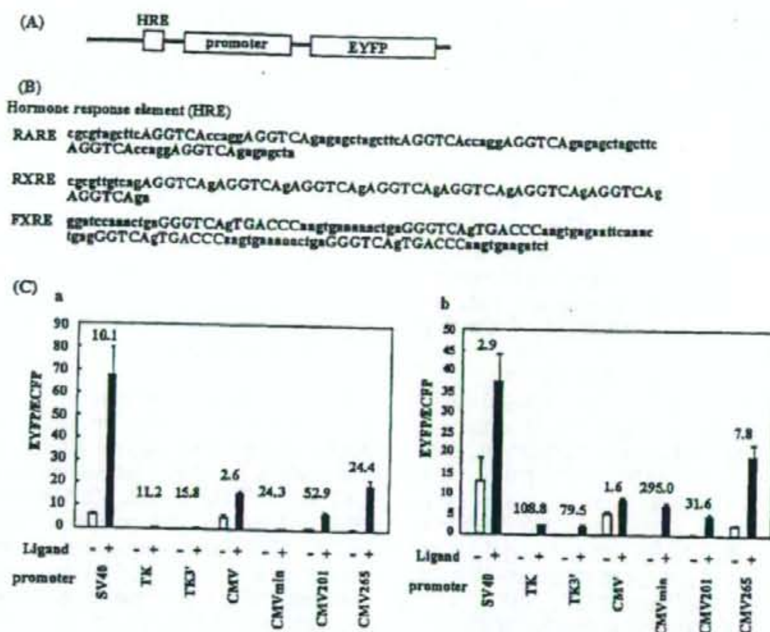


Fig. 1. Reporter plasmids for the assay of nuclear receptors. (A) Model of the constructed reporter plasmids. (B) The sequences for HREs of RAR, RXR and FXR (RARE, RXRE, and FXRE). (C) Effect of different promoters on the reporter assay. Seven species of promoter were employed in the reporter plasmid containing the HRE and EYFP genes. The activations of RARα (a) and FXR (b) are shown. The transfected cells were treated with ligands (black bar), 1 μM of ATRA for the RAR reporter assay or 100 μM of CDCA for FXR, or DMSO as a vehicle (white bar). The vertical axis indicates the ratio of fluorescence of EYFP (signal) to ECFP (internal control). The fold response relative to vehicle-treated cells is shown above the bars. Data are shown as the means + SD derived from six experiments.

Cotransfection and reporter assay

A monkey kidney cell line, COS-7, was kept in DMEM with 10% FBS. Transfections were performed using an Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. The ratio of the reporter plasmid, receptor expression plasmids (for example, the RARα and RXRα expression plasmids for assay of RARα ligands) and the internal control plasmid was 4:1:1:1. The culture medium was replaced with DMEM without phenol red (Gibco BRL, Gaithersburg, MD) supplemented with 10% charcoal-treated FBS (Hyclone, Logan, UT) when the transfections were performed. At 15 h after transfections, the cells were treated with trypsin/penicillin reagent and divided among wells of a black, 96-well plate with 100 μl of the culture medium. At 6 h after division among wells, the cells were treated with chemicals. After a 40-h incubation, the medium was eliminated by decantation, the cells were washed twice with PBS, and the wells were filled with 200 μl PBS. Fluorescence was detected using a microplate reader (ARVO; Perkin Elmer, Fremont, CA, USA). The fluorescence of EYFP was detected with an excitation filter of 485 nm and an emission filter of 545 nm, and that of ECFP was detected with filters of 420 and 486 nm (Perkin Elmer), respectively. The autofluorescence in COS-7 cells was subtracted from each of the detected fluorescences, and the EYFP/ECFP ratio was calculated using the resulting values.

Results

Reporter assay system

In the present reporter assay, EYFP and ECFP were selected as a reporter protein and an internal control for normalization of transfection, respectively. These two fluorescent proteins were chosen, because the peaks of their excitation and emission wavelengths are sufficiently different (a difference of 80 and 50 nm,

respectively) so that they can be detected simultaneously without cross-detection. The considerable cross-detection between EYFP and ECFP could be prevented using a set of optical filters (see Materials and methods). The EYFP/ECFP ratio was calculated after the autofluorescence of COS-7 cells was subtracted from the fluorescence intensities of EYFP and ECFP, because the autofluorescence was not negligible.

The reporter plasmids were constructed as shown in Fig. 1A. As HREs for FXR (FXR-RXR heterodimer), RAR (RAR-RXR heterodimer) and RXR (RXR homodimer), the fragments shown in Fig. 1B were used. In order to amplify signals, we employed three copies of DR5 (direct repeat with 5 bp of spacing) and four copies of DR1 as RAR and RXR response elements (RARE and RXRE). For the FXR response element (FXRE), four copies of the response element (inverted repeat) existing in the upstream region of the phospholipid transfer protein (PLTP) gene were employed. The tandem repeats in HREs elevated the response to a sufficient degree to detect the chemicals that activated the receptor. Then, an appropriate promoter for enhancing the fluorescent signal while retaining the response to the chemicals was selected from among seven promoters (Fig. 1C). Since the SV40 or CMV promoter caused a high fluorescence intensity with or without ligands, the responses to the ligands were not strong. The response of the RAR reporter plasmid with the SV40 promoter was about ten-fold. However, the apparent rate of the response was enhanced by interference of the expression of ECFP by the expression of EYFP, because the same promoter was employed for the reporter plasmid and the internal control plasmid. Therefore, the rate did not reflect a real response, and had a large SD. The TK promoter, the 3′ region of the TK promoter and the minimal CMV promoter caused strong responses, but the expression in the control plasmid was too low for quantitative measurement. The expression of reporter proteins with the 3′ region of the CMV promoter was higher than that with TK or the minimal CMV promoter, maintaining the induction rate by the ligands. Based on a comparison between the 3′ regions of the CMV promoters, we selected the CMV201 (201 bp of the CMV promoter) promoter for use in the experiments below, since the response of CMV201 was stronger than that of CMV265.

In addition to the promoter for reporter plasmids, the promoter for the internal control plasmid and the expression plasmids of nuclear receptors were examined in order to establish an appropriate assay system of the nuclear receptor ligands. When the SV40 promoter was employed for the expression of ECFP in the internal control plasmid, the SV40 promoter for nuclear receptor expression interfered with the expression of ECFP (data not shown). Therefore, the CMV promoter was employed for nuclear receptor expression plasmids. Finally, we established the following plasmid set as the reporter assay system: a reporter plasmid containing the EYFP gene, whose expression was regulated by the HRE and CMV201 promoter; an internal control plasmid containing the ECFP gene expressed by the SV40 promoter; and the expression plasmid of the nuclear receptor containing each nuclear receptor gene expressed by the CMV promoter.

Fig. 2A shows the response to typical agonists for FXR, RARs and RXRα in the screening system. For screening of RAR ligands, three subtypes of RARs (RARα1, RARβ2, RARγ1) were expressed in the cells independently. Although endogenous RARs co-exists in the cell, the preference for the subtype of compounds could be detected. Fig. 2B and C show the dose-dependence of the assay system of FXR and RAR ligands, respectively. RARs were activated by 100 pM of ATRA. ED_{50} values were estimated to be about 1–10 nM for RARα and 0.1–1 nM for RARβ and RARγ (only the result of RARα is shown in Fig. 2B). On the other hand, activation of FXR was seen in 3–10 μM CDCA and greater activation was observed at 100 μM CDCA (Fig. 2C). These dose-dependent response patterns were comparable to those reported previously (Brand et al., 1988; Parks et al., 1999), indicating that these assays could be used for quantitative measurement of the activation by ligands. The established method of the reporter assay was described in Materials and methods.

Screening of a novel ligand for nuclear receptors

Using the established screening system, we found some natural compounds and their derivatives which acted as agonists for RARs and FXR. In the screening, there was a possibility that unexpected factors may have changed the signal responses (in the present assay system, the transcriptional efficiency may be changed irrespective of the nuclear receptor, the tested chemicals may have their own fluorescence, and so forth). Therefore, another reporter plasmid without HRE was also constructed to eliminate these unexpected factors. As this plasmid was used in place of the reporter plasmid, the compounds that regulated the expression of EYFP without HRE were eliminated. Some results of the response for each nuclear receptor are shown in Fig. 3 (RAR, upper panel; FXR, middle panel; control, lower panel). The results for RARβ are presented as representative of those for RARs. Ten millimolar of each compound referring to the stock solution in DMSO was added to the culture medium of the transfected COS-7 cells at a final concentration of 30 μM (Fig. 3, Nos. 1–26). Compound Nos. 27, 28, and 29 were 3 μM ATRA, 30 μM CDCA, and vehicle, respectively. ATRA also slightly activated the FXR-RXR heterodimer, due

to the activation of RXR. Although, for example, Nos. 16, 18, 19, and 25 enhanced the relative EYFP/ECFP ratio, these compounds also enhanced the control that was used with the reporter plasmid without HRE. Thus it was concluded that these compounds were not ligands for the nuclear receptors.

As a result of screening more than 140 compounds (a part of the results is shown in Fig. 3), five compounds were found as ligands for the nuclear receptors. CAPE (compound No. 20 in Fig. 3), geranyl caffeate (No. 21), and farnesyl caffeate (not shown in Fig. 3) were found to be RAR agonists. Ginkgolic acid 15:1 (No. 12), geranyl caffeate (No. 21), and grifolin (No. 26) were found to be FXR agonists.

The structures of the caffeic acid derivatives tested in the screening are shown in Fig. 4A. CAPE, known as an active compound of propolis from honeybee hives, was synthesized from caffeic acid and β-phenylethyl bromide and other caffeic acids were purified and synthesized as described in Materials and methods. Three of these compounds (i.e., all of those tested except for bazzanenyl caffeate) activated RARs (Fig. 4B). The cells treated with over 30 µM of these compounds were removed from wells by washing of the reporter assay, because these compounds were toxic to the cell. Therefore, the results shown are for a reporter assay conducted using lower concentrations. Although the activation of RARs could be hardly detected by a low concentration of caffeic acid-derivatives, the activation by the compounds 10–30 µM was comparable to maximum activation by ATRA. As shown in Fig. 4B, CAPE activated RARβ to a greater degree than RARα or RARγ.

