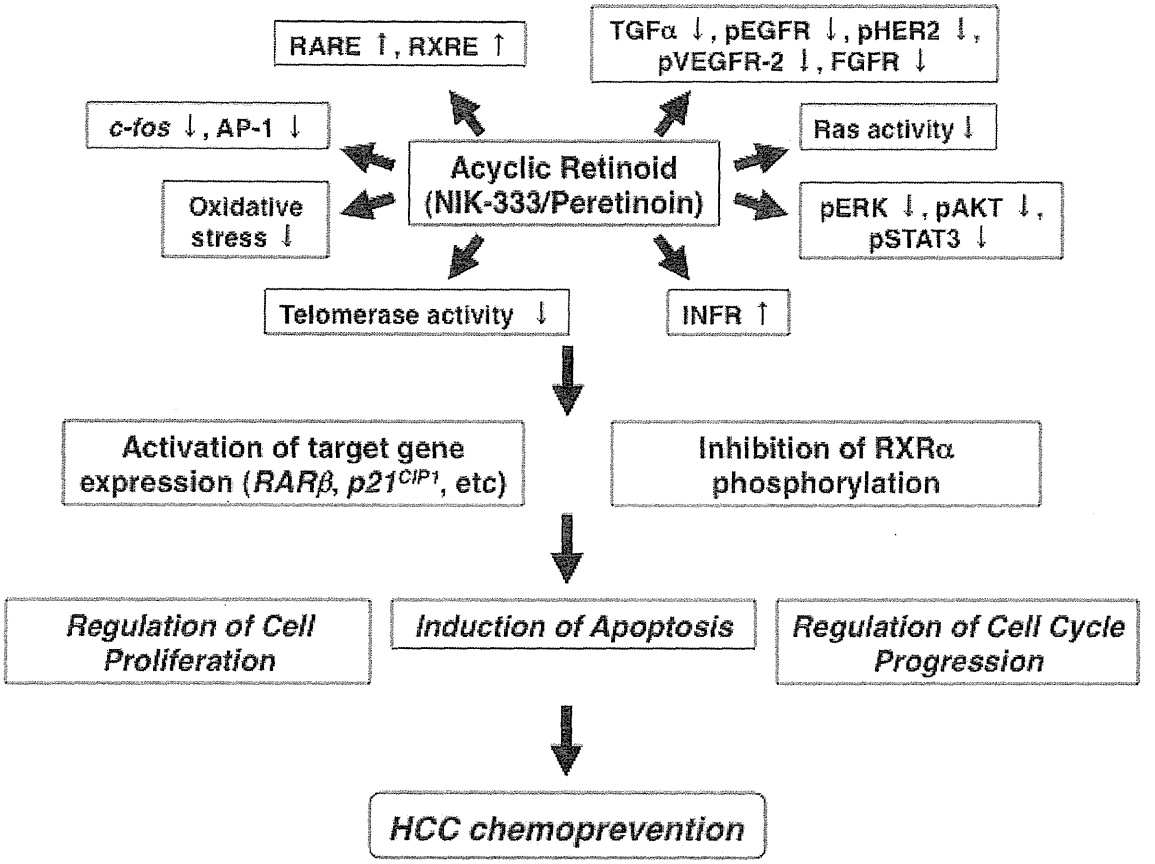

Figure 3. Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes, such as *RARβ* and $p21^{CIP1}$, by upregulating the promoter activity of RARE and RXRE. In addition, ACR suppresses cancer cell growth by inhibiting activation and expression of some types of RTKs, including EGFR, HER2, VEGFR-2, and FGFR, which contribute to the subsequent inhibition of Ras-MAPK activation and RXRα phosphorylation. Phosphorylation of Akt and Stat3 proteins are also inhibited by ACR. Induction of RARβ and restoration of the function of RXRα due to dephosphorylation by ACR leads to cooperative regulation of cell proliferation, cell cycle progression, and induction of apoptosis, thus preventing the development of HCC. ACR also induces the expression of IFN receptor (INFR), inhibits transcriptional activity of *c-fos* and AP-1 promoters, and down-regulates telomerase activity in HCC and squamous cell carcinoma cells. ACR also suppresses liver tumorigenesis by repressing oxidative stress. Detailed discussion of these findings may be found in previous articles (6-8, 11-13, 36-50, 53, 58, 60-62).

particularly relevant to field cancerization. Once a liver is exposed to continuous carcinogenic insults, such as hepatitis viral infection and alcohol toxicity, the whole exposed liver is regarded as a precancerous lesion which possesses multiple as well as independent premalignant or latent malignant clones. Hence, even if the first cancer is diagnosed and removed early, the next clone essentially arises to form a secondary HCC. Therefore, the most effective strategy for HCC chemoprevention is the deletion of latent malignant clones (clonal deletion) and inhibition of the evolution of such clones (clonal inhibition) before they expand into clinically detectable tumors. We have proposed that implementation of this novel concept, "clonal deletion" therapy, which is defined as the removal of latent malignant (or premalignant) clones that are invisible by diagnostic imaging from the liver when it is in a hypercarcinogenic state, is fundamental to the chemoprevention of HCC (Figure 4) (6-8).

ACR has been used to effectively demonstrate this concept in the clinical setting. In the clinical trial, serum levels of lectin-reactive α-fetoprotein factor 3 (AFP-L3), which indicates the presence of latent (*i.e.*, invisible) malignant clones in the remnant liver, were significantly reduced by 12-month administration of ACR (52). This observation indicates that ACR eliminates or removes the AFP-L3 producing premalignant clones from the remnant liver before they expanded into clinically detectable (*i.e.*, visible) tumors, thereby inhibiting secondary HCC. Moreover, ACR suppressed the appearance of serum AFP-

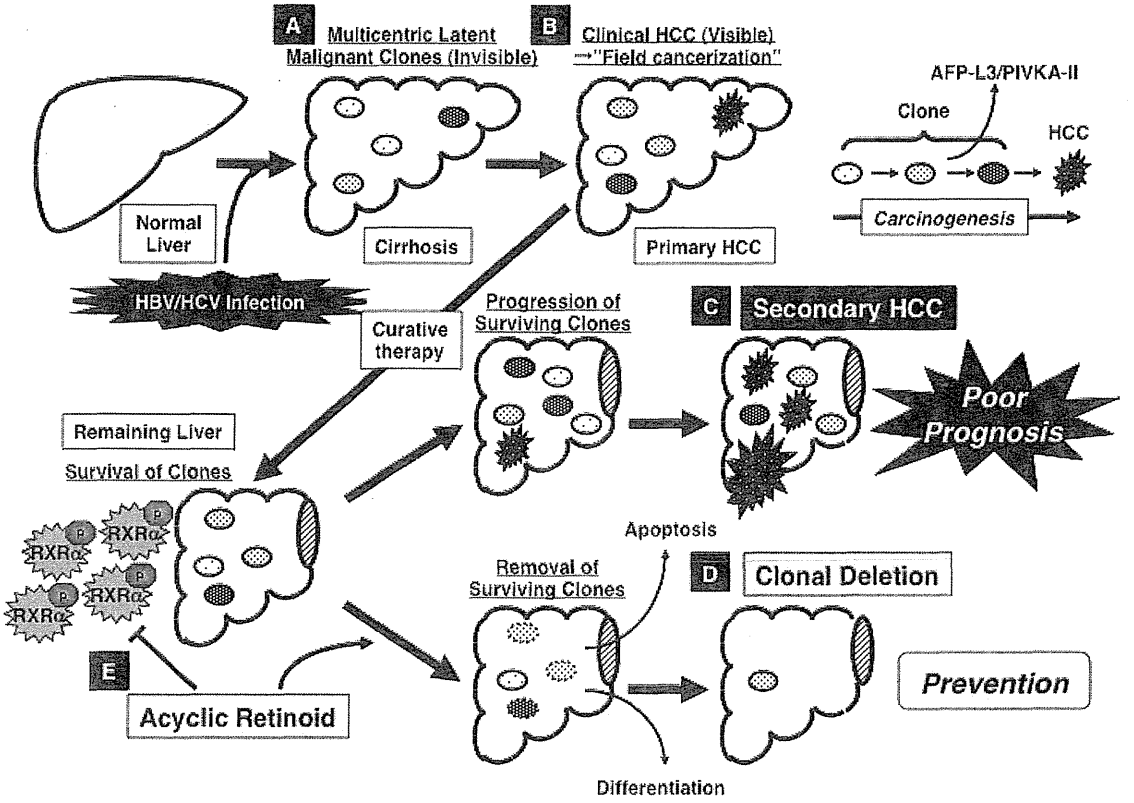

Figure 4. The concept of "clonal deletion" therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a "precancerous field", which consists of multiple latent malignant clones that arise through multicentric carcinogenesis and are clinically undetectable by image analysis (invisible) (A). These multiple clones demonstrate different grades of malignancy in the cirrhotic liver and, at some point, turn into clinical (visible) HCC ("field cancerization") (B). Even when primary HCC is found and removed early, the other clones survive in the remaining liver and grow into secondary HCC, which is a major cause of the poor prognosis for patients with this malignancy (C). Therefore, one of the most promising strategies to prevent secondary HCC is deletion of such transformed clones by inducing cell differentiation or apoptosis before they expand into clinically detectable tumors (the concept of "clonal deletion" therapy) (D). ACR, which targets phosphorylated RXRα (E), prevents the recurrence and development of secondary HCC via the mechanism described by this concept; ACR decreased the serum levels of AFP-L3 and PIVKA-II, which are produced by latent malignant clones, thus demonstrating the eradication and inhibition of these clones. Once such clones are deleted, the preventive effect on HCC lasts several years without continuous administration of ACR. Therefore, ACR can significantly improve the survival rate of such patients.

L3 in patients whose AFP-L3 levels were negative at trial enrollment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group; these patients had a significantly higher risk of secondary HCC (52). This finding suggests that, in addition to elimination, ACR actively inhibits the development of AFP-L3-producing clones, which have the potential to become HCC. This is one of the reasons why only a short-term administration (12 months) of ACR exerted a long-term preventive effect on HCC development for several years after termination of treatment (16). It takes several years for the next cancer clones to arise clinically once they are eliminated or inhibited. Therefore, the promise of clonal deletion seems to be therapeutic rather than preventive, and ACR prevents the development of HCC by this mechanism.

