Biomedical Research **26** (1) 9-14, 2005

Protection of hepatocytes from apoptosis by a novel substance from actinomycetes culture medium

Yang LIU[1], Saori TAKAHASHI[2], Hironobu OGASAWARA[2], Han Geuk SEO[3], Masami KAWAGOE[1], Fujiko HIRASAWA[1], Naxin GUO[1], Yasuharu UENO[1], Takashi KAMEDA[1] and Toshihiro SUGIYAMA[1]*

[1] Department of Biochemistry, School of Medicine, Akita University, 1-1-1 Hondo, Akita 010-8543, Japan, [2] Department of Bioengineering, Akita Research Institute of Food and Brewing (ARIF), 4-26 Sanuki, Araya-machi, Akita 010-1623, Japan, and [3] Department of Pharmacology, College of Medicine, Gyeongsang National University, 92 Chilam-Dong, Chinju 660-751, Korea

(Received 30 November 2004; and accepted 5 January 2005)

ABSTRACT

A novel substance, #675, found from an *Streptomyces* sp. SM675 culture medium, dose-dependently stimulates the proliferation of human functional liver cell 4 (FLC4). When FLC4 cells were incubated under conditions without fetal bovine serum (FBS), typical features of apoptotic cell death such as shrinkage and nuclear condensation appeared; high molecular weight (HMW) DNA fragments were found; and caspase-3 and poly (ADP-ribose) polymerase (PARP) proteins were cleaved. When FLC4 cells were incubated with #675 and without FBS, the cells grew healthy, no HMW DNA fragments were found, and caspase-3 and PARP cleavage weakened, suggesting that #675 protects FLC4 cells from apoptosis induced by FBS-deprivation. The quantitative reverse-transcribed polymerase chain reaction did not show differences in PARP or Bcl-2 mRNA expression in FLC4 cells incubated with or without #675, indicating other genes may be involved in this anti-apoptosis effect. These results show that #675 enhances FLC4 proliferation via an apoptosis-inhibition pathway, implying potential pharmacological and clinical applications.

Apoptosis (programmed cell death) plays a crucial role in the development of multicellular organisms, numerous physiological processes and the maintenance of homeostasis. An imbalance in apoptosis contributes to many pathological states including certain cancers and organ ischemia and perfusion injury. In the liver, apoptosis is responsible for viral hepatitis, alcohol-induced disease, nonalcoholic fatty liver disease, cholestatic diseases, ischemia and reperfusion injury (5, 11, 20, 34, 35) and copper accumulation in Wilson's disease (31). Hepatocytic apoptosis increases significantly in patients with alcoholic hepatitis and nonalcoholic steatohepatitis and correlates with disease severity and hepatic fibrosis (11, 20). Given the important role of apoptosis in liver disease, numerous studies have been conducted on how to protect hepatocytes from apoptosis, but they focused mainly on animals model (14, 18, 27). Due to interspecies differences, however, it is often difficult to extrapolate results in animals directly to human beings.

Actinomycetes are Gram-positive bacilli and grow commonly in soil, marshes and coastal habitats. The most interesting property of actinomycetes is that some may produce secondary metabolites including antibiotics and bioactive compounds valuable in human and veterinary medicine, agriculture and unique biochemical tools (6, 16, 17). During a screening program for novel growth factor from actinomycetes, we found a novel substance #675 which can stimulates the proliferation of human functional liver cell 4 (FLC4). FLC4 cell line reportedly retains functions of drug-metabolizing enzymes and has potential implications for medical and pharmacological studies and for constructing an artificial liver (2–4, 19). We found that #675 enhances FLC4 prolifera-

*Corresponding author: Dr. Toshihiro Sugiyama
Tel:+81-18-884-6074, Fax:+81-18-884-6443
E-mail: sugiyama@med.akita-u.ac.jp

tion by inhibiting apoptosis induced by serum-deprivation.

MATERIALS AND METHODS

Reagents. Cleaved caspased-3 (Asp175) antibody, cleaved poly (ADP-ribose) polymerase (Asp214) (PARP) antibody (human specific), and chaps cell extract buffer were purchased from Cell Signalling Technology (Beverly, MA). RNase A and proteinase K were purchased from Sigma (St. Louis, MO). Bradford assay dye reagents were purchased from Bio-Rad Laboratories (Richmond, CA).