As FXR agonists, geranyl caffeate, ginkgolic acid 15:1 and grifolin were found. Geranyl caffeate, the RAR agonist, highly activated FXR (Fig. 3, No. 21), but the activation of the RXR homodimer was not detected (data not shown). It could not be determined whether or not farnesyl caffeate, a compound similar to geranyl caffeate, activated FXR, because 30 µM of these compounds showed toxicity for cells. The structures of ginkgolic acids and grifolin are shown in Fig. 5A. It has been reported that ginkgolic acid 15:1 was present in ginkgolic leaves (Ahlemeyer et al., 2001), and grifolin in mushrooms (Hirata and Nakanishi, 1949; Sugiyama et al., 1992). The activations of FXR by ginkgolic acid 15:1 and geranyl caffeate were comparable to that by CDCA,

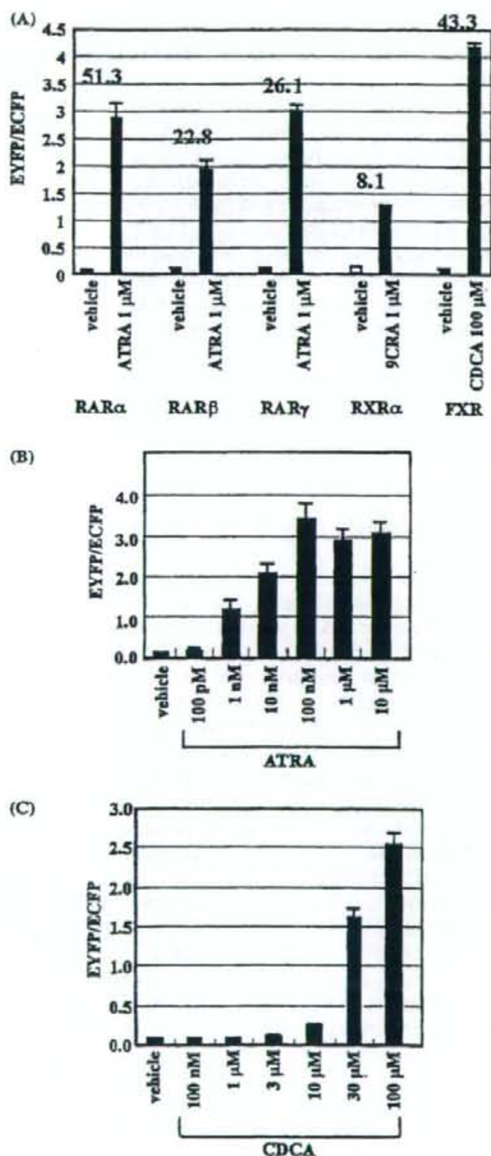


Fig. 2. Response in the reporter expression. (A) The responses in the reporter assay system by typical agonists for RAR, RXR, and FXR. COS-7 cells were transfected with an appropriate set of the plasmids (e.g. for assay of RARα ligand, the reporter plasmid containing RARE, the expression plasmids of RARα and RXRα and the internal control plasmid; for assay of RXRα ligand, the reporter plasmid containing RXRE, the RXRα expression plasmid, and the internal control plasmid). The transfected cells were treated with 1 µM of ATRA, 1 µM of 9CRA, or 100 µM of CDCA as ligands (black bar), or DMSO as a vehicle (white bar). The response rate is shown above the bars. Data are shown as the means + SD derived from three experiments. (B), (C) Dose–response analyses of ATRA and CDCA on the reporter assay of RAR and FXR. Data are shown as the means + SD derived from four experiments.

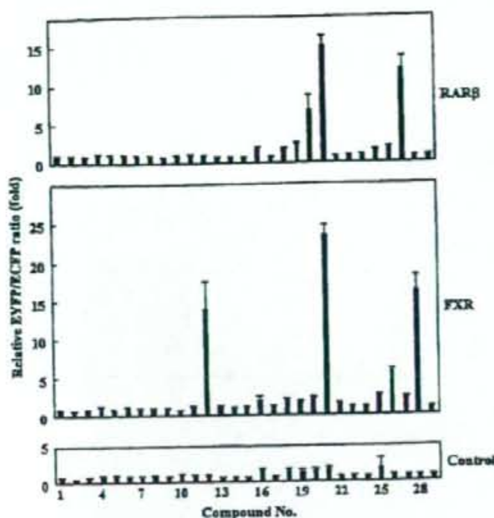
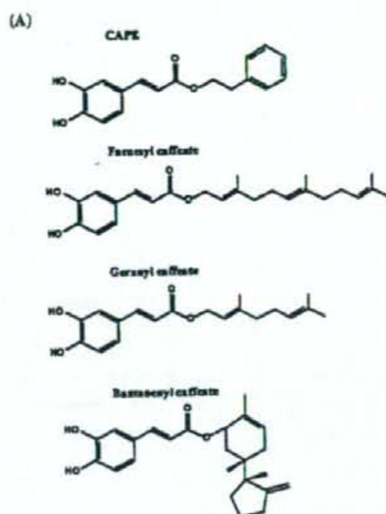


Fig. 3. Screening of ligands for RAR and FXR. COS-7 cells were transfected with the reporter plasmid, the receptor expression plasmid, and the internal control plasmid as shown in Fig. 2. The cells were treated with 30 μM of each compound. The results of the screening for RAR are shown in the upper panel, those for FXR in the middle panel, and those for the control (no HRE) in the lower panel. The results for RARβ are presented as representative of those for RARs (No. 1, hydrangeic acid; No. 2, ethyl 4′-ethylhydrangenate; No. 3, hydrangenol; No. 4, 8,3′-dimethoxyphylodulcin; No. 5, macrophyllaside A; No. 6, yashabashiletodiol A; No. 7, lycogarbin C; No. 8, lycogarbin A; No. 9, polygodial; No. 10, sacculatal; No. 11, ptychantin A; No. 12, ginkgolic acid 15:1; No. 13, 2-methyl ginkgolic acid methyl ester; No. 14, bilobal dimethyl ether; No. 15, 3-tridecanyl-*m*-cresol; No. 16, [11]-cytochalasa-6(12),13-diene-1,21-dione-7,18-dihydroxy-16,18-dimethyl-19-methoxy-10-phenyl-(7*S**,13*E*,16*S**,18*S**,19*R**); No. 17, hispidin; No. 18, costunolide, No. 19, beta-cyclocostanolide; No. 20, caffeic acid phenethyl ester; No. 21, geranyl caffeate; No. 22, atroctylon).



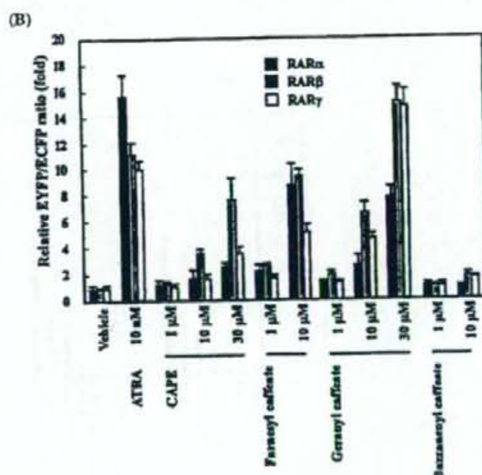


Fig. 4. Ligands for RARs. (A) The structures of caffeic acid derivatives tested in the screening. (B) Response in the RAR reporter assay. The responses in the COS-7 cells expressing RARα, RARβ or RARγ are indicated by black, gray, and white bars, respectively. Data are expressed as the fold response relative to vehicle (0.1% DMSO)-treated cells and are shown as the means + SD derived from four experiments.

the most potent endogenous bile acid. Ginkgolic acids 17:1, 15:0 and 13:0 (described in Fig. 5A) were also investigated as the other ginkgolic acids of ginkgo leaves (Fig. 5B). Ginkgolic acid 17:1 activated FXR more strongly than did 15:1, and ginkgolic acids with an alkyl chain (13:0, 15:0) activated FXR at concentrations of more than 20 μM. It seemed that the double bond and length of the carbon chain had an influence on FXR activation. Moreover, the structures except for the carbon chain were also important for FXR activation, because the methylated compound of ginkgolic acid 15:1 (2-methyl ginkgolic acid methyl ester, Fig. 5A) had no potency for FXR activation (Fig. 3, No. 13).

Discussion

To discover ligands for the nuclear receptors, we developed a battery of reporter assay systems

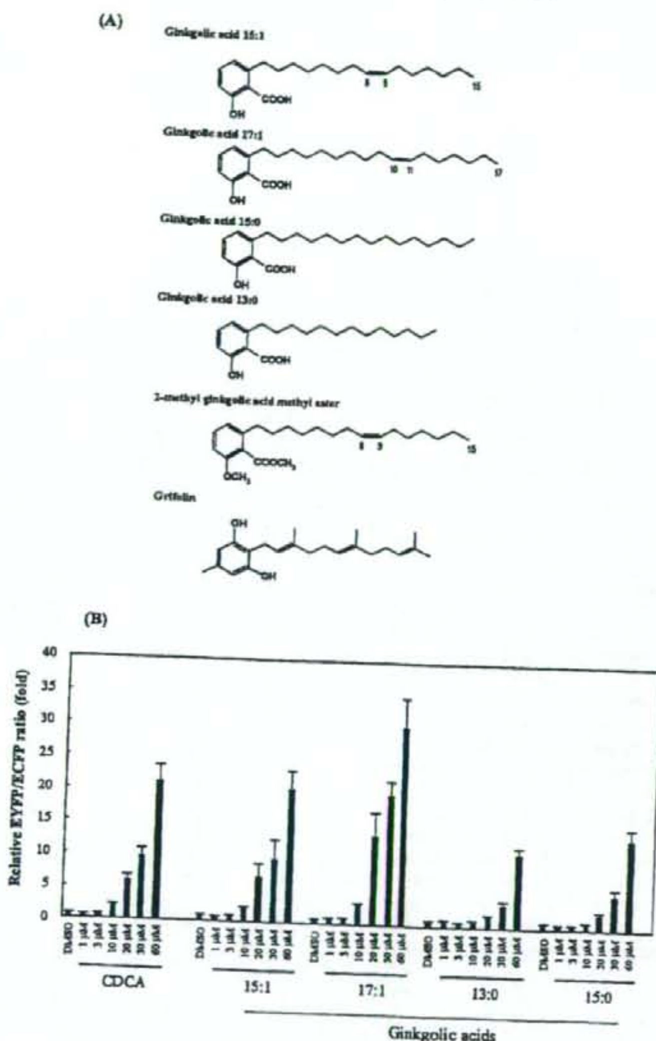


Fig. 5. Ligands for FXR. (A) The structure of candidates for FXR agonists and their related compounds (2-methyl ginkgolic acid methyl ester). (B) The activation of FXR by ginkgolic acids. COS-7 cells were transfected with the reporter plasmid containing FXRE, the expression plasmids of FXR and RXRα and the internal control plasmid. The transfected cells were treated with each compound. Data are shown as the means+SD derived from four experiments.

incorporating the advantages of fluorescent proteins. The disadvantage of GFP (low sensitivity) could be overcome by modifications. The present screening system using fluorescent proteins has clear merits of a high efficiency, convenience and low cost, because the two fluorescent signals can be measured simultaneously without addition of any co-factors. Moreover, the fluorescent signal was stable for more than 2 h after the wash. Considering these merits, this reporter assay system with fluorescent proteins might be advantageous for automatic high-throughput screening. If the expression of the fluorescent protein can be increased, the measurement of fluorescence can be carried out in culture medium, and the signal can be measured by time-course without any treatment. Moreover, the use of three fluorescent proteins (for example, DsRed with EYFP and ECFP) would enable us to carry out more efficient measurement.