8. "COMBINATION CHEMOPREVENTION" OF HCC USING ACR AS THE KEY DRUG

Combination therapy is often advantageous because it provides the potential for synergistic effects between specific drugs; ACR is no exception in this regard. For instance, ACR acts synergistically with interferon (IFN)-β in suppressing growth and inducing apoptosis in human HCC cell lines via upregulation of type 1 IFN receptor and Stat1 expression by ACR (53). The combination of ACR plus vitamin K_2 (VK_2) synergistically inhibits cell growth and induces apoptosis in HCC cells

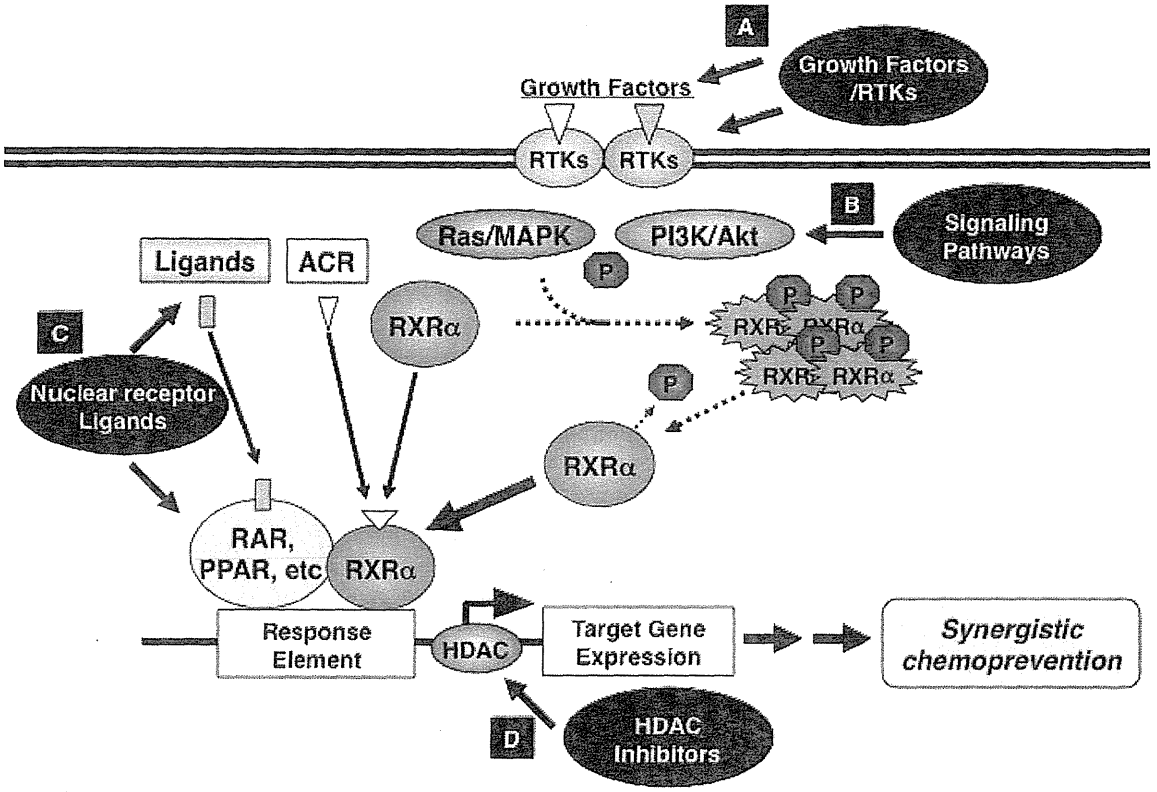

Figure 5. The possibility of "combination chemoprevention" for HCC using ACR as the key agent. Dephosphorylation of RXRα and subsequent restoration of the function of this nuclear receptor are critical to prevent the development of HCC. Therefore, the agents which target growth factor and their corresponding RTKs (A), as well as their related signaling pathways (B), including the Ras-MAPK and PI3K-Akt signaling pathways that phosphorylate RXRα, might be good partners for ACR to exert synergistic effects on the chemoprevention of HCC. The ligands for the nuclear receptors, which form heterodimers with RXR such as RAR and PPAR (C), are also able to enhance the chemopreventive effect of ACR through the activation of target gene expression. HDAC inhibitors increase the expression of ACR-target genes by remodeling the chromatin template and increasing histone acetylation, which suggests that the combination of ACR plus HDAC inhibitors may also be a promising regimen for HCC chemoprevention (D).

without affecting the growth of normal human hepatocytes (12). These findings are significant when considering the clinical use of ACR because both IFN and VK_2 are expected to exert preventive effects on the development and recurrence of HCC (54-57). Therefore, we assume that "combination chemoprevention" using ACR as the key agent may be a useful strategy to prevent the development of HCC.

The expected mechanisms of ACR-based combination chemoprevention are schematically summarized in Figure 5. Initially, specific agents that target the Ras-MAPK signaling pathway and its upstream RTKs are among the most promising partners for ACR because these agents dephosphorylate RXRα. Indeed, ACR and VK_2 cooperatively inhibit activation of the Ras-MAPK signaling pathway, thus suppressing the phosphorylation of RXRα and the growth of HCC cells (12). The combination of 9-*cis* RA (58) or ACR (unpublished data) plus trastuzumab, a humanized anti-human epidermal growth factor receptor-2 (HER2) monoclonal antibody, synergistically inhibits growth and induces apoptosis in HCC cells via cooperative inhibition of the activation of HER2 and its downstream signaling molecules, including ERK and Akt, and subsequent dephosphorylation of RXRα. Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, acts synergistically to induce apoptosis and G_0-G_1 cell cycle arrest in HCC cells by inhibiting phosphorylation of RXRα, ERK, Akt, and GSK-3β proteins (13).

In addition to dephosphorylation of RXRα, induction of nuclear receptors that dimerize RXR, such as RAR and PPAR (33, 59), and recruitment of their ligands may also exert synergistic growth inhibition in cancer cells when combined with ACR. Both valproic acid (13) and OSI-461 (43), a potent derivative of sulindac sulfone, enhance the ability of ACR to raise the cellular levels of

RARβ and $p21^{CIP1}$, thereby markedly increasing the RARE and RXRE promoter activities and inducing apoptosis in HCC cells. Therefore, these combinations may also be an effective regimen for the chemoprevention and chemotherapy of HCC.

9. PERSPECTIVE

The prevention of HCC is an urgent task on a global scale, and one of the most practical approaches to the accomplishment of this purpose is "clonal deletion" therapy. Experimental studies strongly suggest that RXRα phosphorylation is profoundly involved in liver carcinogenesis and thus may be a critical target for HCC chemoprevention. Clinical trials reveal that ACR, which inhibits RXRα phosphorylation but induces RARβ expression, is a promising candidate for HCC chemoprevention by putting the concept of "clonal deletion" in practice. ACR-based combination chemoprevention, which is expected to exert synergism, also holds great promise as a master therapeutic for HCC chemoprevention. In conclusion, ACR may play a critical role in preventing HCC development when it is used alone or combined with other drugs and, therefore, early clinical application of this agent is greatly anticipated.

10. ACKNOWLEDGEMENTS

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11. REFERENCES

1. El-Serag, H. B.; Rudolph, K. L. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 132:2557-2576 (2007)

2. Parikh, S.; Hyman, D. Hepatocellular cancer: a guide for the internist. *Am J Med* 120:194-202 (2007)

3. Ince, N.: Wands, J. R. The increasing incidence of hepatocellular carcinoma. *N Engl J Med* 340:798-799 (1999)

4. Kumada, T.: Nakano, S.: Takeda, I.: Sugiyama, K.: Osada, T.: Kiriyama, S.: Sone, Y.: Toyoda, H.: Shimada, S.: Takahashi, M.: Sassa, T. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 25:87-92 (1997)

5. Koda, M.: Murawaki, Y.: Mitsuda, A.: Ohyama, K.: Horie, Y.: Suou, T.: Kawasaki, H.: Ikawa, S. Predictive factors for intrahepatic recurrence after percutaneous ethanol injection therapy for small hepatocellular carcinoma. *Cancer* 88:529-537 (2000)

6. Shimizu, M.: Takai, K.: Moriwaki, H. Strategy and mechanism for the prevention of hepatocellular carcinoma: phosphorylated retinoid X receptor alpha is a critical target for hepatocellular carcinoma chemoprevention. *Cancer Sci* 100:369-374 (2009)

7. Moriwaki, H.: Shimizu, M.: Okuno, M.: Nishiwaki-Matsushima, R. Chemoprevention of liver carcinogenesis with retinoids: Basic and clinical aspects. *Hepatol Res* 37 Suppl 2:S299-302 (2007)

8. Okuno, M.: Kojima, S.: Akita, K.: Matsushima-Nishiwaki, R.: Adachi, S.: Sano, T.: Takano, Y.: Takai, K.: Obora, A.: Yasuda, I.: Shiratori, Y.: Okano, Y.: Shimada, J.: Suzuki, Y.: Muto, Y.: Moriwaki, H. Retinoids in liver fibrosis and cancer. *Front Biosci* 7:d204-218 (2002)

9. Matsushima-Nishiwaki, R.: Okuno, M.: Adachi, S.: Sano, T.: Akita, K.: Moriwaki, H.: Friedman, S. L.: Kojima, S. Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma. *Cancer Res* 61:7675-7682 (2001)

10. Sporn, M. B.: Newton, D. L. Chemoprevention of cancer with retinoids. *Fed Proc* 38:2528-2534 (1979)

11. Matsushima-Nishiwaki, R.: Okuno, M.: Takano, Y.: Kojima, S.: Friedman, S. L.: Moriwaki, H. Molecular mechanism for growth suppression of human hepatocellular carcinoma cells by acyclic retinoid. *Carcinogenesis* 24:1353-1359 (2003)

12. Kanamori, T.: Shimizu, M.: Okuno, M.: Matsushima-Nishiwaki, R.: Tsurumi, H.: Kojima, S.: Moriwaki, H. Synergistic growth inhibition by acyclic retinoid and vitamin K2 in human hepatocellular carcinoma cells. *Cancer Sci* 98:431-437 (2007)

13. Tatebe, H.: Shimizu, M.: Shirakami, Y.: Sakai, K.: Yasuda, Y.: Tsurumi, H.: Moriwaki, H. Acyclic retinoid synergises with valproic acid to inhibit growth in human hepatocellular carcinoma cells. *Cancer Lett* 285:210-217 (2009)

14. Muto, Y.: Moriwaki, H.: Ninomiya, M.: Adachi, S.: Saito, A.: Takasaki, K. T.: Tanaka, T.: Tsurumi, K.: Okuno, M.: Tomita, E.: Nakamura, T.: Kojima, T. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. *N Engl J Med* 334:1561-1567 (1996)

15. Muto, Y.: Moriwaki, H.: Saito, A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. *N Engl J Med* 340:1046-1047 (1999)

16. Takai, K.: Okuno, M.: Yasuda, I.: Matsushima-Nishiwaki, R.: Uematsu, T.: Tsurumi, H.: Shiratori, Y.: Muto, Y.: Moriwaki, H. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. Updated analysis of the long-term follow-up data. *Intervirology* 48:39-45 (2005)

17. Okita, K.: Matsui, O.: Kumada, H.: Tanaka, K.: Kaneko, S.: Moriwaki, H.: Izumi, N.: Okusaka, T.: Ohashi, Y.: Makuuchi, M. Effect of peretinoin on recurrence of

hepatocellular carcinoma (HCC): Results of a phase II/III randomized placebo-controlled trial. Peretinoin Study Group. *J Clin Oncol* 28 Suppl 7s:4024 (2010)

