increased (not significantly) in comparison to the control. Therefore, the activity of 5-FU-related enzymes were not related to the anti-tumor effects shown in our PEG-IFN-α2b plus 5-FU group.

IFN-α2b exerts its actions through a specific cell surface receptor, Type I IFN receptor, which consists of two subunits IFNAR-1 and IFNAR-2. IFNAR-2 is the binding subunit and is more important than IFNAR-1 for the expression of IFN-α2b activity (25-27). Oie *et al* (17) examined the expression of type I IFN receptor mRNA in 6 HCC cell lines treated with 5-FU. They showed that the expression of type I IFN receptor was markedly increased in the 3 cell lines whose proliferation was suppressed synergistically by the administration of 5-FU and IFN-α than in the other 3 cell lines whose proliferation was suppressed in an additive manner. In our current study, expression of IFNAR-1 and IFNAR-2 increased in the IFN-α2b alone and PEG-IFN-α2b alone groups, whereas the expression levels were markedly lower

in the combination groups of IFN-α plus 5-FU than in the IFN alone groups. These findings differ from those of Oie *et al* which could be the reason why the effects of our combination treatment were additive and not synergistic.

Our results confirmed that in the treatment of RCC, PEG-IFN-α2b presents more potent anti-tumor effects than conventional non-pegylated IFN-α2b, and the effects are augmented when 5-FU is used in combination. The most probable mechanism of this potent effect is apoptosis induction, and the target molecules that induce apoptosis will be determined in future studies. We expect that the addition of another agent to the combination of IFN-α2b and 5-FU would result in more potent anti-tumor effects in the treatment of RCC.

