唆した。また、Gankyrin に対するモノクローナル抗体 3A6 を作成した。この抗体を用いて免疫組織化学的に肝癌組織における Gankyrin の発現を検討すると、癌部では非癌部に比して癌細胞の細胞質で Gankyrin の発現が亢進していた。今年度の検討課題は、Gankyrin 陽性肝癌の臨床的特長を明らかにし、cDNA microarray を用いて 1) Gankyrin がヒトの他の臓器の癌で高発現しているか否かを検討し、2) Gankyrin を高発現させることによって高発現する遺伝子を検討することとした。

B. 研究方法

1. 肝癌組織における Gankyrin の免疫組織化学的検討は HCC 臨床検体 43 例を用いて以下のように行った。すなわち、パラフィン切片を脱パラ後 5 分間オートクレーブ処理。内因性ペルオキシダーゼを阻害し血清で処理した後、一次抗体およびビオチン化した二次抗体、さらに、avidin HRP と DAB で発色させ鏡検した。insulin like grouth factor binding protein 5 (IGFBP5) の肝癌組織における発現も同様の方法で行った。

2. 肝移植レシピエントの摘出肝 (肝不全 2 例、肝癌 3 例、自己免疫性肝炎 1 例、原発性胆汁性肝硬変 1 例) を用いて Western blotting を行い、組織中の Gankyrin の発現量を検討した。

3. 241 例のヒト癌組織 (乳癌、大腸癌、胃癌、肺癌など) より抽出した RNA より作成した cDNA を用いた市販の membrane array で Gankyrin の mRNA 発現を半定量し非癌部と比較検討した。プローブは Gankyrin の 0～386bp を用いて作成した。

4. ヒト osteosarcoma の cell line である U2OS 細胞で Gankyrin を過剰発現させ RNA を抽出し cDNA を作成、3 万個のヒト遺伝子を搭載した市販の microarray で増幅した mRNA を検討した。また、その結果を Western blotting で確認した。今回の検討では insulin like grouth factor に関連した遺伝子発現の検索のみを提示する。

本研究においては、協力者に研究の目的、医学的意義、侵襲の程度を十分に説明する。さらに、研究結果の匿名性のみならず、いつでも同意を撤回できること、研究への同意は診療内容には一切関係しないこと、研究終了後は検体を破棄することについても説明し、予め、同意書に署名・捺印を得た後、研究を始めることとしている。

C. 研究結果

1. 43 例の HCC 臨床検体を用いた免疫組織学的検討で Gankyrin は癌

部では非癌部に比べてその発現が亢進していた。また、Gankyrin は主に肝癌細胞の細胞質に強く発現しており、一部の組織では核にも染色されていた。肝癌 43 例のうち Gankyrin は 27 例の細胞質に陽性であり、16 例では陰性であった。Gankyrin 陽性例は TNM 分類の stage□、stage□に属する割合が陰性例に比べて有意に高く
(P=0.0106)、陰性例に比し被膜浸潤
(P=0.0468)、門脈浸潤 (P=0.0398)、肝内転移 (P=0.0236) の頻度が低かった。IGFBP5 の免疫組織化学的検討では Gankyrin 同様、癌部では非癌部に比べてその発現が亢進していた。

2. 肝移植レシピエント（肝不全 2 例、肝癌 3 例、自己免疫性肝炎 1 例、原発性胆汁性肝硬変 1 例）の摘出肝における Gankyrin の発現を Western blotting で検討した。肝不全 2 例における Gankyrin の発現は僅かであったが肝癌組織においては Gankyrin の発現が強かった。また、肝癌症例では癌部以外の非癌部でも Gankyrin の発現が強かった。

3. 241 例のヒト癌組織より作成した市販の membrane array で行った Gankyrin mRNA の検討では乳癌、大腸癌、直腸癌、胃癌、膵癌、肺癌、甲状腺癌、前立腺癌など多くの癌の癌部でその発現が非癌部に比して亢進していた。

4. U2OS 細胞で Gankyrin を過剰発現させ 3 万個のヒト遺伝子を搭載した市販の microarray で増幅した mRNA を検討すると IGFBP5 の発現がコントロールに比して 5.28 倍亢進しており、今回検索した中で最も亢進していた。また、Western blotting でもその結果が確認された。

Gankyrin は肝癌症例 16 例で陰性であったが、その中で IGFBP5 陽性例は 3 のみであった。また、Gankyrin は肝癌症例 27 例で陽性であり、そのうち 15 例では IGFBP5 が陽性であった。