18. Germain, P.: Chambon, P.: Eichele, G.: Evans, R. M.: Lazar, M. A.: Leid, M.: De Lera, A. R.: Lotan, R.: Mangelsdorf, D. J.: Gronemeyer, H. International Union of Pharmacology. LX. Retinoic acid receptors. *Pharmacol Rev* 58:712-725 (2006)

19. Germain, P.: Chambon, P.: Eichele, G.: Evans, R. M.: Lazar, M. A.: Leid, M.: De Lera, A. R.: Lotan, R.: Mangelsdorf, D. J.: Gronemeyer, H. International Union of Pharmacology. LXIII. Retinoid X receptors. *Pharmacol Rev* 58:760-772 (2006)

20. Michalik, L.: Auwerx, J.: Berger, J. P.: Chatterjee, V. K.: Glass, C. K.: Gonzalez, F. J.: Grimaldi, P. A.: Kadowaki, T.: Lazar, M. A.: O'Rahilly, S.: Palmer, C. N.: Plutzky, J.: Reddy, J. K.: Spiegelman, B. M.: Staels, B.: Wahli, W. International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol Rev* 58:726-741 (2006)

21. Altucci, L.: Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat Rev Cancer* 1:181-193 (2001)

22. Altucci, L.: Leibowitz, M. D.: Ogilvie, K. M.: De Lera, A. R.: Gronemeyer, H. RAR and RXR modulation in cancer and metabolic disease. *Nat Rev Drug Discov* 6:793-810 (2007)

23. de The, H.: Marchio, A.: Tiollais, P.: Dejean, A. A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature* 330:667-670 (1987)

24. Sever, C. E.: Locker, J. Expression of retinoic acid alpha and beta receptor genes in liver and hepatocellular carcinoma. *Mol Carcinog* 4:138-144 (1991)

25. Ando, N.: Shimizu, M.: Okuno, M.: Matsushima-Nishiwaki, R.: Tsurumi, H.: Tanaka, T.: Moriwaki, H. Expression of retinoid X receptor alpha is decreased in 3'-methyl-4-dimethylaminoazobenzene-induced hepatocellular carcinoma in rats. *Oncol Rep* 18:879-884 (2007)

26. Alvarez, S.: Germain, P.: Alvarez, R.: Rodriguez-Barrios, F.: Gronemeyer, H.: de Lera, A. R. Structure, function and modulation of retinoic acid receptor beta, a tumor suppressor. *Int J Biochem Cell Biol* 39:1406-1415 (2007)

27. Matsushima-Nishiwaki, R.: Shidoji, Y.: Nishiwaki, S.: Yamada, T.: Moriwaki, H.: Muto, Y. Aberrant metabolism of retinoid X receptor proteins in human hepatocellular carcinoma. *Mol Cell Endocrinol* 121:179-190 (1996)

28. Adachi, S.: Okuno, M.: Matsushima-Nishiwaki, R.: Takano, Y.: Kojima, S.: Friedman, S. L.: Moriwaki, H.: Okano, Y. Phosphorylation of retinoid X receptor suppresses its ubiquitination in human hepatocellular carcinoma. *Hepatology* 35:332-340 (2002)

29. Yoshimura, K.: Muto, Y.: Shimizu, M.: Matsushima-Nishiwaki, R.: Okuno, M.: Takano, Y.: Tsurumi, H.: Kojima, S.: Okano, Y.: Moriwaki, H. Phosphorylated retinoid X receptor alpha loses its heterodimeric activity with retinoic acid receptor beta. *Cancer Sci* 98:1868-1874 (2007)

30. Lee, H. Y.: Suh, Y. A.: Robinson, M. J.: Clifford, J. L.: Hong, W. K.: Woodgett, J. R.: Cobb, M. H.: Mangelsdorf, D. J.: Kurie, J. M. Stress pathway activation induces phosphorylation of retinoid X receptor. *J Biol Chem* 275:32193-32199 (2000)

31. Solomon, C.: White, J. H.: Kremer, R. Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3-dependent signal transduction by phosphorylating human retinoid X receptor alpha. *J Clin Invest* 103:1729-1735 (1999)

32. Macoritto, M.: Nguyen-Yamamoto, L.: Huang, D. C.: Samuel, S.: Yang, X. F.: Wang, T. T.: White, J. H.: Kremer, R. Phosphorylation of the human retinoid X receptor alpha at serine 260 impairs coactivator(s) recruitment and induces hormone resistance to multiple ligands. *J Biol Chem* 283:4943-4956 (2008)

33. Yamazaki, K.: Shimizu, M.: Okuno, M.: Matsushima-Nishiwaki, R.: Kanemura, N.: Araki, H.: Tsurumi, H.: Kojima, S.: Weinstein, I. B.: Moriwaki, H. Synergistic effects of RXR alpha and PPAR gamma ligands to inhibit growth in human colon cancer cells -phosphorylated RXR alpha is a critical target for colon cancer management. *Gut* 56:1557-1563 (2007)

34. Kanemura, N.: Tsurumi, H.: Okuno, M.: Matsushima-Nishiwaki, R.: Shimizu, M.: Moriwaki, H. Retinoid X receptor alpha is highly phosphorylated in retinoic acid-resistant HL-60R cells and the combination of 9-cis retinoic acid plus MEK inhibitor induces apoptosis in the cells. *Leuk Res* 32:884-892 (2008)

35. Lattuada, D.: Vigano, P.: Mangioni, S.: Sassone, J.: Di Francesco, S.: Vignali, M.: Di Blasio, A. M. Accumulation of retinoid X receptor-alpha in uterine leiomyomas is associated with a delayed ligand-dependent proteasome-mediated degradation and an alteration of its transcriptional activity. *Mol Endocrinol* 21:602-612 (2007)

36. Yamada, Y.: Shidoji, Y.: Fukutomi, Y.: Ishikawa, T.: Kaneko, T.: Nakagama, H.: Imawari, M.: Moriwaki, H.: Muto, Y. Positive and negative regulations of albumin gene expression by retinoids in human hepatoma cell lines. *Mol Carcinog* 10:151-158 (1994)

37. Araki, H.: Shidoji, Y.: Yamada, Y.: Moriwaki, H.: Muto, Y. Retinoid agonist activities of synthetic geranyl geranoic acid derivatives. *Biochem Biophys Res Commun* 209:66-72 (1995)

38. Muto, Y.: Moriwaki, H. Antitumor activity of vitamin A and its derivatives. *J Natl Cancer Inst* 73:1389-1393 (1984)

39. Fukutomi, Y.: Omori, M.: Muto, Y.: Ninomiya, M.: Okuno, M.: Moriwaki, H. Inhibitory effects of acyclic retinoid (polyprenoic acid) and its hydroxy derivative on cell growth and on secretion of alpha-fetoprotein in human hepatoma-derived cell line (PLC/PRF/5). *Jpn J Cancer Res* 81:1281-1285 (1990)

40. Nakamura, N.: Shidoji, Y.: Yamada, Y.: Hatakeyama, H.: Moriwaki, H.: Muto, Y. Induction of apoptosis by acyclic retinoid in the human hepatoma-derived cell line, HuH-7. *Biochem Biophys Res Commun* 207:382-388 (1995)

41. Nakamura, N.: Shidoji, Y.: Moriwaki, H.: Muto, Y. Apoptosis in human hepatoma cell line induced by 4,5-didehydro geranylgeranoic acid (acyclic retinoid) via down-regulation of transforming growth factor-alpha. *Biochem Biophys Res Commun* 219:100-104 (1996)

42. Yasuda, I.: Shiratori, Y.: Adachi, S.: Obora, A.: Takemura, M.: Okuno, M.: Shidoji, Y.: Seishima, M.: Muto, Y.: Moriwaki, H. Acyclic retinoid induces partial differentiation, down-regulates telomerase reverse transcriptase mRNA expression and telomerase activity, and induces apoptosis in human hepatoma-derived cell lines. *J Hepatol* 36:660-671 (2002)

43. Shimizu, M.: Suzui, M.: Deguchi, A.: Lim, J. T.: Xiao, D.: Hayes, J. H.: Papadopoulos, K. P.: Weinstein, I. B. Synergistic effects of acyclic retinoid and OSI-461 on growth inhibition and gene expression in human hepatoma cells. *Clin Cancer Res* 10:6710-6721 (2004)

44. Suzui, M.: Shimizu, M.: Masuda, M.: Lim, J. T.: Yoshimi, N.: Weinstein, I. B. Acyclic retinoid activates retinoic acid receptor beta and induces transcriptional activation of p21(CIP1) in HepG2 human hepatoma cells. *Mol Cancer Ther* 3:309-316 (2004)

45. Suzui, M.: Masuda, M.: Lim, J. T.: Albanese, C.: Pestell, R. G.: Weinstein, I. B. Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. *Cancer Res* 62:3997-4006 (2002)

46. Shimizu, M.: Suzui, M.: Deguchi, A.: Lim, J. T.: Weinstein, I. B. Effects of acyclic retinoid on growth, cell cycle control, epidermal growth factor receptor signaling, and gene expression in human squamous cell carcinoma cells. *Clin Cancer Res* 10:1130-1140 (2004)

47. Kagawa, M.: Sano, T.: Ishibashi, N.: Hashimoto, M.: Okuno, M.: Moriwaki, K.: Suzuki, R.: Kohno, H.: Tanaka, T. An acyclic retinoid, NIK-333, inhibits N-diethylnitrosamine-induced rat hepatocarcinogenesis through suppression of TGF-alpha expression and cell proliferation. *Carcinogenesis* 25:979-985 (2004)

48. Sano, T.: Kagawa, M.: Okuno, M.: Ishibashi, N.: Hashimoto, M.: Yamamoto, M.: Suzuki, R.: Kohno, H.: Matsushima-Nishiwaki, R.: Takano, Y.: Tsurumi, H.: Kojima, S.: Friedman, S. L.: Moriwaki, H.: Tanaka, T. Prevention of rat hepatocarcinogenesis by acyclic retinoid is accompanied by reduction in emergence of both TGF-alpha-expressing oval-like cells and activated hepatic stellate cells. *Nutr Cancer* 51:197-206 (2005)

49. Shao, R. X.: Otsuka, M.: Kato, N.: Taniguchi, H.: Hoshida, Y.: Moriyama, M.: Kawabe, T.: Omata, M. Acyclic retinoid inhibits human hepatoma cell growth by suppressing fibroblast growth factor-mediated signaling pathways. *Gastroenterology* 128:86-95 (2005)

50. Komi, Y.: Sogabe, Y.: Ishibashi, N.: Sato, Y.: Moriwaki, H.: Shimokado, K.: Kojima, S. Acyclic retinoid inhibits angiogenesis by suppressing the MAPK pathway. *Lab Invest* 90:52-60 (2010)

51. Slaughter, D. P.: Southwick, H. W.: Smejkal, W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* 6:963-968 (1953)