Acknowledgements

References

1. Hartmann JT and Bokemeyer C: Chemotherapy for renal cell carcinoma. Anticancer Res 19: 1541-1543, 1999.
2. Atzpodien J, Lopez Hanninen E, Kirchner H, Bodenstein H, Pfreundschuh M, Rebmann U, Metzner B, Illiger HJ, Jakse G, Niesel T, *et al*: Multiinstitutional home-therapy trial of recombinant human interleukin-2 and interferon alfa-2 in progressive metastatic renal cell carcinoma. J Clin Oncol 13: 497-501, 1995.
3. Hernberg M, Pyrhonen S and Muhonen T: Regimens with or without interferon-alpha as treatment for metastatic melanoma and renal cell carcinoma: an overview of randomized trials. J Immunother 22: 145-154, 1999.
4. Dutcher JP, Logan T, Gordon M, Sosman J, Weiss G, Margolin K, Plasse T, Mier J, Lotze M, Clark J and Atkins M: Phase II trial of interleukin 2, interferon alpha, and 5-fluorouracil in metastatic renal cell cancer: a cytokine working group study. Clin Cancer Res 6: 3442-3450, 2000.
5. Wadler S and Wiernik PH: Partial reversal of doxorubicin resistance by forskolin and 1, 9-dideoxyforskolin in murine sarcoma S180 variants. Cancer Res 48: 539-543, 1988.
6. Baker DE: Pegylated interferon plus ribavirin for the treatment of chronic hepatitis C. Rev Gastroenterol Disord 3: 93-109, 2003.
7. Reddy KR, Wright TL, Pockros PJ, Shiffman M, Everson G, Reindollar R, Fried MW, Purdum PP III, Jensen D, Smith C, Lee WM, Boyer TD, Lin A, Pedder S and DePamphilis J: Efficacy and safety of pegylated (40-kd) interferon alpha-2a compared with interferon alpha-2a in noncirrhotic patients with chronic hepatitis C. Hepatology 33: 433-438, 2001.
8. Lindsay KL, Trepo C, Heintges T, Shiffman ML, Gordon SC, Hoefs JC, Schiff ER, Goodman ZD, Laughlin M, Yao R and Albrecht JK: A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. Hepatology 34: 395-403, 2001.
9. Lee SD, Yu ML, Cheng PN, Lai MY, Chao YC, Hwang SJ, Chang WY, Chang TT, Hsieh TY, Liu CJ and Chen DS: Comparison of a 6-month course peginterferon alpha-2b plus ribavirin and interferon alpha-2b plus ribavirin in treating Chinese patients with chronic hepatitis C in Taiwan. J Viral Hepat 12: 283-291, 2005.
10. Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Camozzi M, Rebucci C, Di Bona D, Colombo M, Craxi A, Mondelli MU and Pinzello G: Peginterferon alfa-2b plus ribavirin for naive patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol 41: 474-481, 2004.
11. Yano H, Yanai Y, Momosaki S, Ogasawara S, Akiba J, Kojiro S, Moriya F, Fukahori S, Kurimoto M and Kojiro M: Growth inhibitory effects of interferon-alpha subtypes vary according to human liver cancer cell lines. J Gastroenterol Hepatol 21: 1720-1725, 2006.
12. Motzer RJ, Rakhit A, Ginsberg M, Rittweger K, Vuky J, Yu R, Fettner S and Hooftman L: Phase I trial of 40-kd branched pegylated interferon alfa-2a for patients with advanced renal cell carcinoma. J Clin Oncol 19: 1312-1319, 2001.
13. Yano H, Maruiwa M, Sugihara S, Kojiro M, Noda S and Eto K: Establishment and characterization of a new human renal cell carcinoma cell line (KRC/Y). In Vitro Cell Dev Biol 24: 9-16, 1988.
14. Hisaka T, Yano H, Ogasawara S, Momosaki S, Nishida N, Takemoto Y, Kojiro S, Katafuchi Y and Kojiro M: Interferon-alphaCon1 suppresses proliferation of liver cancer cell lines in vitro and in vivo. J Hepatol 41: 782-789, 2004.
15. Chou T-C and Talalay P: Analysis of combined drug effects: a new look at a very old problem. Trends Pharmacol Sci 4: 450-454, 1983.
16. Kojiro S, Yano H, Ogasawara S, Momosaki S, Takemoto Y, Nishida N and Kojiro M: Antiproliferative effects of 5-fluorouracil and interferon-alpha in combination on a hepatocellular carcinoma cell line in vitro and in vivo. J Gastroenterol Hepatol 21: 129-137, 2006.
17. Oie S, Ono M, Yano H, Maruyama Y, Terada T, Yamada Y, Ueno T, Kojiro M, Hirano K and Kuwano M: The up-regulation of type I interferon receptor gene plays a key role in hepatocellular carcinoma cells in the synergistic antiproliferative effect by 5-fluorouracil and interferon-α. Int J Oncol 29: 1469-1478, 2006.
18. Shang D, Ito N, Watanabe J, Awakura Y, Nishiyama H, Kamoto T and Ogawa O: Synergy of interferon-alpha and 5-fluorouracil in human renal cell carcinoma requires p53 activity. Eur Urol 52: 1131-1139, 2007.
19. Vyas K, Brassard DL, DeLorenzo MM, Sun Y, Grace MJ, Borden EC and Leaman DW: Biologic activity of polyethylene glycol 12000-interferon-alpha2b compared with interferon-alpha2b: gene modulatory and antigrowth effects in tumor cells. J Immunother 26: 202-211, 2003.
20. Sella A, Logothetis CJ, Fitz K, Dexeus FH, Amato R, Kilbourn R and Wallace S: Phase II study of interferon-alpha and chemotherapy (5-fluorouracil and mitomycin C) in metastatic renal cell cancer. J Urol 147: 573-577, 1992.
21. Dinney CP, Bielenberg DR, Perrotte P, Reich R, Eve BY, Bucana CD and Fidler IJ: Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. Cancer Res 58: 808-814, 1998.
22. Oka Y, Naomoto Y, Yasuoka Y, Hatano H, Haisa M, Tanaka N and Orita K: Apoptosis in cultured human colon cancer cells induced by combined treatments with 5-fluorouracil, tumor necrosis factor-alpha and interferon-alpha. Jpn J Clin Oncol 27: 231-235, 1997.
23. Kase S, Kubota T, Watanabe M, Teramoto T, Kitajima M and Hoffman RM: Recombinant human interferon alpha-2a increases 5-fluorouracil efficacy by elevating fluorouridine concentration in tumor tissue. Anticancer Res 14: 1155-1159, 1994.
24. Schwartz EL, Hoffman M, O'Connor CJ and Wadler S: Stimulation of 5-fluorouracil metabolic activation by interferon-alpha in human colon carcinoma cells. Biochem Biophys Res Commun 182: 1232-1239, 1992.
25. Pestka S, Langer JA, Zoon KC and Samuel CE: Interferons and their actions. Annu Rev Biochem 56: 727-777, 1987.
26. Lutfalla G, Holland SJ, Cinato E, Monneron D, Reboul J, Rogers NC, Smith JM, Stark GR, Gardiner K, Mogensen KE, *et al*: Mutant U5A cells are complemented by an interferon-alpha beta receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster. EMBO J 14: 5100-5108, 1995.
27. Domanski P, Witte M, Kellum M, Rubinstein M, Hackett R, Pitha P and Colamonici OR: Cloning and expression of a long form of the beta subunit of the interferon alpha beta receptor that is required for signaling. J Biol Chem 270: 21606-21611, 1995.