Cultivation of Streptomyces sp. SM675. Streptomyces sp. SM675 was aerobically grown at 30°C for 4 days in a medium containing 1% beef extract, 1% polypeptone, 1% glucose, and 0.3% NaCl. The culture supernatant was used for assay.

Cell culture. Functional liver cell 4 (FLC4), kindly provided by Dr. Seishi Nagamori (Kyorin University, Japan), was maintained in Dulbecco's modified Eagle's medium (DMEM)/F12-Ham (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2 and was passed at confluency.

Cell viability measurement and microscopy. FLC4 cells (1×10^4) were seeded in 6 well cell culture clusters (Corning Incorporated, NY) and incubated as described above. One day the seeding, cells were washed with DMEM/F12-Ham (FBS-free) before incubation in DMEM/F12-Ham (FBS-free) containing 1% or 10% #675 while control cells were incubated in DMEM/F12-Ham (FBS-free) without #675. Cells continued to be incubated at 37°C and culture medium was refreshed every 2 days. At different time intervals, cells were harvested by trypsinization and viable cell numbers were assayed by a trypan blue exclusion test and counted using a hemocytometer. Morphological effects of #675 on FLC4 were evaluated by phase contrast microscopy.

DNA fragmentation. Cells incubated in the presence or absence of #675 for an indicated time were collected, washed once with chilled phosphate-buffered saline (PBS) and lysed in 100 µl of lysis buffer (10 mM Tris-HCl (pH7.5), 10 mM EDTA and 0.5% Triton X-100) at 4°C for 30 min. Soluble fraction were treated with 0.4 mg/ml RNase A at 37°C for 1 h and treated further with 0.4 mg/ml protease K at 37°C for 1 h. DNA was precipitated at −20°C with 0.2 volumes of 5M NaCl and 1 volume of isopropanol overnight, pelleted by centrifugation at 12,000 rpm for 10 min and resuspended in TE buffer. DNA was analyzed by electrophoresis in 2% agarose gel in Tris-acetate/EDTA (TAE) and visualized by soaking the gel in TAE buffer containing 1 µg/ml of ethidium bromide.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Cells incubated in the presence or absence of #675 for an indicated time were collected, washed 3 times with chilled PBS and resuspended with 1 volume of chaps cell extract buffer. Cells were frozen and thawed 3 times and centrifuged at 14,000 rpm for 10 min. Protein concentrations were determined by Bradfold assay. Then 40 µg of proteins was separated by SDS-PAGE under a reducing condition. For cleaved PARP, 12.5% gel was used while 15% gel was used for cleaved caspase-3. After SDS-PAGE, proteins were blotted onto PVDF membranes (Millipore, MA). Nonspecific binding to the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS/T) for 1 h. After washing 3 times with TBS/T, membranes were incubated overnight with rabbit polyclonal antibody against human cleaved caspase-3 or PARP antibody (1 : 500) at 4°C followed by secondary antibody horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (1 : 2000, DAKO, Denmark) at room temperature for 1 h. All antibodies were diluted in TBS/T containing 5% nonfat milk. Bands were visualized by ECL Western blotting detection reagents (Amersham Biosciences, UK).

RNA isolation and quantitative real-time reverse-transcribed polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from FLC4, and incubated in the presence or absence of #675 for an indicated time, using ISOGEN-LS (Nippon Gene, Japan) according to the manufacturer's instructions. RNA (1 µg) was reverse-transcribed to cDNA using a First Strand cDNA Synthesis Kit for reverse transcription-PCR (AMV) (Roche, Germany) at 25°C for 10 min, 42°C for 60 min, and 99°C for 5 min with Oligo-dT primers. cDNA was then diluted 2 fold and 2 µl aliquots were added to an 18 µl mixture of LightCycler FastStart DNA Master SYBR Green I mix (Roche, Germany) containing 0.5 µM primers. Primers were as follows: for PARP: 5' -AAGCCCTAAAGGCTCAGAAC-3' (forward) and 5'-TTGGGTGTCTGTGTCTTGAC-3' (reverse); for Bcl-2, 5'-TGCACCTGACGCCCTTCAC-3' (for-