Using this assay system, several compounds that induce expression of the reporter gene for RARs and/or FXR were identified. These compounds were described as ligands in this report, although there is a possibility that these compounds are metabolized and their metabolites bind to the receptors as ligands.

Three new ligands for RARs were identified: CAPE, geranyl caffeate, and farnesyl caffeate. The whole structure of these compounds may be needed for RAR-activation, because caffeic acid, a constituent compound of the compounds, did not activate RARs (data not shown). CAPE has been reported to have antioxidant, antiviral, anti-inflammatory and immunomodulatory activities (Grunberger et al., 1988), and has also been shown to inhibit the growth of different types of oncogene-transformed cells and to induce apoptosis (Grunberger et al., 1988; Burke et al., 1995; Su et al., 1994; Watabe et al., 2004). Since RARs have been reported to mediate many biological processes, it is possible that some of the diverse activities are due to their binding to RARs. Since geranyl and farnesyl caffeate have also been reported to exert antioxidant effects and to inhibit the growth of cancer cells (Inoue et al., 2004), the three compounds may suppress the growth of cancer by at least two pathways: induction of RAR and antioxidant effects. Considering its preferential activation of RARβ (Fig. 4B), CAPE may inhibit cancer (e.g., lung cancer) growth more selectively without substantial toxicity, such as the triglyceride elevation associated with RARα, and the skin, bone and teratogenic toxicity associated with RARγ Thus, especially CAPE could be assumed to be a seed for the development of an anti-cancer drug.

We also found that two natural compounds, ginkgolic acids and grifolin, activated FXR. Grifolin was first isolated as an antibiotic constituent of a mushroom, *Grifola confluens* (Hirata and Nakanishi, 1949). In 1992, it was reported that grifolin decreased liver cholesterol content, plasma total cholesterol levels, and plasma (very low-density lipoprotein (VLDL) + low-density lipoprotein (LDL)) cholesterol levels, and increased plasma high-density lipoprotein (HDL) cholesterol and plasma triglyceride levels (Sugiyama et al., 1992). It has been suggested that the effect of grifolin might be elicited, at least in part, by the augmented excretion of cholesterol into the faces (Sugiyama et al., 1994). On the other hand, FXR controls the expression of critical genes in bile acid and cholesterol homeostasis. In fact, FXR-null mice show elevated serum cholesterol and triglyceride levels (Sinal et al., 2000), and an FXR agonist has been shown to reduce serum triglyceride levels (Maloney et al., 2000). Moreover, FXR induces the expression of the gene of PLTP, which plays a role in HDL metabolism (Urizar et al., 2000). It seems that the cholesterol-lowering and HDL-cholesterol-increasing effects of grifolin are related to FXR activation, although grifolin's enhancement of triglyceride production was not consistent with its down-regulation of FXR agonists.

The FXR agonists found in this study are all non-steroidal compounds, whereas the well-known ligand of FXR, bile acid, is a steroidal one. The common characteristic of the structure of the ligands is their long carbon chains (i.e., geranyl, farnesyl and pentadecenyl), and farnesol has been shown to be a FXR ligand (Forman et al., 1995). However, aspects of the structures other than the carbon chains also appear to be important for FXR activation, because geraniol, a constituent compound of geranyl caffeate, has been reported not to activate FXR (Forman et al., 1995), and the methylated compound of ginkgolic acid 15:1 had no potency for FXR activation in the present study.

Several compounds, such as TTNPB, GW4064, Farnesoid, Forskolin, Fexaramine, AGN29 and AGN31, have been reported as non-steroidal agonists (Maloney et al., 2000; Howard et al., 2000; Downes et al., 2003; Dussault et al., 2003). The non-steroidal ligands may be important tools for studying the pharmacology of the receptor, because they may not have the property of bile acids and are not metabolized to form harmful lithocholic acid (Fischer et al., 1996; Javitt, 1966). In the present study, ginkgolic acids and geranyl caffeate strongly activated FXR, and both had structures quite different from bile acids, so that they could be good tools in this sense. Moreover, the importance of identifying gene-selective modulators that regulate a subset of FXR-specific genes as therapeutic agents has been recognized (Cui et al., 2003; Dussault et al., 2003). The gene-selective modulators of estrogen receptor, selective estrogen receptor modulators (SERMs), have been well studied (reviewed in McDonnell et al., 2002), and some compounds with a structure divergent from that of estrogen have been identified and applied to therapies of breast cancer and osteoporosis. The non-steroidal compounds could also be good tools for studying the selective response of FXR target genes.

In this report, we developed a new method for screening novel nuclear receptor agonists, and used it to identify new candidate ligands for FXR and RARs. We expect that these new ligands will be good pharmacological tools. Since the compound whose structure is much different from bile acids is expected to possess a specific effect as a ligand, we continue to screen various ligands from natural compounds with a wide variety of structures.

Acknowledgements

This work was supported by a grant-in-aid (MF-16) from the Pharmaceuticals and Medical Device Agency, a grant-in-aid for Research on Health Sciences Focusing

on Drug Innovation from the Japan Health Science Foundation, and a grant-in-aid for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan.

References

Ahlemeyer, B., Selke, D., Schaper, C., Klumpp, S., Krieglstein, J., 2001. Ginkgolic acids induce neuronal death and activate protein phosphatase type-2C. Eur. J. Pharmacol. 430, 1–7.

Begum, P., Hashidoko, Y., Islam, M.T., Ogawa, Y., Tahara, S., 2002. Zoosporicidal activities of anacardic acids against *Aphanomyces cochlioides*. Z. Naturforsch. [C] 57, 874–882.

Brand, N., Petkovich, M., Krust, A., Chambon, P., de Thé, H., Marchio, A., Tiollais, P., Dejean, A., 1988. Identification of a second human retinoic acid receptor. Nature 332, 850–853.

Burke Jr., T.R., Fesen, M.R., Mazumder, A., Wang, J., Carothers, A.M., Grunberger, D., Driscoll, J., Kohn, K., Pommier, Y., 1995. Hydroxylated aromatic inhibitors of HIV-1 intergrase. J. Med. Chem. 38, 4171–4178.

Cui, J., Huang, L., Zhao, A., Lew, J.-L., Yu, J., Sahoo, S., Meinke, P.T., Royo, I., Peláez, F., Wright, S.D., 2003. Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. J. Biol. Chem. 278, 10214–10220.

Downes, M., Verdecia, M.A., Roecker, A.J., Hughes, R., Hogenesch, J.B., Kast-Woelbern, H.R., Bowman, M.E., Ferrer, J.-L., Anisfeld, A.M., Edwards, P.A., Rosenfeld, J.M., Alvarez, J.G.A., Noel, J.P., Nicolaou, K.C., Evans, R.M., 2003. A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. Mol. Cell 11, 1079–1092.

Dussault, I., Beard, R., Lin, M., Hollister, K., Chen, J., Xiao, J.H., Chandraratna, R., Forman, B.M., 2003. Identification of gene-selective modulators of the bile acid receptor FXR. J. Biol. Chem. 278, 7027–7033.

Fischer, S., Beuers, U., Spengler, U., Zwiebel, F.M., Koebe, H.G., 1996. Hepatic levels of bile acids in end-stage chronic cholestatic liver disease. Clin. Chim. Acta 251, 173–186.

Fisher, G.J., Voorhees, J.J., 1996. Molecular mechanisms of retinoid actions in skin. FASEB J. 10, 1002–1013.

Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., Perlmann, T., Noonan, D.J., Burka, L.T., McMorris, T., Lamph, W.W., Evans, R.M., Weinberger, C., 1995. Identification of a nuclear receptor that is activated by farnesol metabolites. Cell 81, 687–693.

Grunberger, D., Banerjee, R., Eisinger, K., Oltz, E.M., Efros, L., Caldwell, M., Estevez, V., Nakanishi, K., 1988. Preferential cytotoxity on tumor cells by caffeic acid phenethyl ester isolated from propolis. Experientia 44, 230–232.

Hansen, L.A., Sigman, C.C., Andreola, F., Ross, S.A., Kelloff, G.J., De Luca, L.M., 2000. Retinoids in chemoprevention and differentiation therapy. Carcinogenesis 21, 1271–1279.

Hashimoto, T., Tori, M., Asakawa, Y., Wollenweber, E., 1988. Synthesis of two allergenic constituents of propolis and poplar bud excretion. Z. Naturforsch. [C] 43, 470–472.

Hirata, Y., Nakanishi, K., 1949. Grifolin, an antibiotic from a basidiomycete. J. Biol. Chem. 184, 135–143.

Howard, W.R., Pospisil, J.A., Njolito, E., Noonan, D.J., 2000. Catabolites of cholesterol synthesis pathways and forskolin as activators of the farnesoid X-activated nuclear receptor. Toxicol. Appl. Pharmacol. 163, 195–202.

Inoue, K., Nishitani, N., Tanabe, A., Yamanaka, H., Hashimoto, T., Asakawa, Y., Fujiki, H., 2004. Significance of structural specificity in the antioxidant activity of hydrangenol and caffeic acid-derivatives. Abstract for 124th Congress on Nihon yakugakukai, Osaka, Japan, vol. 2, p. 173.

Ishii, N., Takahashi, A., Kusano, G., Nozoe, S., 1988. Studies on the constituents of *Polyporus dispansus* and *P. confluens*. Chem. Pharm. Bull. 36, 2918–2924.

Javitt, N.B., 1966. Cholestasis in rats induced by taurolithocholate. Nature 210, 1262–1263.

Khorasanizadeh, S., Rastinejad, F., 2001. Nuclear–receptor interactions on DNA-response elements. Trends Biochem. Sci. 26, 384–390.

Krust, A., Kastner, P.H., Petkovich, M., Zelent, A., Chambon, P., 1989. A third human retinoic acid receptor, hRAR-γ. Proc. Natl. Acad. Sci. USA 86, 5310–5314.