D. 考案

1．今回われわれは Gankyrin 陽性肝癌が TNM 分類の stage□、stage□、すなわち早期の肝癌に有意に多いことを明らかにした。また、Gankyrin 陰性の肝癌では被膜浸潤、門脈浸潤、肝内転移が多く臨床的に予後が悪いと考えられた。すなわち Gankyrin は予後良好な肝癌を含めた悪性腫瘍のマーカーとなる可能性があると考えられ、今後他の腫瘍マーカーとの関連を検討することが望まれる。

2．市販の microarray を用いた検討および Western blotting で Gankyrin は IGFBP5 の産生を亢進させること

が明らかになった。IGFBP5 は癌の悪性転化に関わるとの報告もあり、Gankyrin は IGFBP5 の発現を亢進させ肝癌の進展に関わる可能性が示唆された。

3．共同研究者の藤田らはヒトの肝癌組織において Gankyrin が過剰発現しており、Gankyrin が RB や p53 の不活化に関わることを明らかにしている。今回の検討で Gankyrin の肝癌進展への新たなメカニズムが示唆された。

E．結論

1． Gankyrin は初期の肝癌で高発現していた。

2．Gankyrin は IGFBP5 の発現を亢進し肝癌の進展に関わる可能性が示唆された。

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H． 知的所有権の取得状況

なし

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分担研究報告書

B型およびC型肝炎ウイルス関連肝細胞癌患者血清のプロテオーム解析

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研究要旨：プロテインチップシステムは、蛋白質群を質量分析計で網羅的に測定し、分子量と発現量の情報を迅速に得ることができ、蛋白質ディフェレンシャルディスプレイ分析に有用である。本年度はB型肝炎ウイルス（HBV）とC型肝炎ウイルス（HCV）関連肝細胞癌患者血清中に発現変動する蛋白質を比較検討した。SELDIプロテインチップを用いて分子量1万以下の領域を患者血清を用いて比較したところ、血清中の蛋白質発現パターンが両疾患で異なっており、有意差のある蛋白質ピークを14個検出した。また、そのうちHBV関連肝細胞癌に比べてHCV関連肝細胞癌で高かった一つの蛋白ピークはC3a flagmentであることを明らかにし、C3aのピーク値はALT、腫瘍径、AFP、PIVKA-IIと明らかな相関は無いものの、治療後に低下する傾向であった。以上のことから、肝炎ウイルス関連慢性肝疾患患者血清のプロテオーム解析は肝細胞癌診断に有用であるだけでなく、病態進展に関わる分子を同定できる可能性があると思われた。

A. 研究目的

本年度の目的は、B 型肝炎ウイルス（HBV）およびC 型肝炎ウイルス(HCV)関連肝細胞癌患者の血清を用いてプロテオーム解析を行い、肝細胞癌の診断や病態進展に関与する分子を同定することである。

B. 研究方法

1)、HBV 陽性肝細胞癌(HBV-HCC) 25 例、HCV 陽性肝細胞癌(HCV-HCC)45 例、健常者 21 例を対象としての血清を比較検討した。

2) SELDI プロテインチップシステムを用いて解析した。測定範囲は分子量 1 万以下の蛋白質・ペプチド領域とした。

3) 発現に有意差のあったピーク蛋白のひとつは、精製後ゲル泳動し、蛋白断片を Mascot Search で検討した。候補蛋白を Western blot 法、および SELDI immunoaffinity assay で検証した。

4)肝細胞癌患者 70 例の ALT、腫瘍径、AFP、PIVKA-II と同定した蛋白ピーク値の関連を検討した。

5) 肝細胞癌発症前後の保存血清 10 例 36 サンプルを使用し、治療前後における蛋白ピーク値を検討した。

倫理面の配慮

a. 個人の人権の擁護：1) 研究内容について充分な説明を行い、研究への参加は任意であること、研究に参加しない場合でも、従来通り診療を受けることができることを示す。2) 参加者のデータは、厳重な秘密保持のもとに管理され、本研究のデータが参加者に不利益を及ぼすことはないと考えられる。

b. 個人情報の管理：1)ID 番号、氏名、住所、電話番号などの個人を特定できる情報を除いたものを作製し、新たな番号を付与し、本研究にはこの番号のみを用い、個人が特定できる名前などを用いない。2)対象者由来の血液サンプルは個人が同定できる情報を消去して、番号を付与する。

c. 対象者に理解を求め同意を得る方法：担当医より、研究内容について説明を行ない、書面による同意を得る。

d. 研究等によって生じる個人への不利益：超音波検査や静脈穿刺は侵襲性のあるものではなく、被験者に不当な危険が生じることはない。個人のプライバシーに関わる点については上記のように十分な配慮を行い、対象者の不利益が生じないようにする。