52. Moriwaki, H.: Yasuda, I.: Shiratori, Y.: Uematsu, T.: Okuno, M.: Muto, Y. Deletion of serum lectin-reactive alpha-fetoprotein by acyclic retinoid: a potent biomarker in the chemoprevention of second primary hepatoma. *Clin Cancer Res* 3:727-731 (1997)

53. Obora, A.: Shiratori, Y.: Okuno, M.: Adachi, S.: Takano, Y.: Matsushima-Nishiwaki, R.: Yasuda, I.: Yamada, Y.: Akita, K.: Sano, T.: Shimada, J.: Kojima, S.: Okano, Y.: Friedman, S. L.: Moriwaki, H. Synergistic induction of apoptosis by acyclic retinoid and interferon-beta in human hepatocellular carcinoma cells. *Hepatology* 36:1115-1124 (2002)

54. Habu, D.: Shiomi, S.: Tamori, A.: Takeda, T.: Tanaka, T.: Kubo, S.: Nishiguchi, S. Role of vitamin K2 in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. *JAMA* 292:358-361 (2004)

55. Mizuta, T.: Ozaki, I.: Eguchi, Y.: Yasutake, T.: Kawazoe, S.: Fujimoto, K.: Yamamoto, K. The effect of menatetrenone, a vitamin K2 analog, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment: a pilot study. *Cancer* 106:867-872 (2006)

56. Ikeda, K.: Arase, Y.: Saitoh, S.: Kobayashi, M.: Suzuki, Y.: Suzuki, F.: Tsubota, A.: Chayama, K.: Murashima, N.: Kumada, H. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology* 32:228-232 (2000)

57. Kubo, S.: Nishiguchi, S.: Hirohashi, K.: Tanaka, H.: Shuto, T.: Yamazaki, O.: Shiomi, S.: Tamori, A.: Oka, H.: Igawa, S.: Kuroki, T.: Kinoshita, H. Effects of long-term

postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 134:963-967 (2001)

58. Tatebe, H.: Shimizu, M.: Shirakami, Y.: Tsurumi, H.: Moriwaki, H. Synergistic growth inhibition by 9-cis-retinoic acid plus trastuzumab in human hepatocellular carcinoma cells. *Clin Cancer Res* 14:2806-2812 (2008)

59. Shimizu, M.: Moriwaki, H. Synergistic Effects of PPARgamma Ligands and Retinoids in Cancer Treatment. *PPAR Res* 2008:181047 (2008)

60. Suzui, M.: Sunagawa, N.: Chiba, I.: Moriwaki, H.: Yoshimi, N. Acyclic retinoid, a novel synthetic retinoid, induces growth inhibition, apoptosis, and changes in mRNA expression of cell cycle- and differentiation-related molecules in human colon carcinoma cells. *Int J Oncol* 28:1193-1199 (2006)

61. Sakabe, T.: Tsuchiya, H.: Endo, M.: Tomita, A.: Ishii, K.: Gonda, K.: Murai, R.: Takubo, K.: Hoshikawa, Y.: Kurimasa, A.: Ishibashi, N.: Yanagida, S.: Shiota, G. An antioxidant effect by acyclic retinoid suppresses liver tumor in mice. *Biochem Pharmacol* 73:1405-1411 (2007)

62. Nakagawa, T.: Shimizu, M.: Shirakami, Y.: Tatebe, H.: Yasuda, I.: Tsurumi, H.: Moriwaki, H. Synergistic effects of acyclic retinoid and gemcitabine on growth inhibition in pancreatic cancer cells. *Cancer Lett* 273:250-256 (2009)

Abbreviations: ACR, acyclic retinoid; AFP-L3, lectin-reactive α-fetoprotein factor 3; HBV, hepatitis B virus; HCC, Hepatocellular carcinoma; HCV, hepatitis C virus; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor-2; IFN, interferon; MAPK, mitogen-activated protein kinase; PIVKA-II, protein induced by vitamin K absence or antagonist-II; PPAR, peroxisome proliferator-activated receptors; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid receptor responsive element; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; RXRE, retinoid X response element; VK_2, vitamin K_2

Key Words: Retinoid, HCC, chemoprevention, phosphorylated RXR alpha, Review

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Ultraviolet enhances the sensitivity of pancreatic cancer cells to gemcitabine by activation of 5′ AMP-activated protein kinase

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ABSTRACT

Although gemcitabine is recognized as the standard drug for the treatment of advanced pancreatic cancer, the clinical outcome is not satisfactory. We recently reported that relatively high dose ultraviolet-C (UV-C; 200 J) inhibits cell growth by desensitization of epidermal growth factor receptor (EGFR) in human pancreatic cancer cells. In the present study, we investigated the combination effects of low dose UV-C (10 J) and gemcitabine on apoptosis and cell growth in these cells. UV-C enhanced gemcitabine-induced suppression of cell viability. In addition, the combination use clearly induced apoptosis, while neither UV-C nor gemcitabine alone did. Concurrently, combination use caused the decrease in the EGFR protein level and reduced EGF-induced activation of Akt pathway, subsequently resulting in accumulation of β-catenin. The order of the treatment with UV-C and gemcitabine did not affect their synergistic effects on apoptosis and cell growth. Interestingly, combination use synergistically induced phosphorylation of 5′ AMP-activated protein kinase (AMPK) alpha at Thr172 and acetyl-CoA carboxylase at Ser79 as a downstream molecular target of AMPK. AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-riboside, induced apoptosis and suppressed cell growth in these cells, thus suggesting that combination effects of UV-C and gemcitabine is due to the activation of AMPK. Together, our findings could provide a new aspect of pancreatic cancer therapy.

1. Introduction

Because of the difficulty in early diagnose of pancreatic cancer, most patients with this malignancy have already reached an advanced stage when the first symptoms appear. The standard treatment for advanced pancreatic cancer is chemotherapy. Gemcitabine is a nucleoside analogue of deoxycytidine that is enzymatically activated inside the cell where it subsequently inhibits DNA synthesis and induces apoptosis [1] and has been the first line drug for pancreatic cancer. However, the median survival of patients treated with gemcitabine is not satisfactory. Moreover, a number of studies have compared gemcitabine alone with gemcitabine-based combinations, such as fluorouracil, capecitabine, cisplatin, docetaxel, irinotecan, oxaliplatin, or pemetrexed, but they added no clear survival benefit [2]. Therefore, the breakthrough development of treatments for unresectable pancreatic cancer is required urgently.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases [3]. Binding of ligands such as epidermal growth factor (EGF) to the EGFR leads to receptor dimerization and autophosphorylation [4]. The autophosphorylation of the EGFR at tyrosine residues activates downstream signalling, including Akt-glycogen synthase kinase (GSK)-3β pathway, thus resulting in the stimulation of cell proliferation [5]. The EGFR has been reported to be overexpressed in pancreatic cancer [6]. We have recently reported that the blockade of EGF stimulation significantly suppressed pancreatic cancer cell growth, suggesting that the EGFR pathway plays an important role in proliferation of these cells [7]. Therefore, EGFR-mediated pathways appear to be important potential targets for new therapies for this malignancy. Moreover, the addition of EGFR-targeted therapy to gemcitabine in advanced pancreatic cancer has been demonstrated to provide a small, but statistically significant, survival benefit [8].

Ultraviolet (UV) radiation from sunlight is sorted by wavelength regions: long-wavelength UV-A (320–400 nm), medium-wavelength UV-B (280–320 nm) and short-wavelength UV-C (200–280 nm). In general, UV-A and UV-B are recognized as the major carcinogenic components of sunlight [9], and UV-C is used for studying DNA damage and cellular DNA repair process, and commonly applied for equipments such as water sterilization. We recently reported that UV-C irradiation suppresses cell growth via downregulation of EGFR in human pancreatic cancer cells [10].

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Moreover, our recent study shows that UV-C can induce evasion of colon cancer cells from oncogenic stimulation of EGF [11]. Hence, UV-C might be applied for clinical strategy against human cancers.

5′ AMP-activated protein kinase (AMPK) is a central cellular energy sensor which may be a crucial factor in the interaction between metabolism and cancer [12]. AMPK activation results in the restoration of energy levels through regulation of metabolism and growth [13], and the loss of AMPK activity causes cell proliferation, suggesting that AMPK is a potential target for anti-cancer therapy. In this study, we tried the combination use of low dose UV-C and gemcitabine in pancreatic cancer cells and found that this exerts synergistic effects on the induction of apoptosis and suppression of cell growth via activation of AMPK.

2. Materials and methods

2.1. Materials

Gemcitabine was obtained from Eli Lilly Co. (Indianapolis, IN, USA) and 5-aminoimidazole-4-carboxamide-1-β-riboside (AICAR) was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). EGF was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against EGFR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other antibodies were purchased from Cell Signalling (Beverly, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) cell proliferation kit I and Cell Proliferation ELISA (BrdU) were obtained from Roche Diagnostics Co. (Indianapolis, IN, USA).

2.2. Cell culture

Panc1 and KP3 pancreatic cancer cells were provided from American Type Culture Collection (Manassas, VA, USA). They were grown in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, San Diego, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 incubator at 37 °C.

2.3. UV-C exposure

UV-C exposure to cells was performed in UVC 500 UV Crosslinker (GE Healthcare) with 0–500 J/m^2 (J) UV at 254 nm. After aspiration of the growth medium, the cells were exposed to UV-C (0 or 10 J) and then incubated in the growth medium for the indicated periods.

2.4. Cell viability assay and BrdU incorporation assay

In cell viability assay, the cells (5×10^3/well) were seeded onto 96-well plates and 24 h later, the cells were exposed to 10 J of UV-C and/or gemcitabine at the indicated concentrations. These cells were then incubated in RPMI for 48 h and the remaining cells were finally counted by MTT cell proliferation kit. For the latter assay, the cells (7×10^3/well) were seeded onto 96-well plates and 48 h later, the cells were exposed to 10 J of UV-C and/or 10 μM of gemcitabine or 10 mM of AICAR. These cells were then incubated in RPMI without FCS for 48 h. BrdU incorporation was finally measured using Cell Proliferation ELISA. All assays were done at least in triplicate.

2.5. Western blot analysis

The cells were lysed in lysis buffer and were examined by Western blot analysis as previously described [14].

2.6. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously [15]. The cells were first exposed to UV-C (10 J) and incubated for 24 h at 37 °C, followed by exposure to Hoechst 33258 (Wako, Tokyo, Japan) for 30 min. The cells were then examined by fluorescence microscopy, BIOREVO (BZ-9000) (Keyence, Tokyo, Japan).

2.7. Image analysis

The protein band intensities in the Western blot analysis were determined by integrating the optical density over the band area (band volume) using the NIH image software program (Image J ver. 1.32). Based on the intensity of the control protein band on the X-ray film, the protein samples were quantitatively compared.