Liu, Y., Binz, J., Numerick, M.J., Dennis, S., Luo, G., Desai, B., MacKenzie, K.I., Mansfield, T.A., Kliewer, S.A., Goodwin, B., Jones, S.A., 2003. Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. J. Clin. Invest. 112, 1678–1687.

Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., Hull, M.V., Lustig, K.D., Mangelsdorf, D.J., Shan, B., 1999. Identification of a nuclear receptor for bile acids. Science 284, 1362–1365.

Maloney, P.R., Parks, D.J., Haffner, C.D., Fivush, A.M., Chandra, G., Plunket, K.D., Creech, K.L., Moore, L.B., Wilson, J.G., Lewis, M.C., Jones, S.A., Willson, T.M., 2000. Identification of a chemical tool for the orphan nuclear receptor FXR. J. Med. Chem. 43, 2971–2974.

Martin, C.S., Wight, P.A., Dobretsova, A., Bronstein, I., 1996. Dual luminescence-based reporter gene assay for luciferase and β-galactosidase. Biotechniques 21, 520–524.

McDonnell, D.P., Connor, C.E., Wijayaratne, A., Chang, C.Y., Norris, J.D., 2002. Definition of the molecular and cellular mechanisms underlying the tissue-selective agonist/antagonist activities of selective estrogen receptor modulators. Recent Prog. Horm. Res. 57, 295–316.

Morimoto, H., Kawamatsu, Y., Sugihara, H., 1968. Stereostructure of toxin from the fruit of *Ginkgo biloba L.* Chem. Pharm. Bull. 16, 2282–2286.

Nukata, M., Hashimoto, T., Yamamoto, I., Iwasaki, N., Tanaka, M., Asakawa, Y., 2002. Neogrifolin derivatives possessing anti-oxidative activity from the mushroom *Albatrellus ovinus*. Phytochemistry 59, 731–737.

Parks, D.J., Blanchard, S.G., Bledsoe, R.K., Chandra, G., Consler, T.G., Kliewer, S.A., Stimmel, J.B., Willson, T.M., Zavacki, A.M., Moore, D.D., Lehmann, J.M., 1999. Bile acids: natural ligands for an orphan nuclear receptor. Science 284, 1365–1368.

Paul, V.J., Yeddanapalli, L.M., 1956. On the olefinic nature of anacardic acid from Indian cashew nut shell liquid. J. Am. Chem. Soc. 78, 5675–5678.

Petkovich, M., Brand, N.J., Krust, A., Chambon, P., 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330, 444–450.

Sherf, B.A., Navarro, S.L., Hannah, R.R., Wood, K.V., 1996. Dual-luciferase™ reporter assay: an advanced co-reporter technology integrating firefly and *Renilla* luciferase assays. Promega Notes 57, 2.

Sinal, C.J., Tohkin, M., Miyata, M., Ward, J.M., Lambert, G., Gonzalez, F.J., 2000. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell 102, 731–744.

Su, Z.-Z., Lin, J., Grunberger, D., Fisher, P.B., 1994. Growth suppression and toxicity induced by caffeic acid phenethyl ester (CAPE) in type 5 adenovirus-transformed rat embryo cells correlate directly with transformation progression. Cancer Res. 54, 1865–1870.

Sugiyama, K., Kawagishi, H., Tanaka, A., Saeki, S., Yoshida, S., Sakamoto, H., Ishiguro, Y., 1992. Isolation of plasma cholesterol-lowering components from Ningyotake (*Polyporus confluens*) mushroom. J. Nutr. Sci. Vitaminol. 38, 335–342.

Sugiyama, K., Tanaka, A., Kawagishi, H., Ojima, F., Sakamoto, H., Ishiguro, Y., 1994. Hypocholesterolemic action of dietary grifolin on rats fed with a high-cholesterol diet. Biosci. Biotechnol. Biochem. 58, 211–212.

Toyota, M., Asakawa, Y., 1988. Sesquiterpenoids from the liverwort *Bazzania fauriana*. Phytochemistry 27, 2155–2159.

Urizar, N.L., Dowhan, D.H., Moore, D.D., 2000. The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. J. Biol. Chem. 275, 39313–39317.

Wang, H., Chen, J., Hollister, K., Sowers, L.C., Forman, B.M., 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol. Cell 3, 543–553.

Watabe, M., Hishikawa, K., Takayanagi, A., Shimizu, N., Nakaki, T., 2004. Caffeic acid phenethyl ester induces apoptosis by inhibition of NFκB and activation of Fas in human breast cancer MCF-7 cells. J. Biol. Chem. 279, 6017–6026.

Change in Annexin A3 Expression by Regulatory Factors of Hepatocyte Growth in Primary Cultured Rat Hepatocytes

Mizuho HARASHIMA,[a] Shingo NIIMI,*[,b] Hitomi KOYANAGI,[a] Masashi HYUGA,[b] Seiji NOMA,[c] Taiichiro SEKI,[a] Toyohiko ARIGA,[a] Toru KAWANISHI,[b] and Takao HAYAKAWA[d]

[a] *Department of Nutrition and Physiology, Nihon University College of Bioresource Sciences; Kameino, Fujisawa 252–8510 Japan:* [b] *Division of Biological Chemistry and Biologicals, National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan:* [c] *The Japan Health Sciences Foundation; Kyodo Bld. 4F, 13–4 Nihonbashi, Kodenma-cho, Chuo-ku, Tokyo 103–0001, Japan: and* [d] *Pharmaceuticals and Medical Devices Agency; Shinkasumigaseki Bldg. 3–3–2 Kasumigaseki, Chiyoda-ku, Tokyo 100–0013, Japan.*

Received January 30, 2006; accepted April 11, 2006; published online April 18, 2006

We have recently reported that annexin (Anx) A3 expression is necessary for hepatocyte growth in cultured rat hepatocytes seeded at half the subconfluent density on collagen. In the present study, we investigated the effects of various regulatory factors of hepatocyte growth on AnxA3 expression. AnxA3 expression was significantly reduced in hepatocytes cultured under various growth inhibitory conditions such as presence of dexamethasone, culture at subconfluent cell density, and on EHS-Matrigel and lactose-carrying styrene polymer. On the other hand, hepatocyte growth factor and epidermal growth factor, stimulators of hepatocyte growth, significantly increased AnxA3 expression in hepatocytes cultured on EHS-Matrigel. These results show close correlation between known stimulatory or inhibitory actions of various factors to hepatocyte growth and increase or decrease in AnxA3 expression, and suggest the involvement of AnxA3 in their regulation of hepatocyte growth.

Key words annexin A3; hepatocyte growth; primary cultured rat hepatocyte

Annexin (Anx) A3, also called "lipocortin 3" or "placental anticoagulant protein 3" (PAP-III),[1] is a member of the lipocortin/Anx family, which binds to phospholipids and membranes in a Ca^{2+}-dependent manner.[2,3] AnxA3 has been shown to have anticoagulant and anti-phospholipase A_2 properties *in vitro*[4] and to promote the Ca^{2+}-dependent aggregation of isolated specific granules from human neutrophils.[5] However, physiological functions have been completely unknown.[6] AnxA3 has been detected in lung, spleen, placenta, and adrenal medulla, but not in liver and isolated hepatocytes.[7—11]

We have recently reported that AnxA3 is expressed in cultured rat hepatocytes and that inhibition of AnxA3 expression by RNA interference results in a significant inhibition of hepatocyte growth.[12] This evidence indicates that AnxA3 acts as a positive regulator on hepatocyte growth in cultured hepatocytes. In relation to our report, it is noteworthy that hepatocytes placed under culture conditions acquire a growth potential characterized by enhancement of hepatocyte growth dependent on several growth factors,[13—15] whereas adult hepatocytes are normally quiescent *in vivo*.[16,17] The correlation between AnxA3 expression and growth potential of hepatocytes described above suggests that AnxA3 is one of the factors necessary for hepatocytes placed under culture to acquire growth potential.

On the other hand, hepatocyte growth is regulated in cultured hepatocytes by various other factors including cell density,[18] humoral factors such as dexamethasone (Dex),[19—21] hepatocyte growth factor (HGF) and epidermal growth factor (EGF),[14] and cellular substratum such as EHS-Matrigel[22] and lactose-carrying styrene polymer (PVLA).[23] In relation to our report, the question of whether regulation of hepatocyte growth by these factors could be mediated by concurrent change in AnxA3 expression seemed interesting; however, it remained to be elucidated whether these factors cause change in AnxA3 expression.

In the present study, we investigated the effects of various regulators of hepatocyte growth on the AnxA3 expression to examine the involvement of AnxA3 in their regulation of hepatocyte growth.

MATERIALS AND METHODS

Materials Recombinant human hepatocyte growth factor (HGF) was purchased from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). Mouse epidermal growth factor (EGF) was purchased from Wako Pure Chemical, Ltd. (Osaka, Japan). Porcine dermal collagen type 1 (collagen) was purchased from Koken Co. (Tokyo, Japan). Lactose-carrying styrene polymer (PVLA) was purchased from Seikagaku Corp. (Tokyo, Japan). Dishes (10 cm) precoated with Matrigel were purchased from BD Biosciences (Bedford, MA, U.S.A.). Rabbit anti-human AnxA3 serum was a gift from Dr. F. Russo-Marie and Dr. C. Ragueness-Nicol. Rabbit anti-rat albumin IgG and rabbit anti-rat β-actin IgG were purchased from Cappel (Aurora, Ohio, U.S.A.) and Biolegend, Inc. (San Diego, CA, U.S.A.), respectively.

Cell Isolation and Monolayer Cultures Parenchymal hepatocytes were isolated from adult male Wistar rats weighing 180—200 g, by *in situ* perfusion of the liver with collagenase.[24] All animal care and procedure protocols were approved by the institutional animal care committee. The cells were then suspended at a density of 2.5×10^5 cells/ml or 5.0×10^5 cells/ml in Williams medium E (WE) containing 5% fetal bovine serum, 1 nM insulin and 1 μg/ml aprotinin, and cultured at a density of 0.5×10^5 cells/cm^2 or 1.0×10^5 cells/cm^2 in a 10-cm dish precoated with 0.03% collagen. Alternatively, the cells were then suspended at a density of 2.5×10^5 cells/ml in WE containing 1 nM insulin and 1 μg/ml aprotinin and cultured at a density of 0.5×10^5 cells/cm^2 in a 10-

* To whom correspondence should be addressed. e-mail: niimi@nihs.go.jp

© 2006 Pharmaceutical Society of Japan

cm dish precoated with Matrigel or a 10-cm dish precoated with 100 μg/ml PVLA. The cells were cultured in a humidified chamber at 37 °C in 5% CO_2 and 30% O_2 in air. After 2.5 h of culture, the medium was replaced with a serum- and hormone-free medium containing aprotinin (1 μg/ml), and then various humoral factors to be tested were added and the cells were further cultured for 1 d. After 1 d culture, the medium was replaced as described above, and then the humoral factors were again added and the cells were further cultured for 1 d.