ward) and 5'-AGACAGCCAGGAGAAATCAAA CAG-3' (reverse); and for β-actin, 5'-GTGGGGC GCCCCAGGCACCA-3' (forward) and 5'-CTCCT TAATGTCACGCACGATTTC-3' (reverse). RT-PCR was conducted in a light cycler (Roche, Germany) to quantify Bcl-2, PARP and β-actin mRNA. Cycle parameters were 40 cycles: 95°C for 10 sec, 60°C for PARP or 62°C for Bcl-2 and β-actin for 5 sec, 72°C for 20 sec. b-actin mRNA was used to calibrate the original concentration of mRNA. The concentration unit of mRNA in cells was defined as the ratio of target gene mRNA/β-actin mRNA.

PCR products were subjected to 1.5% agarose gel and the gel visualized with ethidium bromide.

Statistical analysis. Student's t test was used and p for statistical significance was set at 0.05 or 0.01.

RESULTS AND DISCUSSION

About 1000 strains of actinomycetes culture stock isolated from soil in Akita Prefecture, Japan were subjected to screen for the ability to produce the effector. Most of actinomyses showed no activity, but one strain No. SM675 was found to produce the effector. The actinomyses was identified as genus *Streptomyces* on the basis of physicochemical properties and its 16s rRNA sequence.

The FLC4 cells were grown in the presence or absence of #675 as described in Materials and Methods. FLC4 proliferation was enhanced by #675, especially 1 week after incubation and this effect was dose-dependent. After 11 days of incubation, cell numbers went from 1×10^4 to 145×10^4, 246×10^4 when FLC4 cells were incubated with 1% and 10% #675, respectively, significantly higher than that of control cells incubated without #675 (Fig. 1). Morphological analysis showed that cells not treated with #675 presented more typical features of apoptotic cell death such as shrinkage, nuclear condensation, than #675-treated cells (Fig. 2).

It has been reported that apoptosis is often induced in serum-deprivation conditions (13, 21, 23), so we wondered whether, in our experimental system, FLC4-stimulation of #675 was due to inhibited apoptosis. First, we checked the DNA ladder, a biochemical hallmark of apoptosis. After incubating FLC4 in FBS-free condition for 11 days, no DNA ladders were found but high molecular weight (HMW) DNA fragments appeared in cells not treated with #675. The DNA ladder is the end point of DNA degradation and does not reflect the full pattern of DNA fragmentation occurring during apoptosis. Many cells do not degrade DNA to this extent. Once apoptosis occurs, HMW DNA fragments appear (12, 22, 24, 33). Fig. 3 shows that FLC4 cells underwent apoptosis under serum-free conditions and that DNA degradation was blocked by #675 even at low concentration (1%).

We analyzed proteins level of cleaved caspase-3 and PARP because caspase activation and PARP cleavage are common feature in the pathway inducing apoptosis (25, 26). Caspase-3 is a key executioners of apoptosis, being responsible either