3. Results

UV-C enhanced gemcitabine-induced cytotoxicity in Panc1 and KP3 pancreatic cancer cells

As shown in Fig. 1A, 10 μM of gemcitabine alone for 48 h caused approximately 20% reduction in cell viability and only 30% reduction was seen even when the cells were treated with 100 μM of gemcitabine alone in KP3 cells (Fig. 1A). However, pretreatment with UV-C significantly increased gemcitabine-induced cytotoxicity in KP3 cells, while 10 J of UV-C alone did not affect KP3 cell viability (Fig. 1A). Ten μM and 30 μM of gemcitabine combined with UV-C caused 50% and 75% reduction in cell viability in KP3 cells, respectively. As well, we used another pancreatic cancer cell line, Panc1, and observed similar effect to that in KP3 cells (Fig. 1B). These results suggest that UV-C enhances gemcitabine-induced suppression of cell viability in pancreatic cancer cells.

3.1. Combination use of UV-C and gemcitabine induced apoptosis in pancreatic cancer cells

In order to elucidate how UV-C enhances gemcitabine-induced suppression of cell viability, we next examined the effects of UV-C and gemcitabine on cell apoptosis in KP3 cells. Apoptotic cells are easily detected by the Hoechst 33258 staining. In addition, PARP helps cells to maintain their viability, while cleavage of PARP induces apoptosis. In Fig. 1C, while 10 J of UV-C alone as well as 10 μM of gemcitabine alone slightly increased the number of Hoechst 33258-positive cells in KP3 cells, combination use caused a marked increase in the number of those cells (Fig. 1C). Similarly, while either UV-C or gemcitabine alone did not induce PARP cleavage, this was clearly observed when the cells were treated with both UV-C and gemcitabine (Fig. 1D). These results suggest that synergistic effect of UV-C and gemcitabine on the suppression of cell viability is, at least in part, due to the induction of cell apoptosis.

3.2. UV-C enhanced gemcitabine-induced cell growth suppression in pancreatic cancer cells

As depicted in Fig. 2A, UV-C had little effect on BrdU incorporation and 10 μM of gemcitabine alone caused a slight suppression of its incorporation (Fig. 2A). As expected, when the cells were exposed to UV-C and then treated with gemcitabine for 48 h, BrdU incorporation was significantly suppressed ($p = 0.0047$), compared with gemcitabine-treated cells (Fig. 3A). We recently reported that the EGFR pathway plays an important role in pancreatic cancer cell proliferation [7]. Therefore, we next examined the effect of UV-C and gemcitabine on the protein level of EGFR in KP3 cells. In Fig. 2B, whereas either UV-C or gemcitabine had little effect on

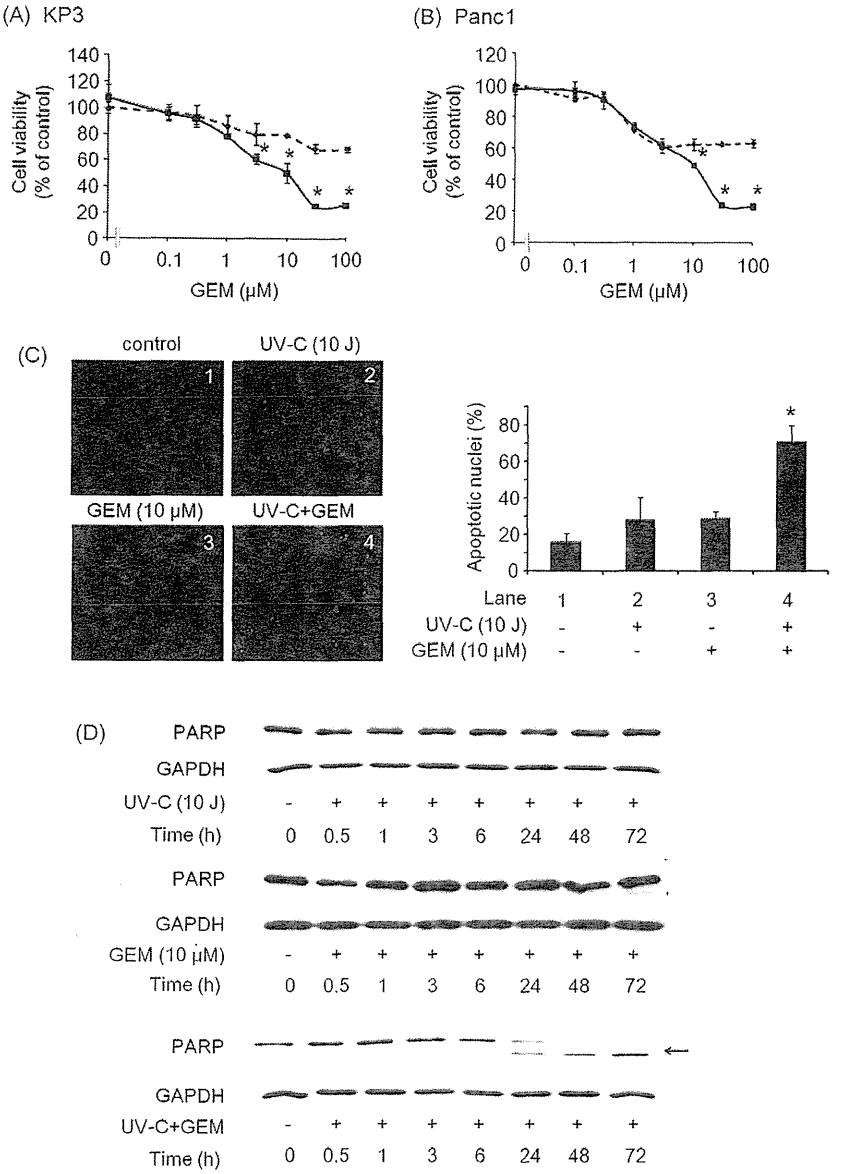

Fig. 1. (A and B) UV-C enhanced the inhibition of cell growth by gemcitabine in pancreatic cancer cells. The KP3 (A) and Panc1 (B) cells were first exposed to UV-C (0 or 10 J) and then incubated with gemcitabine (GEM; 0–100 μM) for 48 h. Cell viability assay was then performed using the MTT cell proliferation kit I. Results are expressed as percentage of growth with 100% representing untreated control cells. Bars designate SD of triplicate assays. Broken line: GEM alone; solid line: UV-C + GEM. (C) The combination use of UV-C and GEM induced DNA fragmentation in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for 48 h. They were then exposed to Hoechst 33258 (blue signal) and were examined by fluorescence microscopy. The numbers of Hoechst 33258-positive cells (apoptotic nuclei) from five randomly chosen fields (×40) were counted, respectively. Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p < 0.05$), as compared with the cells treated with GEM alone (lane 3). (D) The combination use of UV-C and GEM induced PARP cleavage in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without gemcitabine 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against PARP and GAPDH. The arrow indicates cleaved PARP.

the EGFR protein level, combination use markedly decreased EGFR level (Fig. 2B). Therefore, it seems that cell growth suppression by combination use of UV-C and gemcitabine is due to the decrease in EGFR protein level.

3.3. Combination use of UV-C and gemcitabine suppressed EGF-induced activation of Akt-GSK-3β pathway in pancreatic cancer cells

Through EGF-binding to cell surface EGFR, it activates an extensive network of signal transduction pathways including the Akt pathways, which regulates multiple biological processes including survival, proliferation, and cell growth [16]. Therefore, we next examined the effect of UV-C and gemcitabine on EGF-induced phosphorylation of Akt in KP3 cells. EGF induced phosphorylation of Akt within 5 min and this was decreased thereafter (Fig. 2C). UV-C and gemcitabine alone slightly suppressed EGF-induced phosphorylation of Akt (Fig. 2C). Moreover, the combination use markedly suppressed EGF-induced phosphorylation of Akt (Fig. 3C). GSK-3β is a critical downstream element of the Akt pathway, and its activity can be inhibited by Akt-mediated phosphory-

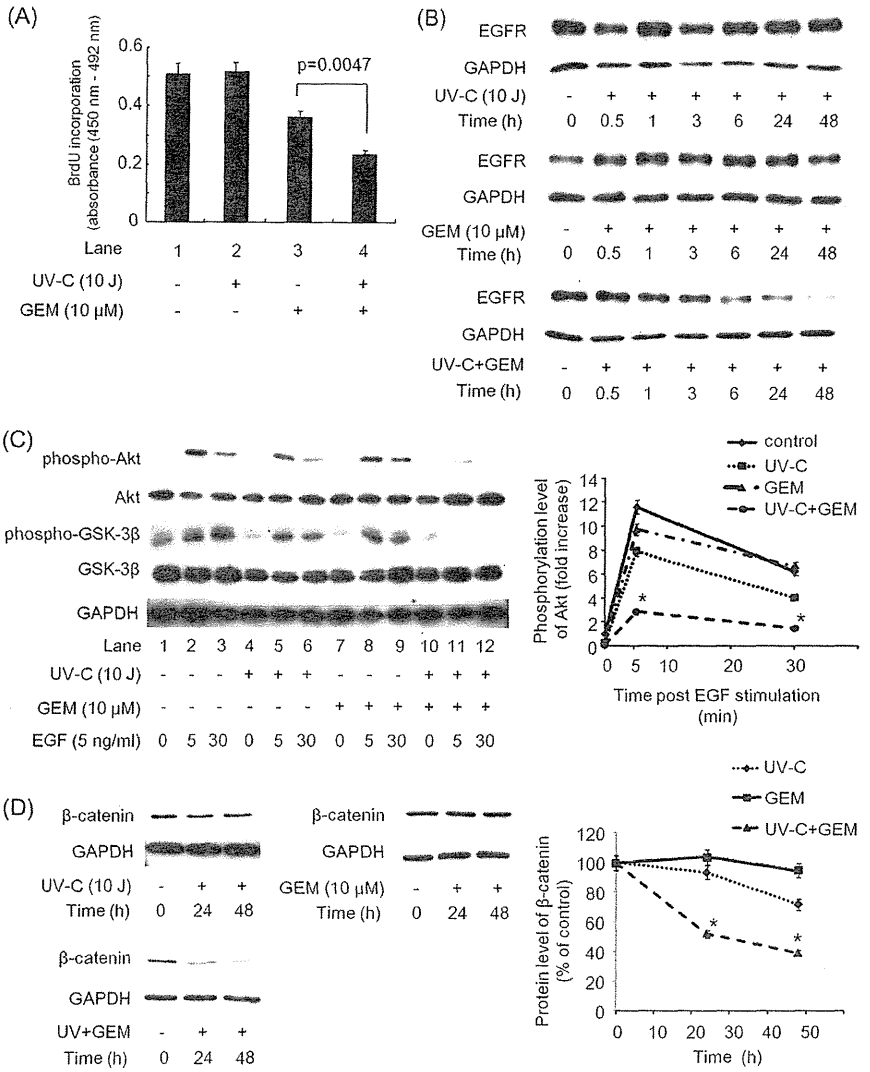

Fig. 2. (A) The combination use of UV-C and gemcitabine (GEM) inhibited BrdU incorporation in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for 48 h and the measurement of BrdU incorporation during DNA synthesis were performed using cell proliferation ELISA (BrdU). Results are expressed as the absorbance (OD 405 nm–492 nm). Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p = 0.0047$), between the indicated pairs. (B) The combination use of UV-C and GEM caused the decrease in the EGFR protein level in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against EGFR and GAPDH. (C) The combination use of UV-C and GEM suppressed EGF-induced Akt pathway in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for 24 h. They were then stimulated with EGF (5 ng/ml) for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against phospho-Akt, Akt, phospho-GSK3β, GSK3β and GAPDH. Right line graph shows the quantification data for the relative phosphorylation levels of Akt, after normalization with respect to total Akt, as determined by densitometry. Bars designate SD of triplicate assays. $^*p < 0.05$: compared to the cells treated with GEM alone. (D) The combination use of UV-C and GEM caused the decrease in the β-catenin protein level in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against β-catenin and GAPDH. Right line graph shows the quantification data for the relative levels of β-catenin, after normalization with respect to GAPDH, as determined by densitometry. Bars designate SD of triplicate assays. $^*p < 0.05$: compared to the cells treated with GEM alone.

lation of GSK-3β at Ser9 [17]. As expected, EGF failed to phosphorylate GSK-3β at Ser9 in the cells treated with UV-C and gemcitabine (Fig. 3C).