Western Blot Analysis Cell lysates were prepared from the cells 2 d after the start of culture by modification of a method previously described.[25] The cells were washed twice with 5 ml of phosphate-buffered saline and then once with 5 ml of buffer A (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 10 mM EDTA). The cells were then harvested after the addition of 20 μl of buffer A. The cells were suspended, shaken for 15 min at room temperature, and sonicated four times for 15 s each time while in an ice bath after the addition of 1/5 [v/v] of 5×buffer A containing 2.5% Triton X-100 and 1/100 [v/v] of a protease inhibitor cocktail (SIGMA). After centrifugation at 100000×$\boldsymbol{g}$, an equal amount of cytosolic protein in each experiment was subjected to SDS-PAGE on a 10% gel and electroblotted to a PVDF membrane (GVHP; Millipore). After blocking the membrane with 5% skimmed milk, a Western blot analysis was performed using rabbit anti-human AnxA3 antibody serum at a dilution of 1 : 21000, rabbit anti-rat albumin IgG at a dilution of 1 : 80000, or rabbit anti-rat β-actin IgG at a dilution of 1 : 500. Detection was performed using an ECL detection system (Amersham Bioscience). We used albumin or β-actin as a housekeeping protein based on the results of the preliminary studies. The intensity of each band was measured over a proportional range in the experiments. A computer assisted-analyzer was used to quantitatively analyze the intensity, and the intensity of each AnxA3 band was normalized to the intensity of the housekeeping protein.

Total RNA Extraction and Real-Time Quantitative PCR Total RNA was extracted from the cells 1 d after the start of culture using Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol. An equal amount of RNA (approximately 1 μg) in each experiment was reverse-transcribed using a THERMOSCRIPT™ RT-PCR System (Invitrogen) and oligo$(dT)_{20}$ at a final volume of 40 μl in accordance with the manufacturer's protocol, and then diluted two-fold with ultrapure water. Subsequently, 2 μl of cDNA was used as a template for real-time PCR analysis in a LightCycler system (Rosche), in accordance with the manufacture's instructions. For AnxA3 and albumin, the PCR program consisted of 40 cycles of 10 s at 94 °C, 10 s at 60 °C, and 12 s at 72 °C. For 18S rRNA, the PCR program consisted of 40 cycles of 10 s at 94 °C, 10 s at 60 °C, and 20 s at 72 °C. The sequences of AnxA3-specific primers were 5′-CAA-ATTCACCGAGATCCTGT-3′ and 5′-TGCTGGAGTGCTG-TACGAAA-3′,[12] those of the albumin-specific primers were 5′-AAGGCACCCCGATTACTCCG-3′ and 5′-TGCGAAGT-CACCCATCACCG-3′,[26] and those of 18S rRNA-specific primers were 5′-CCAGAGCGAAAGCATTTGCCA-3′ and 5′-GGCATCACAGACCTGTTATTGCTC-3′. The 18S rRNA PCR product specificity was confirmed by DNA sequencing using an ABI Prism 377 Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analyses. We used albumin or 18S rRNA as a housekeeping gene based on the results of the preliminary studies. And the AnxA3 levels were normalized to the levels of the housekeeping gene.

RESULTS

Effects of Dex and HGF on AnxA3 Expression by Hepatocytes Cultured on Collagen We investigated the effect of Dex and HGF on AnxA3 expression by hepatocytes cultured on collagen. Dex (100 nM) suppressed the increase of AnxA3 protein level during culture by approximately 80% (Fig. 1). On the other hand, HGF (20 ng/ml) had no effect on the AnxA3 protein level (Fig. 1). Dex (100 nM) suppressed the increase of AnxA3 mRNA level by approximately 80% (Fig. 2).

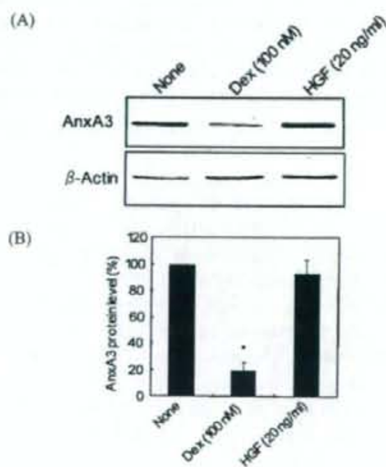


Fig. 1. Effects of Dex and HGF on AnxA3 Protein Level by Hepatocytes Cultured on Collagen

(A) The data shown are representative of the Western blot analysis results. Approximately 7.5 μg of protein was used for the detection of AnxA3 and β-actin. (B) The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen in the absence of humoral factors, shown as None. The data are expressed as the mean±S.D. of 3 experiments. * $p<0.01$, compared with the value of None.

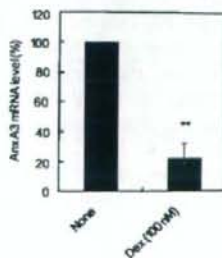


Fig. 2. Effects of Dex on AnxA3 mRNA Level by Hepatocytes Cultured on Collagen

The AnxA3 levels were normalized to the levels of a housekeeping gene, albumin. The data are expressed as the mean±S.D. of 3 experiments. The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen in the absence of humoral factors, shown as None. ** $p<0.05$, compared with the value of None.

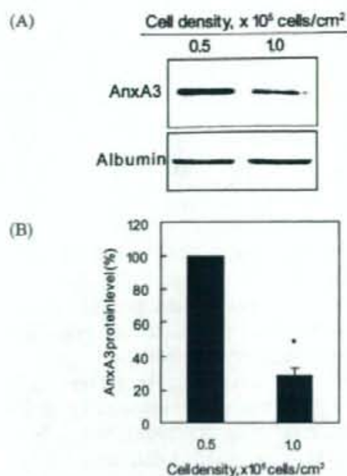


Fig. 3. AnxA3 Protein Level by Hepatocytes Cultured on Collagen at High Density

(A) The data shown are representative of the Western blot analysis results. Approximately 30.0 and 7.5 μg of protein were used for the detection of AnxA3 and albumin, respectively. (B) The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. The data are expressed as the mean$\pm$S.D. of 3 experiments. $*p<0.01$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen.

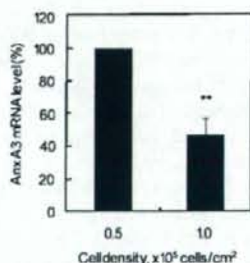


Fig. 4. AnxA3 mRNA Level by Hepatocytes Cultured on Collagen at High Density

The AnxA3 levels were normalized to the levels of a housekeeping gene, albumin. The data are expressed as the mean$\pm$S.D. of 3 experiments. The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. $**p<0.05$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen.

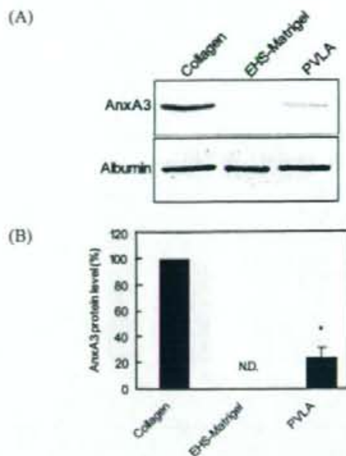


Fig. 5. AnxA3 Protein Level by Hepatocytes Cultured on EHS-Matrigel and PVLA

(A) The data shown are representative of the Western blot analysis results. Approximately 20.0 and 10.0 μg of protein were used for the detection of AnxA3 and albumin, respectively. (B) The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. The data are expressed as the mean$\pm$S.D. of 3 experiments. $*p<0.01$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. N.D.: not detected.

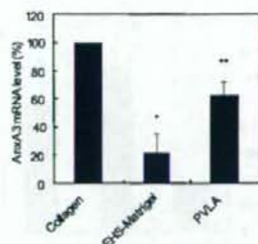


Fig. 6. AnxA3 mRNA Level by Hepatocytes Cultured on EHS-Matrigel and PVLA

The AnxA3 levels were normalized to the levels of a housekeeping gene, albumin. The data are expressed as the mean$\pm$S.D. of 3 experiments. The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. $*p<0.01$, $**p<0.05$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen.

Effect of Cell Density on AnxA3 Expression by Hepatocytes Cultured on Collagen We investigated the effect of cell density on AnxA3 expression by hepatocytes cultured on collagen. AnxA3 protein and mRNA levels produced by the hepatocytes seeded at subconfluent cell density (1×10^5 cells/cm^2) were approximately 70% and 50% lower than those by the hepatocytes seeded at half of subconfluent cell density (0.5×10^5 cells/cm^2), respectively (Figs. 3, 4).

AnxA3 Expression by Hepatocytes Cultured on EHS-Matrigel and PVLA We investigated AnxA3 expression by hepatocytes cultured on EHS-Matrigel and PVLA, and compared the expression with that by hepatocytes cultured on collagen. AnxA3 protein was not detected in hepatocytes cultured on EHS-Matrigel (Fig. 5). And AnxA3 protein level produced by hepatocytes cultured on PVLA was approximately 70% lower than that by hepatocytes cultured on collagen (Fig. 5). mRNA levels produced by hepatocytes cultured on EHS-Matrigel and PVLA were approximately 80% and 40% lower than that by hepatocytes cultured on collagen, respectively (Fig. 6).

Effect of HGF and EGF on AnxA3 Expression by Hepatocytes Cultured on EHS-Matrigel We investigated the effect of HGF and EGF on AnxA3 expression by hepatocytes cultured on EHS-Matrigel. HGF and EGF significantly increased AnxA3 protein level, from an initially undetectable level (Fig. 7). This result suggests that the failure of increase of AnxA3 protein level by HGF, as shown in Fig. 1, is due to maximal stimulation of AnxA3 expression in hepatocytes cultured on collagen. In relation to these findings, it is seen that the stimulation of AnxA3 expression in hepatocytes cultured on collagen is not due the stimulation of HGF synthesis, because hepatocytes do not synthesize HGF.[27] HGF and EGF also increased AnxA3 mRNA levels by approximately 3.2-fold and 2.5-fold (Fig. 8), respectively.