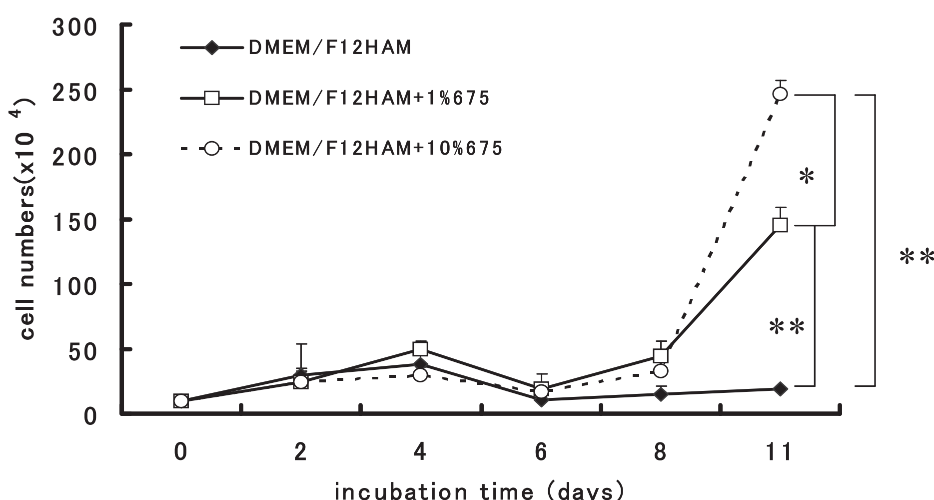

Fig. 1 FLC4 cells proliferation was enhanced by #675 doses dependently. FLC4 (1×10^4) was incubated with 1% or 10% #657 for an indicated time. After 11 days of incubation, cell numbers of 10% and 1% #675-treated FLC4 were significantly higher than those of control cells ($p < 0.01$), 10%#675-treated cells vs. 1%#675-treated, $p < 0.05$. Data is expressed as mean ± SD (n = 3) (*: $p < 0.05$; **: $p < 0.01$).

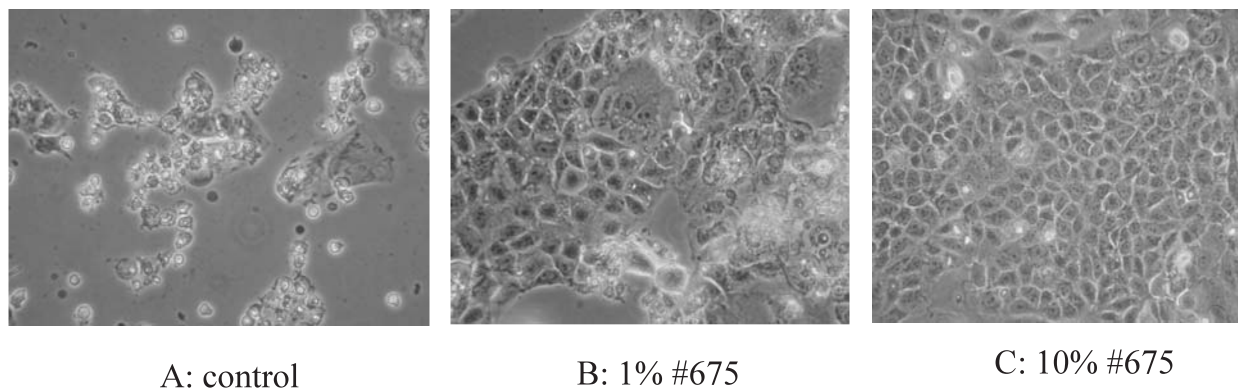

Fig. 2 Morphology of FLC4 after treatment with or without #675 for 11 days. A. FLC4 cells were incubated without #675. B. FLC4 cells were treated with 1% #675. C. FLC4 cells were treated with 10% #675.

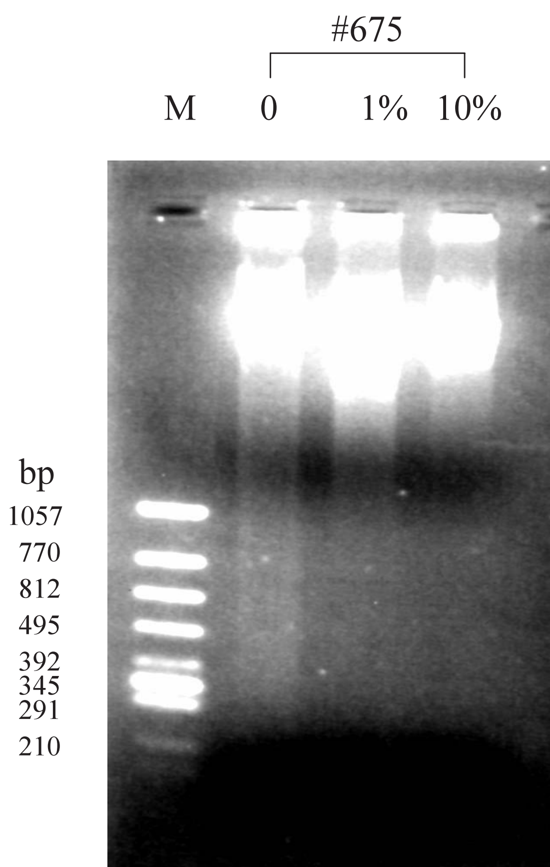

Fig. 3 High molecular weight (HMW) DNA fragments were found in FLC4 cells not treated with #675 (lane 0, size: 1057–345 bp), but not in #675-treated FLC4 cells. FLC4 cells were incubated with or without #675 for 11 days and DNA was isolated and subjected to 2% agarose gel as described in Materials and Methods. Similar results were confirmed in 3 independent experiments.