C. 研究結果

1) HBV-HCC と HCV-HCC を比較したところ、血清中の蛋白質発現パターンが異なっており、有意差のある蛋白質ピークを 14 個検出した。

2) HBV-HCC よりも HCV-HCC で血清中の発現ピーク値が有意に高値であった分子量約 8100 のピーク

蛋白を Complement component 3a (C3a) fragment と同定した。
3) HCV、HBV いずれにおいても C3a fragment のピーク値はALT、腫瘍径、AFP、PIVKA-IIと明らかな相関は無かった。
4) C3a fragment のピーク値は肝細胞癌が明らかになる前から徐々に高値を示し、治療後に低下する傾向であった。

D.考察

本研究ではHBV関連肝細胞癌とHCV関連肝細胞癌で発現に差が見られた約 8100 Dalton の蛋白がC3a fragment であることを明らかにした。このピーク蛋白は、HCV関連肝細胞癌で健常者よりも発現が亢進している蛋白として、すでに我々が候補として挙げていた蛋白であり、各種検討によりこの蛋白が C3a flagment であることを確認した。また、本年度の検討では HBV 関連肝細胞癌患者と比較しても有意に発現が上昇していることを明らかにした。すでに、SELDI プロテインチップシステムを用いて、C3a が肝細胞癌のシングルマーカーであることが報告されているが、我々の報告した C3a と分子量が異なり、また、C3a が大腸癌のマーカーである可能性も報告されている。用いたプロテインチップや患者背景が異なることがこのような差異の原因のひとつと考えられるが、さらに検討が必要である。

E.結論

プロテオーム解析による肝炎ウイルス関連慢性肝疾患患者血清の網羅的タンパク質発現解析は肝細胞癌診断に有用であるだけでなく、病態進展に関わる分子を同定でき、新しい分子標的治療法の開発につながる可能性があると思われた。

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G.知的財産権の出願・登録状況(予定を含む。)

なし

Ⅲ.研究成果の刊行に関する一覧表

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30)T. Takehara, (林)	Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication	J Hepatol	44	267-274	2006
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32)T. Yakushijin (林)	Reduced expression and functional impairment of Toll-like receptor 2 on dendritic cells in chronic hepatitis C virus infection	Hepatol Res	34	156-162	2006
33)M. Inoue, (林)	Enhanced ability of peripheral invariant natural killer T cells to produce IL-13 in chronic hepatitis C virus infection.	J Hepatol	45	190-196	2006

34)N. Hiramatsu, (林)	Should aged patients with chronic hepatitis C be treated with interferon and ribavirin combination therapy?	Hepatol Res	35	185-189	2006
35)N. Hayashi, (林)	Antiviral therapy for chronic hepatitis C: past, present, and future	J Gastroenterol	41	17-27	2006
36)A. Hosui, (林)	Suppressive effect on hepatocyte differentiation of hepatitis C virus core protein	Biochem Biophys Res Commun	346	1125-1130	2006
37)T. Oze, (林)	Early decline of hemoglobin correlates with progression of ribavirin-induced hemolytic anemia during interferon plus ribavirin combination therapy in patients with chronic hepatitis C	J Gastroenterol	41	862-872	2006
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41)T. Tatsumi, (林)	Intrahepatic Delivery of α-Galactosylceramide-Pulsed Dendritic Cells suppresses liver tumor	Hepatology	45	22-30	2007
42)T. Shimakami (金子)	Effect of Hepatitis C Virus (HCV)NS5B-Nucleolin Interaction on HCV Replication with HCV Subgenomic Replicon	Journal of Virology	80	3332-3340	2006

43)E. Mizukoshi (金子)	Cytotoxic T Cell Responses to Human Telomerase Reverse Transcriptase in Patients With Hepatocellular Carcinoma	HEPATOLOGY	43	1284-1294	2006
44)M. Honda, (金子)	Different Signaling Pathways in the Liver of Patients With Chronic Hepatitis B or Chronic Hepatitis C	HEPATOLOGY	44	1122-1138	2006
45)T. Nishikawa (伊藤)	A green tea polyphenol, epigalocatechin-3-gallate, induces apotosis of human hepatocellular carcinoma, possibly throughinhibition of Bcl-2 family proteins	J Hepatol	44	1074-1082	2006
46)K. Yamaguchi (伊藤)	In vivo selection of transduced hematopoietic stem cells and little evidence of their conversion into hepatocytes in vivo	J Hepatol	45	681-687	2006
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48)K. Kusumoto, (坪内)	Interleukin-10 or tumor necrosis factor-alpha polymorphisms and the natural course of hepatitis C virus infection in a hyperendemic area of Japan	Cytokine	34	24-31	2006

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50) H. Uto, (坪内)	Spontaneous elimination of hepatitis C virus RNA in individuals with persistent infection in a hyperendemic area of Japan	Hepatol Res	34	28-34	2006