(A)

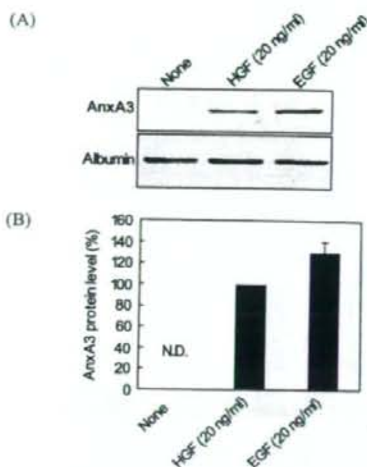


Fig. 7. Effects of HGF and EGF on AnxA3 Protein Level by Hepatocytes Cultured on EHS-Matrigel

(A) The data shown are representative of the Western blot analysis results. Approximately 10.0 μg of protein was used for the detection of AnxA3 and albumin. (B) The value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors is shown as None. The results are shown relative to the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the presence of HGF. The data are expressed as the mean$\pm$S.D. of 3 experiments.

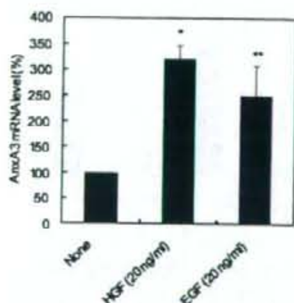


Fig. 8. Effects of HGF and EGF on AnxA3 mRNA Level by Hepatocytes Cultured on EHS-Matrigel

The AnxA3 levels were normalized to the levels of a housekeeping gene, 18S rRNA. The data are expressed as the mean$\pm$S.D. of 3 experiments. The value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors is shown as None. The results are shown relative to the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors. * $p<0.01$, ** $p<0.05$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors.

DISCUSSION

In the present study, we showed that AnxA3 expression is changed by various factors of hepatocyte growth. These factors can be classified as stimulators and inhibitors of hepatocyte growth. EGF and HGF are typical stimulators of hepatocyte growth in cultured hepatocytes.[14] Other factors belong to the inhibitors group, as follows: Dex suppresses hepatocyte growth in cultured hepatocytes.[19—21] Hepatocytes cultured at high density show lower levels of hepatocyte growth compared with those cultured at low density.[18] Hepatocytes cultured on EHS-Matrigel[22] and PVLA[23] show extremely low levels of hepatocyte growth compared with those cultured on collagen and plastic dishes. From the present findings it is evident that AnxA3 expression is increased and decreased in concurrent with enhancement and suppression of hepatocyte growth by growth stimulatory and inhibitory factors, respectively. The close correlation between known actions of various stimulators and inhibitors on hepatocyte growth and change in AnxA3 expression is consistent with our recent finding that AnxA3 acts as a positive regulator of hepatocyte growth in cultured hepatocytes.[12] In addition, we discovered that enhanced expression of AnxA3 was observed in the proliferative hepatocytes after carbon tetrachloride-induced rat liver damage and 70% partial hepatectomy (unpublished observation). This may lead to speculation that regulation of AnxA3 expression by these factors may be involved in their regulation of hepatocyte growth.

The most marked observation in the present study seems to be that AnxA3 protein is not detected in hepatocytes cultured on EHS-Matrigel (Fig. 5). Interestingly, hepatocytes cultured on EHS-Matrigel show small round-shaped morphology compared with those cultured on the collagen, resembling those *in vivo*,[22] whereas hepatocytes cultured on the collagen were uniformly spread flat. This evidence suggests that AnxA3 expression is dramatically reduced in cultured hepatocytes that show round-shaped morphology. This possibility may be supported by the finding that inverse correlation between DNA synthesis and roundness of hepatocytes is observed in cultured hepatocytes showing various morphologies from round shape to flat shape by coating dishes with different concentrations of PVLA.[23] Although hepatocytes cultured on PVLA also show small round-shaped morphology,[23] reduction of AnxA3 protein under this condition is not so marked compared with that using EHS-Matrigel (Fig. 5). Careful microscopic examination showed that morphology of hepatocytes cultured on EHS-Matrigel is smaller and rounder compared with that of hepatocytes cultured on PVLA (data not shown).

There are many reports showing that various factors regulate expression of other Anxs. For example, Dex and other glucocorticoids stimulate AnxA1 expression *in vitro*[28—32] and *in vivo*,[33—36] in contrast to the present finding. This inconsistency suggests that the mode of regulation by glucocorticoids differs among species of Anxs, tissues, and cells. HGF and EGF stimulate expression of AnxA1, AnxA2, AnxA5, and AnxA6 in primary cultured rat hepatocytes.[37] Interleukin-6[38] and 12-*O*-tetradecanoylphorbor β-acetate[39] stimulate AnxA1 expression in A549 cells and cultured astrocytes, respectively.

In conclusion, the present study shows a close correlation between the known actions of various factors to hepatocyte growth and change in AnxA3 expression, and suggests that the changes in AnxA3 expression associated with these factors could be involved in their regulation of hepatocyte growth.

Acknowledgement This work was supported in part by the Gant-in-Aid for Cancer Research (15-2) from the Ministry of Health, Labor and Welfare.

REFERENCES

1) Crumpton M. J., Dedman J. R., *Nature* (London), **345**, 212 (1990).

2) Gerke V., Moss S. E., *Physiol. Rev.*, **82**, 331—371 (2002).
3) Raynal P., Pollard H. B., *Biochim. Biophys. Acta*, **1197**, 63—93 (1994).
4) Tait J. F., Sakata M., McMullen B. A., Miao C. H., Funakoshi T., Hendrickson L. E., Fujikawa K., *Biochemistry*, **27**, 6268—6276 (1988).
5) Ernst J. D., Hoye E., Blackwood R. A., Jaye D., *J. Clin. Invest.*, **85**, 1065—1071 (1990).
6) Rand J. H., *N. Engl. J. Med.*, **340**, 1035—1036 (1999).
7) Kaetzel M. A., Hazarika P., Dedman J. R., *J. Biol. Chem.*, **264**, 14463—14470 (1989).
8) Pepinsky R. B., Tizard R., Mattaliano R. J., Sinclair L. K., Miller G. T., Browning J. L., Chow E. P., Burne C., Huang K. S., Pratt D., Walchter L., Hession C., Frey A. Z., Wallner B. P., *J. Biol. Chem.*, **263**, 10799—10811 (1988).
9) Comera C., Rothhut B., Cavadore J. C., Vilgrain I., Cochet C., Chambaz E., Russo-Marie F., *J. Cell. Biochem.*, **40**, 361—370 (1989).
10) Kristensen B. I., Kristensen P., Johnsen A. H., *Int. J. Biochem.*, **25**, 1195—1202 (1993).
11) Niimi S., Oshizawa T., Yamaguchi T., Harashima M., Seki T., Ariga T., Kawanishi T., Hayakawa T., *Biochem. Biophys. Res. Commun.*, **300**, 770—774 (2003).
12) Niimi S., Harashima M., Gamou M., Hyuga M., Seki T., Ariga T., Kawanishi T., Hayakawa T., *Biol. Pharm. Bull.*, **28**, 424—428 (2005).
13) Fausto N., Laird A. D., Webber E. M., *Faseb. J.*, **9**, 1527—1536 (1995).
14) Michalopoulos G. K., DeFrances M. C., *Science*, **276**, 60—66 (1997).
15) McGowan J. A., "Isolation and Cultured Hepatocytes," ed. by Guillouzo A., Gugeun-Guillouzo C., John Libey, London, 1986, p. 14.
16) Bucher N. L. R., Malt R. A., "Regeneration of Liver and Kidney," Little, Brown and Co., Boston, 1971.
17) Fausto N., Webber E., "The Liver Biology and Pathology," ed. by Arias I., Boyer J., Fausto N., Jakoby W., Schachter D., Shafritz D., Raven Press, New York, 1994, pp. 1059—1094.
18) Nakamura T., Yoshimoto K., Nakayama Y., Tomita Y., Ichihara A., *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 7229—7233 (1983).
19) Baribault H., Marceau N., *J. Cell. Physiol.*, **129**, 77—84 (1986).
20) Richman R. A., Claus T. H., Pilkis S. J., Friedman D. L., *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3589—3593 (1976).
21) Thoresen G. H., Refsnes M., Dajani O. F., Johansen E. J., Christoffersen T., *Pharmacol. Toxicol.*, **76**, 163—170 (1995).
22) Ben-Ze'ev A., Robinson G. S., Bucher N. L., Farmer S. R., *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2161—2165 (1988).
23) Kobayashi A., Goto M., Sekine T., Masumoto A., Yamamoto N., Kobayashi K., Akaike T., *Artif. Organs*, **16**, 564—567 (1992).
24) Tanaka K., Sato M., Tomita Y., Ichihara A., *J. Biochem.* (Tokyo), **84**, 937—946 (1978).
25) Romisch J., Schuler E., Bastian B., Burger T., Dunkel F. G., Schwinn A., Hartmann A. A., Paques E. P., *Blood Coagul. Fibrinolysis*, **3**, 11—17 (1992).
26) Gordon G. J., Coleman W. B., Grisham J. W., *Am. J. Pathol.*, **157**, 771—786 (2000).
27) Kinoshita T., Tashiro K., Nakamura T., *Biochem. Biophys. Res. Commun.*, **165**, 1229—1234 (1989).
28) Browning J. L., Ward M. P., Wallner B. P., Pepinsky R. B., "Cytokines and Lipocortins in Inflammation and Differentiation," ed. by Melli M., Parente L., Wiley-Liss, New York, 1990, pp. 27—45.
29) Croxtall J. D., Flower R. J., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3571—3575 (1992).
30) Solito E., Raugei G., Melli M., Parente L., *FEBS Lett.*, **291**, 238—244 (1991).
31) Blackwell G. J., Carnuccio R., Di Rosa M., Flower R. J., Parente L., Persico P., *Nature* (London), **287**, 147—149 (1980).
32) Perretti M., Flower R. J., *Br. J. Pharmacol.*, **118**, 605—610 (1996).
33) Goulding N. J., Godolphin J. L., Sharland P. R., Peers S. H., Sampson M., Maddison P. J., Flower R. J., *Lancet*, **335**, 1416—1418 (1990).
34) Peers S. H., Smillie F., Elderfield A. J., Flower R. J., *Br. J. Pharmacol.*, **108**, 66—72 (1993).
35) Fuller P. J., Verity K., *J. Immunol.*, **143**, 1015—1017 (1989).
36) Peers S. H., Flower R. J., *Eur. J. Pharmacol.*, **196**, 37—41 (1991).
37) Della Gaspera B., Braut-Boucher F., Bomsel M., Chatelet F., Guguen-Guillouzo C., Font J., Weinman J., Weinman S., *Dev. Dyn.*, **222**, 206—217 (2001).
38) Solito E., de Coupade C., Parente L., Flower R. J., Russo-Marie F., *Cytokine*, **10**, 514—521 (1998).
39) Gebicke-Haerter P. J., Schobert A., Dieter P., Honegger P., Hertting G., *J. Neurochem.*, **57**, 175—183 (1991).