partially or totally for the proteolytic cleavage of many proteins including PARP (7, 10), resulting in the conversion of PARP from its native 113 kDa to an inactive 89 kDa fragment (32). The profile of processing is specific to cells undergoing apoptosis because a different PARP degradation is observed with necrotic cells (15, 28), so the PARP cleavage pattern is used as an assay to confirm apoptosis and to assess caspase activation. Fig. 4 shows that both caspase-3 and PARP were cleaved in cells not treated with #675, indicating that these cells underwent apoptosis in this condition. The cleaved caspase-3 and PARP bands were also found in #675-treated cells, but were weaker than those in control cells. Cleaved caspase-3 and PARP bands in cells incubated in 10% #675 were weaker than those in cells incubated in 1% #675, suggesting that #675 does inhibit apoptosis in this cell line.

To clarify possible mechanisms behind the decrease in PARP, we attempted to confirm the expression of PARP, and Bcl-2 mRNA by quantitative real-time PCR. Bcl-2, located in mitochondria, and endoplasmic reticular and nuclear membranes, is known to remain antiapoptotic (1, 29, 30) by regulating caspase activation (8, 9). In our study, the expression of PARP and BCL-2 mRNA did not changed significantly when FLC4 cells were incubated with #675 (Figs. 5A, B, C), indicating that PARP is post-transcriptionally regulated by #675, and that other members of the Bcl-2 family may be involved in this effect because many other relatives of the Bcl-2 family such as Bcl-x, and Bcl-w are also involved in antiapoptotic effect (8), a finding meriting further investigation.

In conclusion, our results demonstrated that #675 has an FLC4 protective effect by inhibiting apoptosis induced by serum-deprivation. It inhibits apoptosis processes by blocking DNA degradation, and decreases in caspase 3 and PARP cleavage. Our

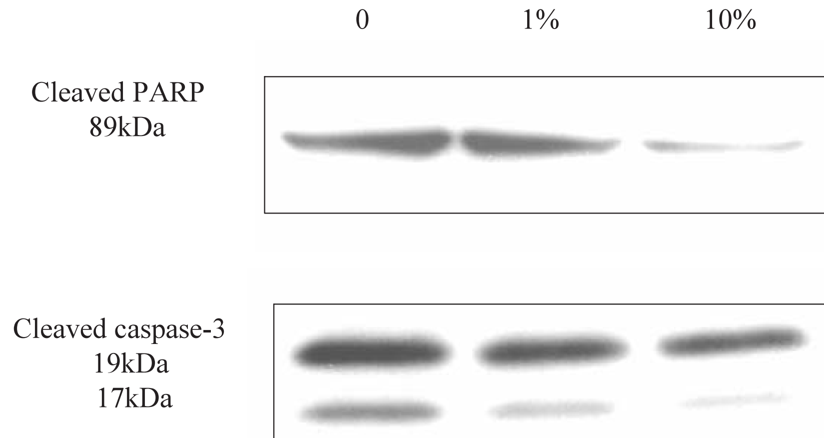

Fig. 4 Inhibition of caspase-3 and PARP cleavage by #675 was dose-dependent. Western blot analysis was conducted after FLC4 cells were incubated with or without #675 for 11 days. The blotting shown is representative of 3 similar experiments.