Ⅳ.研究成果の刊行物・別刷

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Clinical Studies

Zinc is a negative regulator of hepatitis C virus RNA replication

Yuasa K, Naganuma A, Sato K, Ikeda M, Kato N, Takagi H, Mori M. Zinc is a negative regulator of hepatitis C virus RNA replication.
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Abstract: *Background/Aims:* Hepatitis C virus (HCV) infection is a significant global public health problem. In clinical studies, zinc has been closely related to the pathogenesis of chronic hepatitis C. However, the role of zinc in both viral replication and the expression of viral proteins remains unclear. We aimed to clarify the effect of zinc on the replication of HCV *in vitro*. *Methods:* We incubated subgenomic HCV replicon cells (sO) and genome-length HCV RNA-replicating cells (O) treated with several chemicals including trace elements. Total RNAs were collected and subjected to real-time reverse-transcriptase polymerase chain reaction in order to examine the level of HCV RNA replication, and Western blotting was performed to confirm the expression of viral proteins. *Results:* Iron salts and interferon-α suppressed HCV RNA replication and protein expression in both sO and O cells. Zinc salts effectively reduced the viral replication in the genome-length HCV RNA replication system but not in the subgenomic HCV replicon system. *Conclusions:* We demonstrated that zinc may play an important role as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells. Zinc supplementation thus appears to offer a novel approach to the development of future strategies for the treatment of intractable chronic hepatitis C.

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Hepatitis C virus (HCV) infection is a significant global public health problem. Persistent HCV infection eventually develops into liver cirrhosis or hepatocellular carcinoma (1). A sustained viral response (SVR) to anti-HCV therapy has been demonstrated to prevent the progression of liver disease and even to promote the regression of pathologic changes (2). Peginterferon plus oral ribavirin, currently the most powerful therapy for chronic hepatitis C, has successfully induced SVR in about half of treated patients of genotype 1b with high viral load (3, 4). However, there are still a number of non-responders to interferon (IFN)-based therapy. As a result, the treatment efficacy still needs to be improved.

HCV is a positive-polarity, single-stranded RNA virus, a member of the *Hepacivirus* genus of the Flaviviridae family (5). The HCV genome consists of an ~9.6 kb RNA molecule containing a large open reading frame flanked by structured 5′- and 3′-non-translated regions (NTR). Located within the 5′-NTR is an internal ribosome entry site (IRES) directing the translation of an approximately 3000-amino-acid polyprotein that is co- and posttranslationally cleaved by cellular and viral proteases into the following 10 products (listed from the N to the C termini): core, envelope protein 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. The NS2–NS3 cleavage is performed by NS2, and the remaining processing of the NS3–NS4A–NS4B–NS5A–NS5B fragment depends on the NS3/NS4A protease, which is similar to chymotrypsin-like serine protease (6).

Zinc is an essential nutrient for a broad range of biological activities and for cell proliferation (7) and it also functions as an antioxidant (8). It also plays an important role in the function and maintenance of the crystal structures of such HCV proteins as NS2–NS3 (9–11) and NS5A (12, 13). The virus-encoded NS2–NS3 protease that is responsible for autocatalytic cleavage at the NS2–NS3 site is stimulated by $ZnCl_2$ (9, 10). The NS3 protease domain contains a zinc atom (11). These observations have led researchers to propose that zinc plays an important role in the NS2–NS3 protease activity. Several studies have examined the direct inhibitory effects of zinc on viruses, such as human immunodeficiency virus (14), rhinovirus (15), herpes simplex virus (16),

and respiratory syncytial virus (17) *in vitro*. However, the direct effect of zinc on the replication of HCV *in vitro* has never been previously reported.

Despite the clinical significance of HCV, molecular investigations of the virus have been hampered due to the lack of cell culture systems that efficiently support HCV replication, although a reproducible HCV proliferation system in cell culture has very recently been reported (18). In 1999, the situation changed for the better when a subgenomic HCV replicon cell culture system was introduced (19). The replicon RNA is composed of the HCV 5′-NTR containing an HCV IRES, a neomycin phosphotransferase (Neo) gene, and the HCV NS3 through NS5B under the control of an encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3′-NTR. The Neo gene is expressed under the control of the HCV IRES, thereby inducing G-418 resistance to cells that contain replicon RNA. As the replicon RNA proliferates autonomously in cultured cells, this replicon system is thus considered to be a powerful tool for the analysis of molecular mechanisms underlying HCV replication and also for the screening of anti-HCV reagents (20). However, the subgenomic HCV replicon system may be insufficient because it lacks HCV structural proteins. A genome-length HCV RNA replication system may reflect the phenomenon that the HCV-infected human liver undergoes. To date, four genome-length HCV RNA replication systems, using N, Con-1, H77, and O strains, have so far been reported (21–24).