4 ICHガイドライン

川西　徹*

4.1 ICHの歴史

日本・米国・ヨーロッパでは，それぞれの地域で時期は異なるものの，医薬品に関する事件や事故を経験した結果，医薬品は販売開始前に政府による評価・承認が必要であるという同様な認識をもつようになり，それぞれ独自に法制度を整備してきた。特に1960年代から1970年代に，各国で法令やガイドラインが急速に整備され，新医薬品の品質，有効性および安全性についてのデータ報告・評価の体制が整った。

しかし，品質，有効性，安全性を評価するという基本では共通していたものの，承認申請の際の詳細な技術的要件は地域によって異なっていた。一方，この間に，製薬企業は国際的に医薬品の製造・販売を行うようになってきており，新しい医薬品を地域を越えて上市するためには，地域間で異なる規制要件を満たすため，品質，有効性，安全性の評価という同じ目的であるにもかかわらず，時間とコストのかかる重複した試験を数多く行う必要があった。このことは，医薬品開発コスト，さらには健康管理コストの上昇を招くばかりでなく，何よりも必要な患者への安全で有効な新医薬品の供給を妨げる要因となり，各地域の医薬品承認審査の基準の合理化・標準化の必要性が叫ばれるようになった。

このような背景の中で，世界的な規模で医薬品の承認に必要な技術的要件を調和するという動きは1980年代中頃に始まったが，1989年にパリで開催された国際保健機関（WHO）の医薬品規制当局者会議において日・米・EU医薬品規制調和国際会議（ICH）を創設することが決められ，1990年4月，日本，米国，ヨーロッパの各医薬品規制当局と業界団体の6者によりICHが発足した。この会議でICHの運営方針を決定するICH運営委員会が作られ，少なくとも年2回の会合を行うとともに，ICH国際会議も2〜3年に1回程度行われている。

ICH発足以来，50を超えるガイドラインが合意（調和）に至り，各地域で実施されてきた。新医薬品の品質，有効性，安全性の評価にかかわる技術的なガイドラインだけでなく，最近では承認申請資料の形式，市販後安全性体制などにもその対象は広がっている。またICHに参加していない地域との交流，情報の共有化も進んでいる。

4.2 ICHの組織

ICHは日本，米国，EUの各医薬品規制当局（日本：厚生労働省，米国：食品医薬品庁（FDA），EU：欧州委員会（EC））および各極の産業界（日本：日本製薬協会（JPMA），米国：米国製薬

* Toru Kawanishi　国立医薬品食品衛生研究所　薬品部　部長

工業協会（PhRMA），EU：欧州製薬団体連合会（EFPIA））の6者から構成されており，さらに，オブザーバーとして世界保健機構（WHO），カナダ保健省，欧州自由貿易連合（EFTA）が参加している。

ICHでは品質，有効性，安全性，あるいは複合領域のトピックごとに各極の専門家が専門家作業部会で協議し，ガイドライン等を作成する。また各局からの代表者によって構成される運営委員会では，各トピックの進行管理やガイドラインの最終決定等の管理・運営を行う。

4.3　ICH品質ガイドライン

ICH国際調和ガイドラインは，品質，有効性，安全性および複合領域の大きく4群に分けられる。以下に品質に関連するガイドラインを簡単に紹介する。個々のガイドラインは医薬品医療機器総合機構のHPに掲載されており（http://www.pmda.go.jp/ich/ich_index.html），調和原文および国内通知のダウンロードが可能である（表1参照）。

4.3.1　新有効成分含有医薬品の品質データ作成に関する技術課題を扱ったガイドライン（化学合成医薬品）

ICH品質分野ガイドラインの国際調和作業は，新医薬品申請にあたって必要とされる品質データに関する技術的ガイドラインから開始された。即ち，安定性試験ガイドライン（Q1），分析バリデーションガイドライン（Q2），原薬不純物ガイドライン（Q3A）である。

安定性試験ガイドラインとしてはその後光安定性試験（Q1B），新投与経路医薬品への安定性試験の適用（Q1C），ブラッケンティング法及びマトリキシング法の適用（Q1D），長期保存安定性試験からのリテスト期間又は製剤有効期間の外挿（Q1E）等が追加され，整備された。

分析バリデーションガイドラインについては，分析バリデーションの実施項目ガイドライン（Q2A）がまず作成されたが，その後改訂が行われ，分析バリデーションの実施方法に関するガイドライン（Q2B）が追加され，完成された。

純度試験ガイドラインでは，原薬不純物ガイドライン（Q3A）に続いて，製剤不純物ガイドライン（Q3B）が作成され，さらに医薬品中の残留溶媒ガイドライン（Q3C）が整備された。残留溶媒ガイドラインについては，その後も科学的知見の増加に伴い，限度値の見直し等が行われている。

このように品質試験に関する特定な技術課題を扱った3群のガイドラインシリーズと平行して，医薬品品質管理の主要な方策である，規格及び試験方法に関するガイドライン（Q6A）が作成された。

以上の品質試験の技術的課題に関するガイドラインに引き続くものとして，今現在，国際調和作業中の品質ガイドラインに，国際調和薬局方試験の評価および推奨に関するガイドライン

表1　ICH 国際調和品質ガイドラインおよびその国内通知一覧

（平成 20 年 9 月 30 日現在）

(1) 安定性試験	
Q1A (R2)	安定性試験ガイドライン（平成 15 年 6 月 3 日 医薬審発第 0603001 号 厚生労働省医薬局審査管理課長通知）："Stability Testing of New Drug Substances and Products"
Q1B	新原薬及び新製剤の光安定性試験ガイドライン（平成 9 年 5 月 28 日 薬審第 422 号 厚生省薬務局審査課長通知）："Stability Testing: Photostability Testing of New Drug Substances and Products"
Q1C	新投与経路医薬品等の安定性試験成績の取扱いに関するガイドライン（平成 9 年 5 月 28 日 薬審第 425 号 厚生省薬務局審査課長通知）："Stability Testing for New Dosages Forms"
Q1D	原薬及び製剤の安定性試験へのブラケッティング法及びマトリキシング法の適用（平成 14 年 7 月 31 日 医薬審発第 0731004 号 厚生労働省医薬局審査管理課長通知）："Bracketing and Matrixing Designs for Stability Testing of New Drug Substances and Products"
Q1E	安定性データの評価に関するガイドライン（平成 15 年 6 月 3 日 医薬審発第 0603004 号 厚生労働省医薬局審査管理課長通知）："Evaluation of Stability Data"
Q1F	「気候区域 III 及び IV における承認申請のための安定性試験成績に関するガイドライン」の廃止（平成 18 年 7 月 3 日 薬食審査発第 0703001 号 厚生労働省医薬食品局審査管理課長通知）："Explanatory Note on the Withdrawal of ICH Q1F for the ICH Website"
(2) 分析バリデーション	
Q2 (R1)	Q2A 分析法バリデーションに関するテキスト（実施項目）（平成 7 年 7 月 20 日 薬審第 755 号 厚生省薬務局審査課長通知，平成 9 年 10 月 28 日 医薬審第 338 号 一部改正）
Q2B	分析法バリデーションに関するテキスト（実施方法）（平成 9 年 10 月 28 日 医薬審第 338 号 厚生省医薬安全局審査管理課長通知）："Validation of Analytical Procedure: Methodology: Text and Methodology"
(3) 不純物の試験	
Q3A (R2)	新有効成分含有医薬品のうち原薬の不純物に関するガイドライン（平成 14 年 12 月 16 日 医薬審発第 1216001 号 厚生労働省医薬局審査管理課長通知） 新有効成分含有医薬品のうち原薬の不純物に関するガイドラインの一部改定（平成 18 年 12 月 4 日 薬食審査発第 1204001 号 厚生労働省医薬食品局審査管理課長通知）："Impurities in New Drug Substances"
Q3B (R2)	新有効成分含有医薬品のうち製剤の不純物に関するガイドライン（平成 15 年 6 月 24 日 医薬審発第 0624001 号 厚生労働省医薬局審査管理課長通知）新有効成分含有医薬品のうち製剤の不純物に関するガイドラインの一部改定（平成 18 年 7 月 3 日 薬食審査発第 0703004 号 厚生労働省医薬食品局審査管理課長通知）："Impurities in New Drug Products"
Q3C (R3)	Q3C 医薬品の残留溶媒ガイドライン（平成 10 年 3 月 30 日 医薬審第 307 号 厚生省医薬安全局審査管理課長通知）
Q3C (M)	医薬品の残留溶媒ガイドライン N-メチルピロリドン（N-Methylpyrrolidone）の PDE 値について／テトラヒドロフラン（Tetrahydrofuran）の PDE 値について（平成 14 年 12 月 25 日 医薬審発第 1225006 号 厚生労働省医薬局審査管理課長通知）："Impurities: Guideline for Residual Solvents" 医薬品残留溶媒の限度値について（平成 14 年 12 月 3 日 厚生労働省医薬局審査管理課事務連絡）
(4) 生物薬品の品質	
Q5A (R1)	ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウィルス安全性評価（平成 12 年 2 月 22 日 医薬審第 329 号 厚生省医薬安全局審査管理課長通知）："Viral Safety Evaluation on Biotechnology Products Development from Cell Lines of Human or Animal Origin"
Q5B	組換え DNA を応用したタンパク質生産に用いる細胞中の遺伝子発現構成体の分析（平成 10 年 1 月 6 日 医薬審第 3 号 厚生省医薬安全局審査管理課長通知）："Quality of Biotechnological Products analysis of the Expression Construct in Cells Used for Production of R-DNA Derived Protein Products"