Deregulation of Wnt-β-catenin cascades has been reported in many types of cancers, including pancreatic cancer [18] and phosphorylated form of GSK-3β caused the accumulation of β-catenin. As shown in Fig. 2D, combination use of UV-C and gemcitabine caused the decrease in the protein level of β-catenin, whereas either UV-C or gemcitabine alone failed to affect. Together, our results suggest that combination use of UV-C and gemcitabine induces the suppressive effect on cell growth, at least in part, by inhibiting the Akt pathway.

3.4. The order of the treatment with UV-C and gemcitabine did not affect their synergistic effects on apoptosis and cell growth

We next examined which is more effective treatment to enhance the sensitivity to gemcitabine, pre- or post-treatment with UV-C. Either gemcitabine or UV-C did not affect the protein level of EGFR as well as cleavage of PARP (Fig. 3A). When the cells were

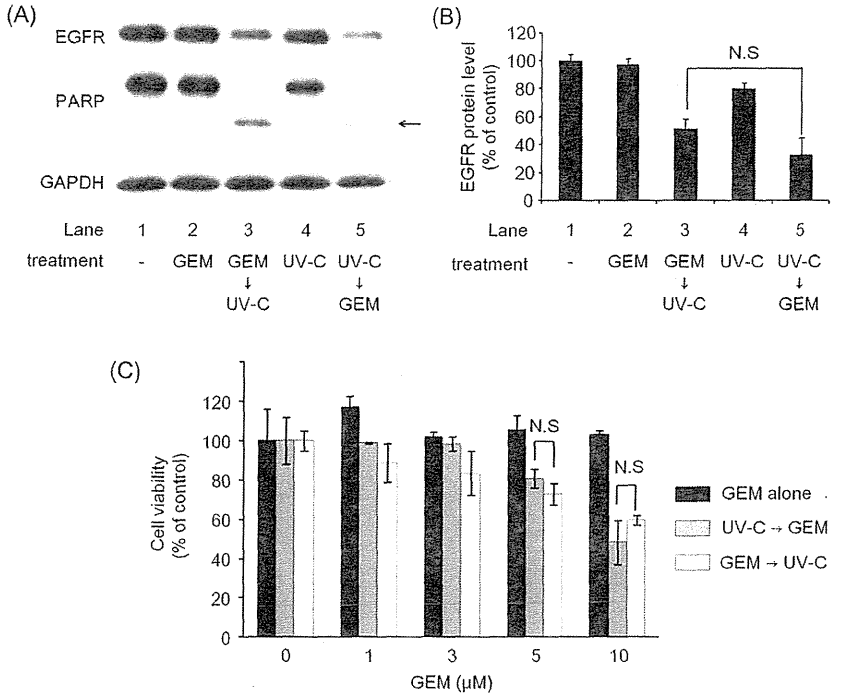

Fig. 3. The order of treatment with UV-C and gemcitabine (GEM) is not critical for their combination effect in KP3 cells. (A) The cells (lanes 4 and 5) were exposed to 10 J of UV-C and then treated with (lane 5) or without (lane 4) GEM (10 μM) for 8 h. The media were then exchanged to the fresh one and incubated for another 40 h. The other cells were first treated with (lanes 2 and 3) or without (lane 1) GEM (10 μM) for 8 h and then exposed to 10 J of UV-C (lane 3) or not (lanes 1 and 2), and subsequent incubation for another 40 h in the fresh media. Protein extracts were then harvested and examined by Western blotting using antibodies against EGFR, PARP and GAPDH. The arrow indicates cleaved PARP. (B) The bar graph shows the quantification data for the relative levels of EGFR after normalization with respect to GAPDH, as determined by densitometry. Bars designate SD of triplicate assays. No significant difference exists between the indicated pairs. (C) Some cells (grey bar) were first exposed to 10 J of UV-C and then treated with GEM at the indicated concentrations for 8 h. The media were then exchanged to the fresh one and incubated for another 40 h. The other cells (black and white bars) were first treated with GEM at the indicated concentrations for 8 h and then exposed to 10 J of UV-C (white bar) or not (black bar), and subsequent incubation for another 40 h in the fresh media. Cell viability assay was then performed using the MTT cell proliferation kit I. Results are expressed as percentage of growth with 100% representing untreated control cells. Bars designate SD of triplicate assays.

pretreated with gemcitabine for 8 h and then exposed to UV-C (gemcitabine → UV-C treatment), we observed the decrease in EGFR protein level in addition to the induction of PARP cleavage, which are similar to the results obtained from UV-C → gemcitabine treatment (Fig. 3A and B). Moreover, similar results were observed in cell viability assay (Fig. 3C). Taken together, our findings strongly suggest that the order is not important for combination use of UV-C and gemcitabine.

3.5. The combination use of UV-C and gemcitabine induced activation of AMPKα in pancreatic cancer cells

In order to investigate the mechanism underlying combination effects of UV-C and gemcitabine, we next examined several kinase cascades including AMPK. Interestingly, whereas either UV-C or gemcitabine had little effect on phosphorylation of AMPKα (Thr172) and acetyl-CoA carboxylase (ACC; Ser 79) as a downstream molecular target of AMPK, the combination use caused a marked phosphorylation of both proteins (Fig. 4A). These results led us to speculate that activation of AMPK induced by UV-C and gemcitabine exert anti-cancer effect, such as the induction of apoptosis and the suppression of cell growth.

To verify these findings, we used AMPK activator, AICAR and found that increasing doses of AICAR caused cleavage of PARP (Fig. 4B) and this increased Hoechst 33258-positive cells (Fig. 4C), suggesting that activation of AMPK leads to apoptosis in pancreatic cancer cells. Moreover, since AICAR suppressed BrdU incorporation (Fig. 4D), indicating that activation of AMPK inhibits cell cycle. Taken together with the results shown above, it is most likely that the synergistic effects of UV-C and gemcitabine are exerted through the activation of AMPK.

4. Discussion

We have recently reported the availability of UV-C for the treatment of human pancreatic cancers [10]. In that article, we used relatively high dose UV-C (200 J), and showed that UV-C has a suppressive effect on cell proliferation in pancreatic cancer cells, but not normal pancreas epithelial cells. In the present study, we demonstrated the synergistic effects of low dose UV-C (10 J) and gemcitabine. First, we showed that UV-C enhanced gemcitabine-induced cytotoxicity in Panc1 and KP3 cells (Fig. 1A and B). This combination use caused cell apoptosis (Fig. 1C and D) as well as inhibition of cell proliferation (Fig. 2A), concurrent with downregulation of EGFR which exerts oncogenic signalling, such as the Akt pathway (Fig. 2B–D). These results indicate that the synergistic effect against pancreatic cancer cells on cell viability is due to the induction of cell apoptosis and suppression of cell proliferation.

Gemcitabine is now regarded as the first-line agent for advanced pancreatic cancer, but the median survival time of patients treated with gemcitabine is not satisfactory. Importantly, it has been reported that gemcitabine induces cell apoptosis by activating p38 mitogen-activated protein kinase (MAPK) in PK-1 and PCI-43 human pancreatic cancer cell lines [19]. However, in this study, the combination use of UV-C and gemcitabine did not induce phosphorylation of p38 MAPK in Panc1 and KP3 cells (data not shown). Therefore, it is likely that the synergistic effect of UV-C and gemcitabine is independent of the p38 MAPK pathway.

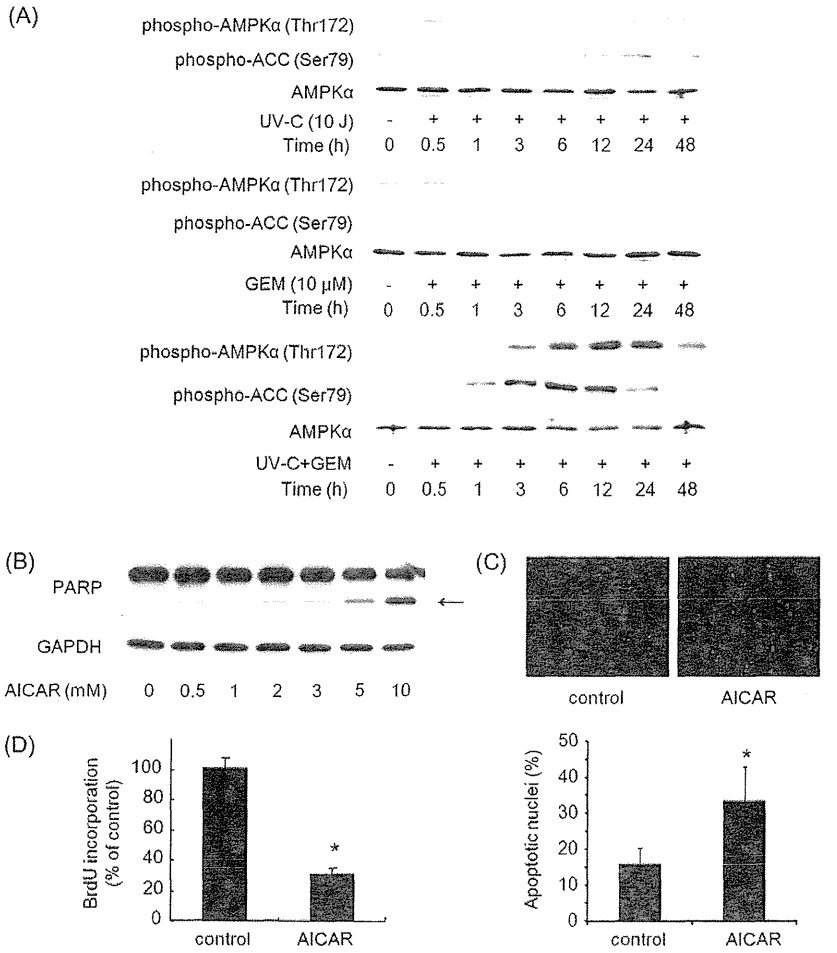

Fig. 4. (A) The combination use of UV-C and gemcitabine (GEM) caused the activation of AMPK in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against phospho-AMPKα (Thr172), phospho-acetyl-CoA carboxylase (ACC; Ser79) and AMPKα. (B) AMPK activator, AICAR, induced apoptosis in KP3 cells. The KP3 cells were treated with AICAR at the indicated concentrations for 24 h. Protein extracts were then harvested and examined by Western blotting using antibodies against PARP and GAPDH. The arrow indicates cleaved PARP. (C) The KP3 cells were treated with 10 mM of AICAR for 24 h and were then exposed to Hoechst 33258 (blue signal) and were examined by fluorescence microscopy. The numbers of Hoechst 33258-positive cells (apoptotic nuclei) from five randomly chosen fields (×40) were counted, respectively. Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p < 0.05$), as compared with the control cells. (D) The KP3 cells were treated with 10 mM of AICAR for 24 h and the measurement of BrdU incorporation during DNA synthesis were performed using cell proliferation ELISA (BrdU). Results are expressed as the absorbance (OD 405 nm–492 nm). Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p < 0.05$), as compared with the control cells.