Clinical data suggest that the trace element metabolism is tightly linked to the pathogenesis of chronic hepatitis C (25, 26). We previously showed zinc supplementation to increase the therapeutic response of IFN-α for intractable chronic hepatitis C with genotype 1b (27, 28). However, it remains unclear as to whether or not zinc interferes with viral replication or the expression of viral proteins. We therefore examined the effect of zinc supplement on viral replication using HuH-7 cells harboring subgenomic HCV replicons (29) or genome-length HCV RNAs (24) derived from the HCV-O strain. We herein showed that zinc effectively suppressed the replication of genome-length HCV RNA but not that of the subgenomic HCV replicon.

Materials and methods

Cell culture systems

We incubated sO (previously described as 1B-2R1) cells (29) replicating the subgenomic HCV replicon and O cells (24) replicating the genome-length HCV RNA in a real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. The sequences of HCV RNA replicating in sO and O cells are derived from HCV RNA in non-neoplastic human hepatocytes PH5CH8 inoculated with HCV-O, and the basal replication levels of both O and sO cells were almost the same as those described previously (24, 29). In a luciferase reporter assay system, we incubated ORN/3-5B/KE cells supporting the subgenomic HCV replicon encoding the luciferase reporter gene, and ORN/C-5B/KE cells supporting genome-length HCV-RNA encoding the luciferase reporter gene (24). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM) and maintained in the presence of G418 (300 μg/ml; Geneticin, Invitrogen). We passaged these cells twice a week at a 5:1 split ratio and used them within six to 10 passages for the experiments in this study.

Reagents

Iron sulfate ($FeSO_4$), iron chloride ($FeCl_3$), zinc sulfate ($ZnSO_4$), and zinc chloride ($ZnCl_2$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The purities of both reagents exceeded 99%. Purified human lymphoblast IFN-α (OIF) was kindly provided by the Otsuka Pharmaceutical Co. (Tokushima, Japan).

Cell viability

As it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth (30), we examined the cytotoxicities of $ZnSO_4$ and $ZnCl_2$ to sO or O cells. In brief, the cells were seeded at a density of 4×10^5 cells per dish onto dishes with a diameter of 95 mm. After a 24-h culture, the cells were treated with or without zinc salts at final concentrations of 50, 100, and 150 μM for 72 h in the absence of G418. Next, the number of viable cells was counted using an improved Neubauer-type hematocytometer after trypan blue dye (Invitrogen) treatment. The effect of zinc salts was calculated as a percentage of the number of control cells to which no reagent was added. All assays were conducted more than three times.

Quantification of HCV RNA by real-time RT-PCR

The subgenomic HCV replicon (29) and replicable genome-length HCV RNA (24) are both well known to be highly sensitive to IFN-α and

recently iron has been reported to suppress the subgenomic HCV replicon (31). To confirm that our subgenomic HCV replicon and genome-length HCV RNA replication system are useful for evaluating antiviral reagents, we examined the established inhibitory effects of IFN-α and iron on the replication of the subgenomic HCV replicon and genome-length HCV RNA using sO and O cells. Next, the effect of zinc salts on the replication of subgenomic HCV replicon and the genome-length HCV RNA was observed by real-time RT-PCR. In brief, sO or O cells seeded on six-well plates (1×10^5 cells per well) were treated with IFN-α, $FeSO_4$, $FeCl_3$, $ZnSO_4$, or $ZnCl_2$ at several concentrations. The total RNAs from cells were harvested at different time points using ISOGEN extraction kits (Nippon Gene Co., Tokyo, Japan) and subjected to a real-time RT-PCR analysis. The 5′-NTR of HCV genomic RNA was quantified using the ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA) as described previously (32), using the 5′-CGGGAG-AGCCATAGTGG-3′ (forward) and 5′-AGTACCACAAGGCCTTT CG-3′ (reverse) primers and the fluorogenic probe 5′-CTGCG-GAACCGGTGAGTACAC-3′. As an internal control, the level of human GAPDH mRNA was quantified using TaqMan hGAPDH reagents (Applied Biosystems). All experiments were conducted more than three times.

Western blot analysis

The cell lysates and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared, and an immunoblotting analysis with a polyvinylidene difluoride membrane was performed as described previously (33). The antibodies used in this study were those against NS3 (Novocastra Laboratories, Newcastle, UK) and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science). Anti-β-actin antibody (Sigma-Aldrich, Tokyo, Japan) was also used to detect β-actin as the internal control. The immunocomplexes on the membranes were detected by an enhanced chemiluminescence assay (Amersham Co., Tokyo, Japan). Image scanning was analyzed using the Scion Image software program (Beta 4.0.2., Scion Corporation, NIH, Frederick, MD).