（続く）

（続き）

Q5C	生物薬品（バイオテクノロジー応用製品／生物起源由来製品）の安定性試験（平成10年1月6日 医薬審第6号 厚生省医薬安全局審査管理課長通知）："Quality of Biotechnological Products: Stability Testing of biotechnological/Biological Products"
Q5D	生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）製造用細胞基剤の由来，調製及び特性解析（平成12年7月14日 医薬審第873号 厚生省医薬安全局審査管理課長通知）："Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products"
Q5E	生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）の規格および試験方法の設定（平成17年4月26日 薬食審査発第0426001号 厚生労働省医薬食品局審査管理課長通知）："Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process."
(5) 規格及び試験方法	
Q6A	新医薬品の規格及び試験方法の設定（平成13年5月1日 医薬審発第568号 厚生労働省医薬局審査管理課長通知）："Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products"
Q6B	生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）の規格及び試験方法の設定（平成13年5月1日 医薬審発第571号 厚生労働省医薬局審査管理課長通知）："Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products"
(6) GMP（医薬品の製造管理および品質管理に関する基準）	
Q7	原薬GMPのガイドライン（平成13年11月2日 医薬発第1200号 厚生労働省医薬局長通知）："Good Manufacturing Practice Guide for Active Pharmaceutical ingredients"
(7) 製剤開発	
Q8	製剤開発に関するガイドライン（平成18年9月1日 薬食審査発第0901001号 厚生労働省医薬食品局審査管理課長通知）："Pharmaceutical Development"
Q8 Annex	ICH Q8 Annex：製剤開発付属書："Q8-Annex Pharmaceutical Development"（平成20年9月現在ステップ2）
(8) 品質リスクマネジメント	
Q9	品質リスクマネジメントに関するガイドライン（平成18年9月1日 薬食審査発第0901004号 厚生労働省医薬食品局審査管理課長，薬食監麻発第0901005号厚生労働省医薬食品局監視指導・麻薬対策課長）："Quality Risk Management"
品質リスクマネジメントICHQ9ブリーフィング・パック："Quality Risk Management ICH Q9 Briefing Pack"	
(9) 医薬品品質システム	
Q10	医薬品品質システムに関するガイドライン（国内通知準備中）："Pharmaceutical Quality System"（Step 4 at June 4, 2008）
(10) 薬局方国際調和の評価関係	
Q4B	国際調和薬局方一般試験法の評価および推奨："Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions"（Step 4 at November 1, 2007）
Q4B-Annex 1	強熱残分試験法："Residue on ignition/Sulphate Ash General Chapter"（Step 4 at November 1, 2007）
Q4B-Annex 2	注射剤の採取容量試験法："Evaluation and Recommendation of Pharmacopoeial Texts for use in the ICH region on Test for Extractable volume of Pharenteral Preparations General Chapter"（Step 4 at June 5, 2008）
Q4B-Annex 3	注射剤の不溶性微粒子試験法："Evaluation and Recommendation of Pharmacopoeial Texts for use in the ICH Regions on Test for Particulate Contamination: Sub-Visible Particles General Chapter"（Step 4 at June 5, 2008）

（続く）

（続き）

Q4B-Annex 4	非無菌製品の微生物学的試験："Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbial Examination of Non-Sterile Products General Chapter"（Step 2）
Q4B-Annex 5	崩壊試験法："Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Disintegration Test General Chapter"（Step 2）
(11) 承認申請書に添付すべき国際共通化資料（コモン・テクニカル・ドキュメント）	
	新医薬品の製造又は輸入の承認申請に際し承認申請書に添付すべき資料の作成要領について（平成13年6月21日 医薬審発第899号 平成13年6月21日厚生労働省医薬局審査管理課長）：「新医薬品の製造又は輸入の承認申請に際し承認申請書に添付すべき資料の作成要領について」の一部改正について（平成15年7月1日 薬食審発第0701004号 厚生労働省医薬食品局審査管理課長通知）
M4	別紙1：医薬品の承認申請のための国際共通化資料コモン・テクニカル・ドキュメント（CTD）の構成："Organization of the Common Technical Document for the Registration of Pharmaceuticals for Human Use"
M4Q	別紙3：医薬品の承認申請のための国際共通化資料（コモン・テクニカル・ドキュメント）CTD－品質に関する文書の作成要領に関するガイドライン："The Common Technical Document for the Registration of Pharmaceuticals for Human Use: MODULLE 2 : Quality of Overall Summary（QOS）; MODULE 3 : Quality"
M4Q に関する Q&A	
	CTD-品質に関する文書の作成要領に関するガイドラインに対する Q&A（平成13年10月22日 事務連絡 厚生労働省医薬局審査管理課）："Q&A on the CTD-Quality Guideline"
	CTD-品質に関する文書　Q&A／記載箇所に関する事項（平成15年11月15日 事務連絡 厚生労働省医薬食品局審査管理課）：Common "Technical Document-Quality Question and Answers/Location Issues"

（Q4B）がある。

これは Q6A ガイドライン作成の過程で，医薬品の規格及び試験法の国際調和を実効あるものとするために極めて重要と指摘された，薬局方一般試験法（含量均一性試験法，重量偏差試験法，溶出試験法，崩壊試験法，微生物限度試験法，不溶性微粒子試験法，注射剤の採取容量試験法，強熱残分試験法，着色度及び澄明度試験法，無菌試験法，エンドトキシン試験法）の国際調和に関連するガイドラインである。局方試験法の国際調和は薬局方検討会議（PDG）が行っているが，各局方に取り込まれた後，各極の規制当局が受入なければ実効あるものとならない。

そこで，PDG で国際調和され局方に取り込まれた試験法を，ICH の Q4B 専門家が順次評価，確認し，必要に応じて PDG が調整を行った上で，3極の規制当局が相互受入する上で考慮すべき事項をまとめるというものである。Q4B ガイドライン本体は Q4B の専門家会議の運営方針，評価方法等を記したものであるが，現在国際調和一般試験法個々（強熱残分試験法，注射剤の採取容量試験法，注射剤の不溶性微粒子試験法等）について検討し，その結果を補遺（Annex）としてまとめ，順次追加している。

4. 3. 2　新有効成分含有医薬品の品質データ作成に関する技術課題を扱ったガイドライン（生物薬品）

生物薬品（バイオテクノロジー応用製品／生物起源製品）では，遺伝子組換え製品の生産に用いる遺伝子発現構成体の分析（Q5B），生物薬品の製造用細胞に関するガイドライン（Q5D），生物薬品の安定性試験ガイドライン（Q5C），生物薬品のウィルス安全性評価ガイドライン（Q5A）の各ガイドラインが作成され，これらに引き続いて，生物薬品の規格及び試験方法ガイドライン（Q6B）が作成された。

以上の生物薬品の開発過程における特定の課題を対象とした技術的ガイドラインに引き続き，製造工程の変更の際に実施すべき同等性／同質性評価に関するガイドライン（Q5E）が作成された。

4. 3. 3　医薬品の製造管理に関するガイドライン

上記の医薬品品質データ作成に関する技術ガイドラインとともに，化学合成医薬品および生物薬品を適用対象として，医薬品の製造施設，および製造管理を扱った医薬品原薬GMPガイドライン（Q7A）が作成された。

4. 3. 4　新医薬品の承認申請にあたって添付すべき国際共通化資料に関するガイドライン

医薬品の承認申請にあたっての技術的要件と同様に，承認申請にあたって添付すべき資料が国際間で異なっていることが，地域を超えた医薬品の迅速な普及の障害の一因であることが指摘されていたため，ICHでは承認申請に添付すべき国際共通化資料（コモンテクニカルドキュメント：CTD）に関するガイドラインの作成が行われた。これは品質データのみならず，非臨床，臨床データについても同様に作成されたが，品質については「CTD-品質に関する文書の作成要領に関するガイドライン（M4Q）」が作成され，概要資料（モジュール2），品質に関する文書（モジュール3）の作成にあたって，記載すべき項目とその配列順序が示された。

4. 4　新しいICH品質ガイドライン—医薬品のライフサイクル全般にわたる品質管理システム構築をめざして—

ICH品質分野の国際調和活動では，上記のように医薬品開発あるいは新医薬品の承認申請に関する技術課題に関するガイドライン，および新医薬品の製造販売承認申請に際して添付資料として提出すべきCTDの国際調和が一段落した。そこで，その後に続くべき新たな医薬品品質関連のテーマが検討され，2003年7月のブラッセル会議GMPワークショップにおいて，「製造科学とリスク管理手法を統合したアプローチによる，医薬品のライフサイクル（開発から市販後）全般に適用する新しい品質管理システムの構築」が提案され，品質の中心テーマとして取り上げることに三極は合意した。その後この合意に従い，「製剤開発に関するガイドライン（Q8）」，「

品質リスク管理に関するガイドライン（Q9）」および「医薬品品質システムに関するガイドライン（Q10）」が国際調和され，現在さらに「Q8-R 製剤開発ガイドライン付属書」の国際調和作業が行われている。続いてこの方向で「原薬の製造方法に関するガイドライン（Q11）」がテーマとして取り上げられた。

このような新しい品質システム構築が提唱された背景としては，米国 FDA の医薬品品質管理についての危機意識があるものと思われる。即ち医薬品は製造開発企業にとって工業製品であるが，通常ヒトの体内に投与され健康に直接的に係わるがゆえに，歴史的に極めて厳しい規制が行われてきた。そのため，医薬品の開発製造コストが高騰し，承認までの時間も延長し，医薬品開発は困難なものとなりつつある。また一度開発，承認されても，品質の向上あるいは製造コストの改善等を目指した製法変更にあたっては，規制当局による承認あるいは届出が科せられ，実施までに時間，経費がかかる。そのため製造方法の変更を避ける傾向にあり，工業製品の中でも製造管理は旧態依然のシステムで行われていることが少なくない。

一方規制側からみると，製法変更に関する承認審査，あるいは GMP 査察のために大きなリソースが必要とされるため，規制コストの増大を招いている。このような問題を解決するために，医薬品の開発・品質管理に製造科学と品質リスク管理の考えを導入し，品質管理システムを近代化させる必要がある。このような FDA の方向は，巨大化，グローバル化の方向にある先進的医薬品開発企業との利害とも一致し，ICH においても推進すべきテーマとしてクローズアップされたものと考えられる。

4. 4. 1　Q8 製剤開発に関するガイドライン

Q8 ガイドラインは，製品および製造工程の開発に際してとられたアプローチを説明するための国際共通化資料（CTD）の第 3 部 3. 2. P.2 項「製剤開発の経過」の項で推奨される記載内容に関するガイドラインとして作成されたものである。しかし本ガイドラインは上記の新しい開発手法の実践を推奨するものである。すなわち，承認申請者は，製剤開発において「経験に基づく（旧来の）アプローチ（「最小限のアプローチ approach at a minimum」，あるいは「基本的アプローチ basement approach」と表現されている）」，あるいは「より体系的な（新しい）アプローチ」のどちらでも選択できるということは三極間で合意された見解であり，後者のアプローチの選択は義務的なものではないが，本ガイドラインでは記述のほぼ 100% を後者のアプローチの説明に割いている。

この体系的アプローチは一般には Quality by Design（QbD）アプローチと呼ばれている。このガイドラインにおいて「より体系的なアプローチ」の意味するところは，環境要因，工程上の要因，原材料，品質特性といった工程上の重要な要素を確認し，これら要素が医薬品の性能や品質へ及ぼす影響をリスクアセスメント手法を用いて解析，その結果に基づいて品質管理システム