AMPK is a serine/threonine protein kinase, which serves as an energy sensor in eukaryotic cell types. Increasing evidence shows that AMPK activation strongly suppresses cell proliferation in normal cells as well as in cancer cells, indicating that AMPK functions as a suppressor of cell proliferation by controlling a variety of cellular events in these cells [20]. Metformin, which is widely used as an anti-diabetic drug, has been recently reported to be associated with a reduced risk of cancer associated with insulin resistance [12,21]. The effects of metformin are explained by the activation of AMPK, which regulates cellular energy metabolism [22]. Of interest, in the present study, the combination use of UV-C and gemcitabine induced activation of AMPK (Fig. 4A). The order of the treatment with UV-C and gemcitabine is not critical for their combination effect (Fig. 3). Moreover, we observed the activation of AMPK in the cells, which were first treated with gemcitabine for 8 h and then exposed to UV-C, subsequent incubation for another 40 h (data not shown), consistently with the results shown in Fig. 3. However, further investigation is necessary to elucidate how the combination use of UV-C and gemcitabine activates AMPK.

In summary, we presented that UV-C enhances the sensitivity to gemcitabine in pancreatic cancer cells via activation of AMPK. Our novel findings could provide a fresh development for human pancreatic cancer therapy, although the development of devices that supply UV-C efficiently, for example with endoscopic approach, is also required for future clinical application.

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References

[1] W. Plunkett, P. Huang, C.E. Searcy, V. Gandhi, Gemcitabine: preclinical pharmacology and mechanisms of action, Semin. Oncol. 23 (1996) 3–15.
[2] A. Stathis, M.J. Moore, Advanced pancreatic carcinoma: current treatment and future challenges, Nat. Rev. Clin. Oncol. 7 (2010) 163–172.

[3] J. Marshall, Clinical implications of the mechanism of epidermal growth factor receptor inhibitors, Cancer 107 (2006) 1207–1218.
[4] R. Zandi, A.B. Larsen, P. Andersen, M.T. Stockhausen, H.S. Poulsen, Mechanisms for oncogenic activation of the epidermal growth factor receptor, Cell. Signal. 19 (2007) 2013–2023.
[5] T. Lahusen, M. Fereshteh, A. Oh, A. Wellstein, A.T. Riegel, Epidermal growth factor receptor tyrosine phosphorylation and signaling controlled by a nuclear receptor coactivator, amplified in breast cancer 1, Cancer Res. 67 (2007) 7256–7265.
[6] K. Tobita, H. Kijima, S. Dowaki, H. Kashiwagi, Y. Ohtani, Y. Oida, H. Yamazaki, M. Nakamura, Y. Ueyama, M. Tanaka, S. Inokuchi, H. Makuuchi, Epidermal growth factor receptor expression in human pancreatic cancer: significance for liver metastasis, Int. J. Mol. Med. 11 (2003) 305–309.
[7] M. Nakashima, S. Adachi, I. Yasuda, T. Yamauchi, J. Kawaguchi, T. Hanamatsu, T. Yoshioka, Y. Okano, Y. Hirose, O. Kozawa, H. Moriwaki, Inhibition of Rho-associated coiled-coil containing protein kinase enhances the activation of epidermal growth factor receptor in pancreatic cancer cells, Mol. Cancer 10 (2011) 79.
[8] B.A. Faller, B. Burtness, Treatment of pancreatic cancer with epidermal growth factor receptor-targeted therapy, Biologics 3 (2009) 419–428.
[9] L. Latonen, M. Laiho, Cellular UV damage responses-functions of tumor suppressor p53, Biochim. Biophys. Acta 1755 (2005) 71–89.
[10] T. Yamauchi, S. Adachi, I. Yasuda, M. Nakashima, J. Kawaguchi, Y. Nishii, T. Yoshioka, Y. Okano, Y. Hirose, O. Kozawa, H. Moriwaki, UV-C irradiation induces downregulation of EGF receptor via phosphorylation at serine 1046/1047 in human pancreatic cancer cells. Radiat. Res. (in press).
[11] S. Adachi, I. Yasuda, M. Nakashima, T. Yamauchi, J. Kawaguchi, M. Shimizu, M. Itani, M. Nakamura, Y. Nishii, T. Yoshioka, Y. Hirose, Y. Okano, H. Moriwaki, O. Kozawa, Ultraviolet irradiation can induce evasion of colon cancer cells from stimulation of epidermal growth factor, J. Biol. Chem. 286 (2011) 26178–26187.
[12] M. Jalving, J.A. Gietema, J.D. Lefrandt, S. de Jong, A.K. Reyners, R.O. Gans, E.G. de Vries, Metformin: taking away the candy for cancer?, Eur J. Cancer 46 (2010) 2369–2380.
[13] M.C. Towler, D.G. Hardie, AMP-activated protein kinase in metabolic control and insulin signaling, Circ. Res. 100 (2007) 328–341.
[14] S. Adachi, T. Nagao, H.I. Ingolfsson, F.R. Maxfield, O.S. Andersen, L. Kopelovich, I.B. Weinstein, The inhibitory effect of (-)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells, Cancer Res. 67 (2007) 6493–6501.
[15] S. Adachi, M. Shimizu, Y. Shirakami, J. Yamauchi, H. Natsume, R. Matsushima-Nishiwaki, S. To, I.B. Weinstein, H. Moriwaki, O. Kozawa, (-)-Epigallocatechin gallate downregulates EGF receptor via phosphorylation at Ser1046/1047 by p38 MAPK in colon cancer cells, Carcinogenesis 30 (2009) 1544–1552.
[16] E.S. Henson, S.B. Gibson, Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy, Cell. Signal. 18 (2006) 2089–2097.
[17] C. Sutherland, I.A. Leighton, P. Cohen, Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling, Biochem. J. 296 (Pt 1) (1993) 15–19.
[18] J.P.t. Morris, S.C. Wang, M. Hebrok, KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma, Nat. Rev. Cancer 10 (2010) 683–695.
[19] A. Habiro, S. Tanno, K. Koizumi, T. Izawa, Y. Nakano, M. Osanai, Y. Mizukami, T. Okumura, Y. Kohgo, Involvement of p38 mitogen-activated protein kinase in gemcitabine-induced apoptosis in human pancreatic cancer cells, Biochem. Biophys. Res. Commun. 316 (2004) 71–77.
[20] H. Motoshima, B.J. Goldstein, M. Igata, E. Araki, AMPK and cell proliferation-AMPK as a therapeutic target for atherosclerosis and cancer, J. Physiol. 574 (2006) 63–71.
[21] M. Zakikhani, R. Dowling, I.G. Fantus, N. Sonenberg, M. Pollak, Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells, Cancer Res. 66 (2006) 10269–10273.
[22] R.J. Dowling, M. Zakikhani, I.G. Fantus, M. Pollak, N. Sonenberg, Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells, Cancer Res. 67 (2007) 10804–10812.

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Renin–angiotensin system inhibitors suppress azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice

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Oxidative stress

ABSTRACT

Obesity-related metabolic abnormalities, including chronic inflammation and oxidative stress, increase the risk of colorectal cancer. Dysregulation of the renin–angiotensin system (RAS) also plays a critical role in obesity-related metabolic disorders and in several types of carcinogenesis. In the present study, we examined the effects of an angiotensin-converting enzyme (ACE) inhibitor and angiotensin-II type 1 receptor blocker (ARB), both of which inhibit the RAS, on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice. Male *db/db* mice were given 4 weekly subcutaneous injections of AOM (15 mg/kg body weight), and then, they received drinking water containing captopril (ACE inhibitor, 5 mg/kg/day) or telmisartan (ARB, 5 mg/kg/day) for 7 weeks. At sacrifice, administration of either captopril or telmisartan significantly reduced the total number of colonic premalignant lesions, i.e., aberrant crypt foci and β-catenin accumulated crypts, compared to that observed in the control group. The expression levels of TNF-α mRNA in the colonic mucosa of AOM-treated *db/db* mice were decreased by captopril and telmisartan. Captopril lowered the expression levels of TNF-α, IL-1β, IL-6, and PAI-1 mRNAs, while telmisartan lowered the expression levels of COX-2, IL-1β, IL-6, and PAI-1 mRNAs in the white adipose tissues of these mice. In addition, these agents significantly reduced the levels of urinary 8-OHdG, a surrogate marker of oxidative damage to DNA, in the experimental mice. These findings suggested that both ACE inhibitor and ARB suppress chemically-induced colon carcinogenesis by attenuating chronic inflammation and reducing oxidative stress in obese mice. Therefore, targeting dysregulation of the RAS might be an effective strategy for chemoprevention of colorectal carcinogenesis in obese individuals.

1. Introduction

Mounting evidence indicates that obesity, a result of a positive energy balance, and its related metabolic abnormalities raise the risk of colorectal cancer (CRC) [1,2]. Obesity is regarded as a state of chronic inflammation, which is closely associated with colorectal carcinogenesis [3]. Increased levels of adipose tissue lead to the expression of a variety of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) [4], which stimulates tumor promotion and progression of carcinogenesis [5]. Oxidative stress, which is induced by increased energy availability [6], has also been suggested to play an important role in the development of CRC [1,2]. Thus, these findings suggest that targeting inflammation and oxidative stress may be an effective strategy for preventing the development of CRC, especially in overweight individuals. For instance, a recent study shows that administration of pitavastatin, a hypolipidemic drug, prevents obesity-related colorectal tumorigenesis by attenuating chronic inflammation [7].