Luciferase reporter assay

To confirm the effect of zinc salts on the replication of HCV RNA by the different assay system, we performed the experiment while utilizing the luciferase reporter assay system using ORN/3-5B/KE cells and ORN/C-5B/KE cells with or without zinc salt. In brief, the cells were plated onto 24-well plates (1.5×10^4 cells per well) and cultured for 24 h. Next, the cells were treated with $ZnSO_4$ or $ZnCl_2$ at several concentrations for 24 h, and then the cells were subjected to the luciferase reporter assay using the *Renilla* luciferase assay system (Promega, Madison, MI) (24). Briefly, after removing the medium, the cells were washed twice with phosphate-buffered saline. The cells were extracted with 100 µl of *Renilla* lysis reagent, and the relative luciferase unit value in 10 µl of lysates was measured by adding 50 µl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. Flash'n Glow LB 955 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect the luciferase activity.

Statistical analysis

All data were expressed as the mean ± standard deviation. The differences between groups were evaluated with Student's *t*-test or one-way analysis of variance $P<0.01$ was considered to be significant.

Results

Inhibitory effects of IFN-α on HCV RNA replication in sO and O cells

IFN-α efficiently inhibited the replication of the subgenomic HCV replicon and genome-length HCV RNA in a dose-dependent manner (Fig. 1). Based on the dose–response curve, the

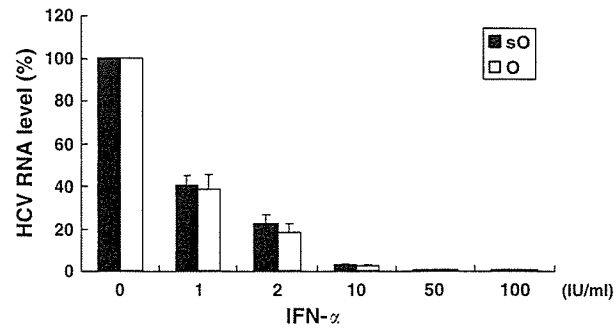

Fig. 1. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with interferon-α (IFN-α). IFN-α sensitivity of HCV RNA replication in sO (black bars) and O cells (white bars). Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials and methods. sO and O cells were treated for 48 h with IFN-α (0, 1, 2, 10, 50, and 100 IU/ml). The replication level of HCV RNA of the respective non-treated cells was assigned as 100%. The replication level of HCV RNA was normalized to the respective GAPDH mRNA expression levels. The data indicate the mean ± SD of triplicates from three independent experiments.

concentrations of IFN-α required for a 50% reduction (IC_{50}) of the subgenomic HCV replicon and genome-length HCV RNA were calculated to be almost equal (0.7 IU/ml). These values were comparable to the previous findings obtained using another HCV-strain-derived subgenomic HCV replicon system (34) or an O-strain-derived HCV RNA replication system (35).

Inhibitory effects of iron salts on HCV RNA replication in sO and O cells

$FeSO_4$ or $FeCl_2$ significantly suppressed the replication of genome-length HCV RNA to the same extent as the subgenomic HCV replicon in a dose-dependent manner (Fig. 2). We demonstrated for the first time the inhibitory effect of iron via a genome-length HCV replication system. Both IFN-α and iron salts inhibited HCV RNA replication in sO and O cells in a dose-dependent manner, thus suggesting that our subgenomic HCV replicon and genome-length HCV RNA replication systems are useful for the evaluation of anti-HCV reagents.

Cytotoxicity of zinc salts to sO and O cells

Although 150 μM and higher of $ZnSO_4$ or $ZnCl_2$ was cytotoxic to sO and O cells, $ZnSO_4$ or $ZnCl_2$ at a concentration of 100 μM or lower had no significant cytotoxic effect on both cells in this assay (Fig. 3A and B). We therefore examined the inhibitory effects of zinc salts at a concentration of 100 μM or lower.

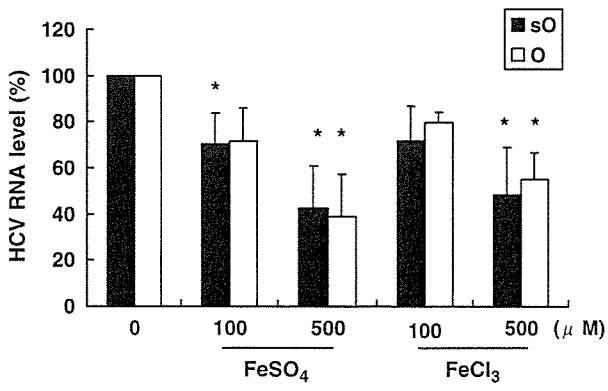

Fig. 2. Inhibition of hepatitis C virus (HCV) RNA replication in sO and O cells treated with iron. Iron inhibition of HCV RNA replication in sO (black bars) and O cells (white bars). sO and O cells were treated for 48 h with iron sulfate (100 and 500 μM) or iron chloride (100 and 500 μM). The control cells without iron salts (0 μM) were treated similarly. The quantification of HCV RNA was performed as described in Fig. 1. The data indicate the mean ± SD of triplicates from three independent experiments. The asterisk (*) indicates a significant inhibition of HCV RNA replication by iron sulfate or iron chloride ($P<0.01$).