N-myc downstream regulated gene 1 (NDRG1)/Cap43 enhances portal vein invasion and intrahepatic metastasis in human hepatocellular carcinoma

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Abstract. N-myc downstream regulated gene 1 (NDRG1)/
Cap43 is a 43 kDa protein that is widely distributed in the
body. Its expression is regulated by nickel, cobalt, hypoxic
condition and others; it is reported to be weaker in tumors
than normal tissues; and NDRG1/Cap43 is considered to act
suppressively to tumor metastasis. This current study immuno-
histochemically examined NDRG1/Cap43 expression in
hepatocellular carcinoma (HCC), and analyzed its relationship
to clinicopathologic factors and prognosis. The samples were
105 surgically resected HCC tissue blocks, i.e., 18 well-
differentiated HCC, 61 moderately differentiated HCC, 10
poorly differentiated HCC, 9 'nodule-in-nodule' type HCC,
and 7 sarcomatous HCC. In all cases, NDRG1/Cap43 was not
expressed in normal liver cells. Strong expression was found
in 65 of the 105 cases (62%), i.e., in 11.1% of well-differ-
entiated HCC, 72.1% of moderately differentiated HCC,
80.0% of poorly differentiated HCC, and 71.4% of
sarcomatous HCC. In the 'nodule-in-nodule' type, its
expression was found in 55.6% of their well-differentiated
component, and this frequency was significantly higher than
that in well-differentiated HCC (11.1%). In the cases showing
strong NDRG1/Cap43 expression, frequency of portal vein
invasion and of intrahepatic metastasis was significantly
high. No clear relationship between the expression and
prognosis was observed. NDRG1/Cap43 expression that was
found in advanced HCC was thought to accelerate tumor
invasion and metastasis. NDRG1/Cap43 could act as a useful
biomarker of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth commonest
malignancy worldwide and is the third most common cause of
cancer related death. The geographic areas at highest risk
are located in Eastern Asia, Middle Africa, and some countries
of Western Africa. HCC most commonly develops in patients
with chronic liver disease, the etiology of which includes
alcohol, viral infection (hepatitis B and C), metabolic diseases
and aflatoxin (1,2). Surgery, including transplantation,
remains the only curative modality for HCC. The prognosis of
HCC is generally poor, and even after surgery, the 5-year
survival rate is limited to 25-29% (3). The ability to predict
patients at higher risk of recurrence and with a poor prognosis
would help to guide surgical and chemotherapeutic treatment.
Efforts have been made to predict recurrence and poor
prognosis in patients with HCC after hepatectomy using
clinicopathological parameters. Tumor size, tumor number,
vascular invasion and the presence of satellite lesion were
reported to be useful predictors (4-6). With the development
of molecular biology, many biomarkers related to invasion,
metastasis, recurrence and survival have been explored.

N-myc downstream regulated gene 1 (NDRG1, also known
as Drd-1, Cap43 or RTP) was identified as a homocysteine-
responsive gene that was induced by sulfhydryl regents such as
cysteine and 2-mercaptoethanol in human umbilical vein
endothelial cells (7). The NDRG1/Cap43 gene is mapped to
chromosome 8q24 (8) and encodes a 3.0 kb mRNA that is
translated into a protein with a molecular weight of 43 kDa
(7,9). NDRG1/Cap43 expression is regulated with nickel,
cobalt, oxidative stress, hypoxia, phorbol esters, vitamin A
and D, steroids, histone deacetylase-targeting drugs, lysophos-
phatidylcholine, oncogene, and tumor suppressor gene (p53
and VHL) products (7,8,10-12). NDRG1/Cap43 acts in
maintenance and differentiation (13) of myelin sheath; and in
exocytosis (14), maturation (15) and degranulation (16) of
mast cells. The NDRG1/CAP43 mRNA is widely expressed in
the non-neoplastic tissues and its expression is especially high
in the prostate (17), brain (18), kidney (13,19), placenta and

intestine (1,3,4,7,9,10,14,15,20,21). Sincce NDRG1/Cap43 expression level in neoplastic cells of breast cancer, prostatic cancer and colon cancer is lower in comparison to that in non-neoplastic tissues, NDRG1/Cap43 is reported to be suppressive to tumor metastasis (8,9,17,22-24). On the other hand, NDRG1/Cap43 is also reported to be overexpressed in tumor tissue than normal tissue (25), and to act as an accelerating factor of metastasis (26). At present, actions of NDRG1/Cap43 in tumor remain elusive.