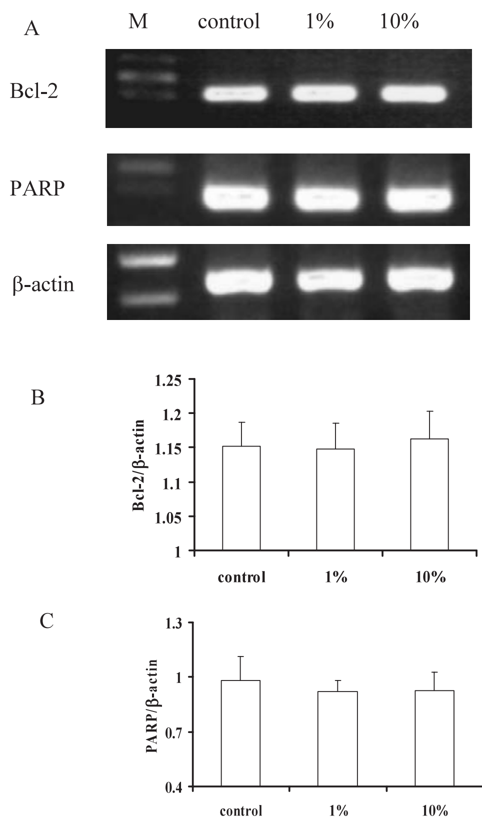

Fig. 5 PARP and Bcl-2 mRNA showed no difference after FLC4 was incubated with or without #675. FLC4 cells were incubated with or without #675 for 11 days and RNA was isolated and real-time reverse-transcribed PCR was conducted as presented in Materials and Methods. A. PCR products were subjected to 1.5% agarose gel and gel was visualized with ethidium bromide. B. Relative concentration of Bcl-2 mRNA. C. Relative concentration of PARP mRNA. Data is expressed as mean ± SD (n = 5).

findings imply that #675 has potential applications in pharmacological development, and clinical medicine although many problems, such as sample purification, the understanding of mechanisms behind its effect, and whether this sample affects other cell lines in the same way, require further investigation.

Acknowledgments

This work was supported by The Japan-Korea Basic Scientific Cooperation Program from the Japan Society for the Promotion of Science and the Grant-in-Aid from the Tokyo Biochemical Research Foundation.

REFERENCES

1. Agarwal N and Mehta K (1997) Possible involvement of Bcl-2 pathway in retinoid X receptor alpha-induced apoptosis of HL-60 cells. *Biochem Biophys Res Commun* **230**, 251–253.
2. Aoki Y, Aizaki H, Shimoike T, Tani H, Ishii K, Saito I, Matsuura Y and Miyamura T (1998) A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. *Virology* **250**, 140–150.
3. Arad U, Axelrod J, Ben-nun-Shaul O, Oppenheim A and Galun E (2004) Hepatitis B virus enhances transduction of human hepatocytes by SV40-based vectors. *J Hepatol* **40**, 520–526.
4. Babu E, Kanai Y, Chairoungdua A, Kim DK, Iribe Y, Tangtrongsup S, Jutabha P, Li Y, Ahmed N, Sakamoto S, Anzai N, Nagamori S and Endo H (2003) Identification of a novel system L amino acid transporter structurally distinct from heterodimeric amino acid transporters. *J Biol Chem* **278**, 43838–43845.
5. Canbay A, Friedman S and Gores GH (2004) Apoptosis: the