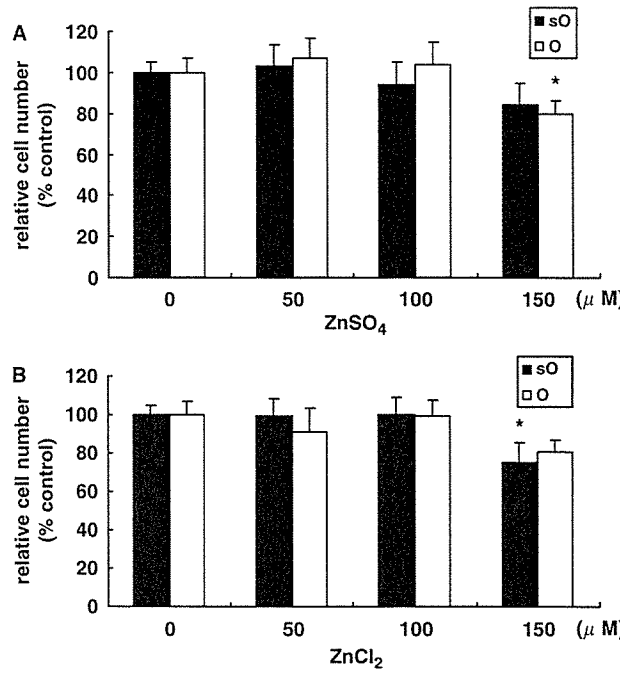

Fig. 3. Cytotoxicity of zinc salts to sO and O cells. (A) sO and O cells were cultured in the absence or presence of zinc sulfate (50, 100, and 150 μM each) for 72 h, and then the cell number was determined as described under the Materials and methods. The relative cell number (% control) calculated at each point, when the cell number of non-treated cells was assigned to be 100%, is presented herein. The data indicate the mean ± SD of three independent experiments. (B) sO and O cells were cultured in the absence or presence of zinc chloride as described in (A). The asterisk (*) indicates significant cytotoxicity by zinc sulfate or zinc chloride ($P<0.01$).

Different effects of zinc salts on the HCV RNA replication between sO and O cells

$ZnSO_4$ or $ZnCl_2$ significantly suppressed the genome-length HCV RNA replication in a dose-dependent manner. The IC_{50} values of $ZnSO_4$ and $ZnCl_2$ were calculated to be 76 and 89 μM, respectively. In contrast, only slight inhibitory effects on the subgenomic HCV replicon were observed in sO cells by 100 μM $ZnSO_4$ and $ZnCl_2$ (Fig. 4A and B). Zinc salts reduced the replication of the genome-length HCV RNA more markedly than that of the subgenomic HCV replicon. To determine whether the inhibitory effect of zinc on the genome-length HCV RNA replication is time dependent or not, O cells were incubated with 100 μM $ZnSO_4$ or $ZnCl_2$ and harvested at three different time points (24, 48, and 72 h) after treatment. The maximum inhibitory effect of zinc salts in O cells occurred at 48 h after treatment (Fig. 4C).

Effects of zinc salts on NS3 and NS5B protein expression

The expression levels of NS3 and NS5B proteins, which are the essential proteins for HCV RNA