To date, NDRG1/Cap43 expression in the liver (25) and its relationship with hepatocellular carcinoma (HCC) were reported (27,28). Chua *et al* showed high NDRG1/Cap43 expression in HCC correlated with shorter overall survival, late tumor stage, vascular invasion, large tumor size, and high histological grade and NDRG1/Cap43 could be a useful predictor (27). In the current study we investigated NDRG1/Cap43 expression and its histopathological features on surgically resected HCC of humans, and evaluated NDRG1/Cap43 as a possible biomarker of HCC.

Materials and methods

Tissue samples. Immunohistochemical examination was performed on formalin-fixed paraffin sections of cancerous and non-cancerous tissues of 105 HCC livers that were surgically resected at Kurume University Hospital in the period between 1989 and 2007. The 105 patients did not receive preoperative anticancer therapies such as a trans-catheter arterial embolization and radiofrequency are summarized in Table I. Among them, 89 cases had a nodule

Table I. Clinicopathological characteristics of 105 HCC cases.

| Clinicopathological factors	No. of cases (%)
Age (years, mean ± SD)	64.3±8.5
Sex (M/F)	80/25
Tumor size (mm, mean ± SD)	38.2±25.2
Differentiation	
Well-differentiated	18 (17.1)
Moderately differentiated	61 (58.1)
Poorly differentiated	10 (9.5)
HCC with sarcomatous change	7 (6.7)
Nodule-in-nodule appearance	9 (8.6)
Portal vein invasion	46 (43.8)
Venous invasion	7 (6.7)
Bile duct invasion	4 (3.8)
Intrahepatic metastasis	8 (26.7)
Virus marker	
Hepatitis B virus (HBV) associated	10 (9.5)
Hepatitis C virus (HCV) associated	75 (71.4)
HBV and HCV associated	9 (8.6)
HBV and HCV negative	6 (5.7)
Unknown	5 (4.8)
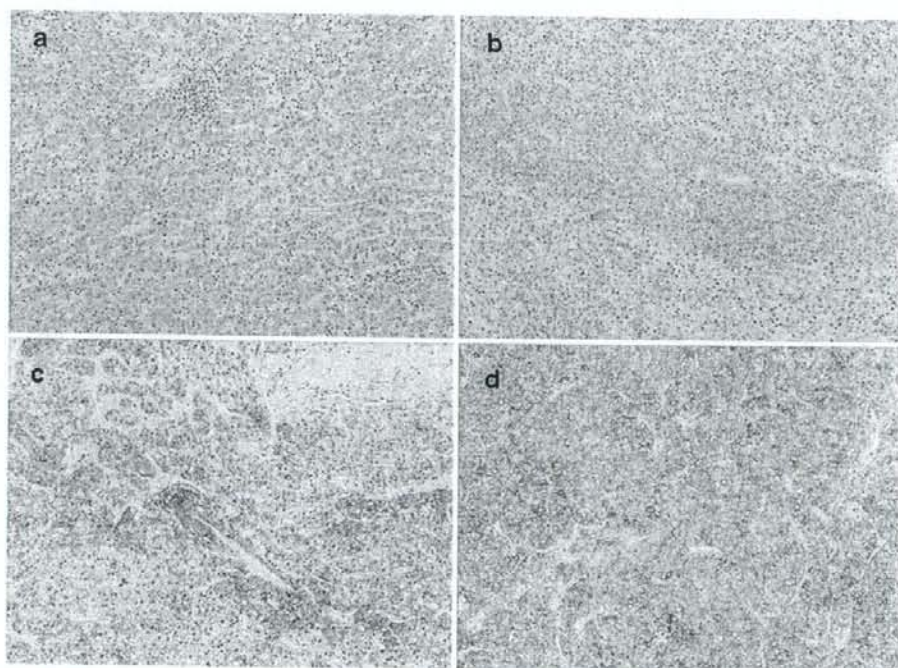
Figure 1. Grading of NDRG1/Cap43 staining distribution. (a) Grade 0: NDRG1/CAP43 positive cells were present <10% of the entire area. (b) Grade +1: the area was 10-40%. (c) Grade +2: the area was 40-70%. (d) Grade +3: the area was 70-100%.