nexus of liver injury and fibrosis. *Hepatology* **39**, 273–278.
6. Champness W (2000) Actinomycete development, antibiotic production, and phylogeny: questions and challenges. In: *Prokaryotic Development*. (Brun YV and Shimkets LJ, eds), pp11–31, American Society for Microbiology, Washington D.C.
7. Cohen GM (1997) Caspase: the executioners of apoptosis. *Biochem J* **326**, 1–16.
8. Cory S and Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**, 647–656.
9. Cory S, Huang DC and Adams JM (2003) The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* **22**, 8590–8607.
10. Denis F, Rheaume E, Aouad SM, Alam A, Sekaly RP and Rohen LY (1998) The role of caspases in T cell development and the control of immune responses. *Cell Mol Life Sci* **54**, 1005–1019.
11. Feldstein AE, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD and Gores GJ (2003) Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* **125**, 437–443.
12. Filipski J, Leblanc J, Youdale T, Sikorska M and Walket PR (1990) Periodicity of DNA folding in higher order chromatin structures. *EMBO J* **9**, 1319–1327.
13. Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami K, Wakeham A, Hakem R, Yoshida H, Kong YY, Mark TW, Zuniga-Pflucker JC, Kroemer G and Penninger JM (2001) Essential role of the mitochondria apoptosis inducing factors in programmed cell death. *Nature* **410**, 549–554.
14. Kasai K, Sato S and Suzuki K (2001) A novel prostaglandin E receptor subtype agonist, 0N0-4819, attenuates acute experimental liver injury in rats. *Hepatol Res* **21**, 252–260.
15. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE and Poirier GG (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* **53**, 3976–3985.
16. Kawasaki T, Kuzuyama T, Furihata K, Itoh N, Seto H and Dairi T (2003) A relationship between the mevalonate pathway and isoprenoid production in actinomycetes. *J Antibiot (Tokyo)* **56**, 957–966.
17. Lemriss S, Laurent F, Couble A, Casoli E, Lancelin JM, Saintpierre-Bonaccio D, Rifai S, Fassouane A and Boiron P (2003) Screening of nonpolyenic antifungal metabolites produced by clinical isolates of actinomycetes. *Can J Microbiol* **49**, 669–674.
18. Maher JJ (2004) What doesn't kill you makes you stronger: how hepatocytes survive prolonged cholestasis. *Hepatology* **39**, 1141–1143.
19. Nagamori S, Hasumura S, Matsuura T, Aizaki H and Kawada M (2000) Developments in bioartificial liver research: concepts, performance, and applications. *J Gastroenterol* **35**, 493–503.
20. Natori S, Rust C, Stadheim LM, Srinivasan A, Burgart LJ and Gores GJ (2001) Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis. *J Hepatol* **34**, 248–253.
21. Navas P, Fernandez-Ayala DM, Martin SF, Lopez-Lluch G, De Caboa R, Rodriguez-Rodriguez-Aguilera JC and Villaba JM (2002) Ceramide-dependent caspase 3 activation is prevented by coenzyme Q from plasma membrane in serum-deprived cells. *Free Rad Res* **36**, 369–374.
22. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR and Sikorska M (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J* **12**, 3679–3684.
23. Pandey S, Lopez C and Jammu A (2003) Oxidative stress and activation of proteasome protease during serum deprivation-induced apoptosis in rat hepatoma cells; inhibition of cell death by melatonin. *Apoptosis* **8**, 497–508.
24. Pandey S, Smith B, Walker PR and Sikorska M (2000) Caspase-dependent and independent cell death in rat hepatoma 5123tc cells. *Apoptosis* **5**, 265–275.
25. Salvesen GS and Dixit VM (1997) Caspases: intracellular signaling by proteolysis. *Cell* **91**, 443–446.
26. Salvesen GS and Dixit VM (1999) Caspase activation: the induced-proximity model. *Proc Natl Acad Sci USA* **96**, 10964–10967.
27. Schoemaker MH, Conde de la Rosa L, Buist-Homan M, Vrenken TE Havinga R, Poelstra K, Haisma HJ, Jansen PL and Monshage H (2004) Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways. *Hepatology* **39**, 1563–1573.
28. Shah GM, Shah RG and Poirier GG (1996) Different cleavage pattern for poly(ADP-ribose) polymerase during necrosis and apoptosis in HL-60 cells. *Biochem Biophys Res Commun* **229**, 838–844.
29. Shimizu T and Pommier Y (1996) DNA fragmentation induced by protease activation in p53-null human leukemia HL60 cells undergoing apoptosis following treatment with the topoisomerase I inhibitor camptothecin: cell-free system studies. *Exp Cell Res* **226**, 292–301.
30. Shimizu S, Eguchi Y, Kamiike W, Funahashi Y, Mignon A, Lacronique V, Matsuda H and Tsujimoto Y (1998) Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. *Proc Natl Acad Sci USA* **95**, 1455–1459.
31. Strand S, Hofmann WJ, Grambihler A, Hug H, Volkmann M, Otto G, Wesch H, Mariani SM, Hack V, Stremmel W, Krammer PH and Galle PR (1998) Hepatic failure and liver cell damage in acute Wilson's disease involve CD95 (APO-1/Fas) mediated apoptosis. *Nat Med* **4**, 588–593.
32. Tewari M, Quan LT, Orourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS and Dixit VM (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**, 801–809.
33. Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF and Sikorska M (1991) Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res* **51**, 1078–1085.
34. Walsh MJ, Vanags DM, Clouston AD, Richardson MM, Purdie DM, Jonsson JR and Powell EE (2004) Steatosis and liver cell apoptosis in chronic hepatitis C: a mechanism for increased liver injury. *Hepatology* **39**, 1230–1238.
35. Yoon JH and Gores GJ (2002) Death receptor-mediated apoptosis and the liver. *J Hepatol* **37**, 400–410.