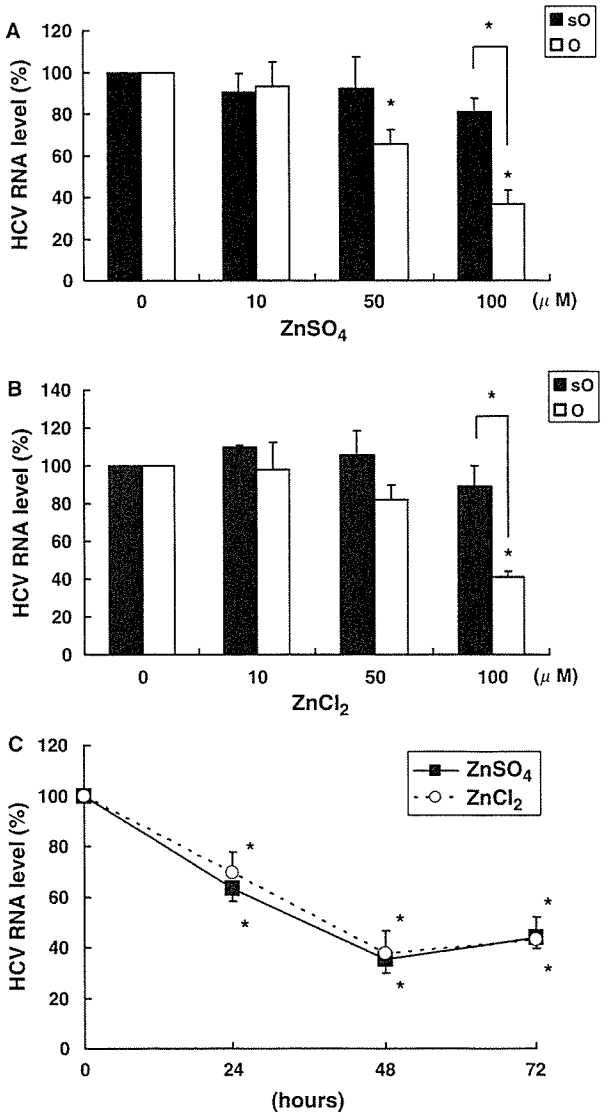

Fig. 4. Different effect of zinc salts between subgenomic hepatitis C virus (HCV) replicon and genome-length HCV RNA replication systems. (A) The sO and O cells were treated for 48 h with zinc sulfate (0, 10, 50, and 100 μM). The quantification of HCV RNA was performed as described in Fig. 1. (B) sO and O cells were treated for 48 h with zinc chloride (0, 10, 50, and 100 μM). The quantification of HCV RNA was performed as described in Fig. 1. (C) Time–response curve of zinc salts. O cells were treated with a fixed concentration (100 μM) of zinc sulfate or zinc chloride for 24, 48, and 72 h. Real-time reverse-transcriptase polymerase chain reaction was performed as described in the Materials and methods. We herein show the replication level of HCV RNA (%) calculated at each point, when the replication level of HCV RNA of the respective non-treated cells at 0 h was assigned as 100%. The replication level of HCV RNA was normalized by the level of GAPDH mRNA. The data indicate the mean ± SD of triplicates findings from three independent experiments. The asterisk (*) indicates a significant inhibition of HCV RNA replication by zinc sulfate or zinc chloride and a significant difference of the inhibitory effect of zinc salts between sO and O cells ($P<0.01$).

replication, did not decrease in the sO cells treated with $ZnSO_4$ or $ZnCl_2$ (100 μM), whereas the expression levels of NS3 and NS5B proteins were clearly decreased in the sO cells treated with $FeSO_4$, $FeCl_3$ (100 or 500 μM), or IFN-α (Fig. 5A and B). However, the expression levels of the NS3 and NS5B proteins both significantly decreased in the O cells treated with $ZnSO_4$ or $ZnCl_2$ (100 μM) as well as $FeSO_4$ or $FeCl_3$ (100 μM) (Fig. 5C and D). These results were consistent with those of the quantification analysis of HCV RNA as described above.

Anti-HCV activity of zinc salts on luciferase reporter assay system

Zinc salts significantly inhibited the *Renilla* luciferase activity in a dose-dependent manner but the extents of the suppressive effects were found to be rather weak depending on real-time RT-PCR (Fig. 6A and B). Zinc salts tend to reduce the replication of genome-length HCV RNA more markedly than that of the subgenomic HCV replicon even though the difference in chemical sensitivity to zinc salts was not significant.

Discussion

We demonstrated that zinc supplementation inhibited the replication of genome-length HCV RNA in O cells without causing cell toxicity, and the effects of zinc supplementation on HCV replication were significantly different between the genome-length HCV RNA replication system and the subgenomic HCV replicon system. On the other hand, IFN-α and iron supplementation suppressed the replication of HCV RNA almost equally between the subgenomic HCV replicon and genome-length HCV RNA replication system. However, other divalent cations, such as magnesium salts, did not suppress the replication of genome-length HCV RNA (data not shown). Therefore, the inhibition of the replication of HCV RNA is not an ubiquitous phenomenon caused by the divalent cations, but a specific phenomenon caused by certain divalent cations such as zinc and iron.

We showed the inhibitory effect of zinc salts in real-time RT-PCR and Western blotting on genome-length HCV RNA systems. In real-time RT-PCR, zinc inhibited the replication of HCV RNA as strongly as that of iron salts, whereas in Western blotting, the inhibitory effect of zinc salts was weaker than that of iron salts. There was a discrepancy in the inhibitory effects of zinc salts on RNA replication and protein expression in both systems. One possible reason is that zinc may affect the function of NS3 proteins of HCV through structural or NS2 proteins and consequently inhibit the replication of genome-length