Hyperactivity of the renin–angiotensin system (RAS), an endocrine system with critical roles in cardiovascular function, has been implicated in the etiology of high blood pressure, obesity, and metabolic syndrome [8]. In addition, there is strong evidence that the RAS is frequently dysregulated in human malignancies, which correlates with poor patient outcomes. Abnormalities in the RAS influences cancer cell migration, invasion, and metastasis, all of which are closely associated with chronic inflammation and angiogenesis [9,10]. In cancer tissues, the RAS is upregulated through systemic

Abbreviations: ACE, angiotensin converting enzyme; ACF, aberrant crypt foci; AOM, azoxymethane; ARB, angiotensin-II type-1 receptor blocker; BCAC, β-catenin accumulated crypt; COX-2, cyclooxygenase-2; CRC, colorectal cancer; *db/db* mice, C57BL/KsJ-*db/db* mice; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IL, interleukin; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PAI-1, plasminogen activator inhibitor-1; RAS, renin–angiotensin system; RT-PCR, reverse transcription-PCR; TNF-α, tumor necrosis factor-α.

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oxidative stress and hypoxia mechanisms, which triggers chronic inflammatory processes to remodel the surrounding environment [11].

Drugs that reduce the synthesis (angiotensin-converting enzyme [ACE] inhibitors) or action (angiotensin-II type-1 receptor blockers [ARBs]) of angiotensin-II, the active product of RAS, are widely used for the treatment of hypertension. These agents have also been expected to exert beneficial effects that improve the symptoms of metabolic disorders [12,13]. In addition, retrospective studies have shown that patients taking ACE inhibitors or ARBs had decreased risk of developing some types of cancers, including CRC [14–16]. The expression levels of ACE are higher in colorectal adenomas and CRC epithelial cells than in the corresponding non-neoplastic crypt and surface epithelia [17]. In a mouse model of CRC liver metastasis, administration of an ACE inhibitor and ARB significantly reduced tumor volume by blocking the RAS activity [18]. These reports suggest that the RAS might be a critical target for the treatment and/or prevention of certain types of human malignancies, including CRC. However, the possibility of CRC chemoprevention by targeting the RAS is yet to be considered.

The C57BL/KsJ-*db/db* (*db/db*) mouse is one of the most widely used models of type 2 diabetes. The development of diabetes in *db/db* mice results in the activation of RAS and induction of oxidative stress, which promotes progressive inflammation [19]. In the present study, we used male *db/db* mice injected with azoxymethane (AOM) to examine the effects of captopril (ACE inhibitor) and telmisartan (ARB) on the development of aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma [20,21], by focusing on the attenuation of inflammation and reduction of oxidative stress. This preclinical animal model is useful for investigating specific agents for their ability to prevent inflammation-related colorectal carcinogenesis caused by obesity [7].

2. Materials and methods

2.1. Animals, chemicals, and diet

Male homozygous *db/db* mice aged 4 weeks (Japan SLC, Inc., Shizuoka, Japan) were maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. AOM, captopril, and telmisartan were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Experimental procedure

The animal experiment, as described previously [7,22,23], was approved by the Committee of the Institutional Animal Experiments of Gifu University. A total of 45 male *db/db* mice were divided into six groups. To induce colonic preneoplastic lesions, at 5 weeks of age, the mice in Groups 4 (10 mice), 5 (10 mice), and 6 (10 mice) were given 4 weekly subcutaneous injections of AOM (15 mg/kg body weight). The mice in Groups 1 (5 mice), 2 (5 mice), and 3 (5 mice) were subcutaneously injected with saline once a week for 4 weeks. Groups 2 and 5 received drinking water containing captopril (5 mg/kg/day) for 7 weeks, starting 1 week after the last injection of AOM. Similarly, the mice in Groups 3 and 6 were given drinking water containing telmisartan (5 mg/kg/day). Captopril and telmisartan intake was maintained by adjusting the concentration of these agents in drinking water, whose volume was measured three times a week. Groups 1 and 4 were given tap water throughout the experiment. At the end of the study (16 weeks of age), all the mice were sacrificed by CO_2 asphyxiation for colon resection. The third portion of excised colons (cecum side) was used to extract RNA, and the remaining part was used to determine the numbers of colonic ACF and BCAC.

2.3. Counting the number of ACF and BCAC

The frequency of ACF and BCAC was determined according to the standard procedures [7,22,23]. The colon samples fixed with 10% buffered formalin were stained with methylene blue (0.5% in distilled water), and the number of ACF was counted under a light microscope. To identify BCAC intramucosal lesions, the distal part (1 cm from the anus) of the colon (mean area: 0.7 cm^2/colon) was embedded in paraffin, and 20 serial sections (4-μm thick) per mouse were created by an *en face* preparation. The sections were then subjected to H&E staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC. The anti-β-catenin primary antibody was purchased from BD Transduction Laboratories (San Jose, CA, USA), and immunohistochemical staining was performed using a labeled streptavidin–biotin method (DAKO, Glostrup, Denmark). β-Catenin-stained BCACs were counted and the values were expressed as per cm^2 of mucosa [7,22,23].

2.4. RNA extraction and quantitative real-time reverse transcription-PCR

The expression levels of TNF-α and interleukin (IL)-6 genes in the colonic mucosa and those of TNF-α, cyclooxygenase (COX)-2, IL-1β, IL-6, and plasminogen activator inhibitor-1 (PAI-1) genes in the white adipose tissues of AOM-treated *db/db* mice were determined by quantitative real-time reverse transcription-PCR (RT-PCR) analysis [7,24]. Total RNA was isolated using the RNAqueous-4PCR Kit (Applied Biosystems, Austin, TX, USA). cDNA was synthesized from 0.2 μg of total RNA by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The specific primers used for the amplification of TNF-α, COX-2, IL-1β, IL-6, and β-actin genes were as previously described [24]. The specific primers used for amplification of the PAI-1 gene were as follows: sense 5′-TTC AGC CCT TGC TTG CCT C-3′ and antisense 5′-ACA CTT TTA CTC CGA AGT CGG T-3′. Real-time RT-PCR was performed using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) with the SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan). The expression level of each gene was normalized to that of the β-actin gene by using the standard curve method.

2.5. Measurement of urinary 8-OHdG levels

Urine samples were collected at the time of sacrifice, and the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were determined by using an enzyme-linked immunosorbent assay (ELISA) kit (NIKKEN SEIL, Shizuoka, Japan) according to the manufacturer's protocol.

2.6. Statistical analyses

The statistical analyses were performed using the JMP 8 software program (SAS Institute, Cary, NC, USA), and the results are presented as mean (SD). Statistical significance was evaluated using Dunnet's *t*-test for multiple comparisons. Differences were considered statistically significant when the two-tailed *p*-value was less than 0.05.

3. Results

3.1. General observations

As listed in Table 1, the average body weight and relative liver weight of the AOM-injected groups (Groups 4–6) at the end of the experiment were significantly ($p < 0.01$) lower than those of

Table 1
Body, liver, and kidney weights of the experimental mice.

Group No.	Treatment	No. of mice	Body weight (g)	Relative weight (g/100 g body weight) of:	
				Liver	Kidney
1	Saline	5	54.6 ± 8.8[a]	5.95 ± 0.92	0.90 ± 0.26
2	Saline + captopril	5	58.2 ± 1.8	6.28 ± 0.44	0.79 ± 0.07
3	Saline + telmisartan	5	62.0 ± 3.6	7.65 ± 1.05	0.76 ± 0.10
4	AOM alone	10	40.9 ± 5.5[b]	4.65 ± 0.60[b]	0.93 ± 0.27
5	AOM + captopril	10	37.4 ± 8.4[b]	4.53 ± 0.64[b]	1.05 ± 0.22
6	AOM + telmisartan	10	42.2 ± 8.8[b]	4.49 ± 0.55[b]	0.96 ± 0.15

[a] Mean ± SD.
[b] Significantly different from Group 1 ($p < 0.01$).

the saline-injected group (Group 1). This might be caused by the toxicity of AOM, as observed in previous experiments [7,22,23]. No significant differences were observed in the mean relative weight of the kidney among the groups. No histopathological findings suggesting toxicity of captopril or telmisartan in the liver, kidney, and spleen of the mice were obtained (data not shown).

3.2. Effects of captopril and telmisartan on AOM-induced ACF and BCAC in db/db mice

Table 2 summarizes the total number of ACF (Fig. 1A and B) and BCAC (Fig. 1C and D) in the mice from all groups. Both ACF and BCAC developed in the colons of all mice that received AOM (Groups 4–6), but not in those without AOM treatment (Groups 1–3). When compared with Group 4 (AOM alone), administration of either captopril or telmisartan in drinking water significantly reduced ACF frequency; the inhibition rates were 43% in Group 5 (AOM + captopril, $p < 0.01$) and 39% in Group 6 (AOM + telmisartan, $p < 0.01$). Similarly, both captopril- (76% reduction, $p < 0.01$) and telmisartan- (71% reduction, $p < 0.01$) treatment groups had significantly decreased numbers of BCAC than the AOM alone-treated group.

3.3. Effects of captopril and telmisartan on the expression levels of TNF-α and IL-6 mRNA in the colonic mucosa of AOM-treated db/db mice

TNF-α is an important tumor promoter involved in obesity, inflammation, and carcinogenesis [3–5]. As shown in Fig. 2A, quantitative real-time RT-PCR analyses showed that both captopril and telmisartan significantly decreased the expression levels of TNF-α mRNA in the colonic mucosa of AOM-treated mice ($p < 0.05$). On the other hand, the expression levels of IL-6 mRNA in the colonic mucosa (Fig. 2B), which also are possibly involved in obesity- and inflammation-related colorectal carcinogenesis [3,25], were not significantly lowered by treatment with these agents.

3.4. Effects of captopril and telmisartan on the expression levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 mRNA in the white adipose tissues of AOM-treated db/db mice

In the white adipose tissues of AOM-treated *db/db* mice, the expression levels of TNF-α (Fig. 3A), IL-1β (Fig. 3C), IL-6 (Fig. 3D), and PAI-1 (Fig. 3E) mRNAs were significantly inhibited by captopril administration compared to the control mice ($p < 0.05$ for each). Drinking telmisartan also caused a decrease in the expression levels of COX-2 (Fig. 3B), IL-1β (Fig. 3C), IL-6 (Fig. 3D), and PAI-1 (Fig. 3E) mRNAs in the white adipose tissues of AOM-treated mice ($p < 0.05$). These findings (Figs. 2 and 3) indicated that administration of these agents attenuates the inflammatory response in the colonic mucosa and in the white adipose tissues of obese mice.

3.5. Effects of captopril and telmisartan on the urinary levels of 8-OHdG in AOM-treated db/db mice

Urinary 8-OHdG levels in AOM-treated *db/db* mice were determined using ELISA method (Fig. 4). The mice treated with either captopril (6.5 ± 2.0 ng/mL) or telmisartan (7.3 ± 2.2 ng/mL) showed a significant decrease in the urinary levels of 8-OHdG compared to the untreated mice (17.9 ± 3.5 ng/mL; $p < 0.01$ for each comparison). These findings indicated that captopril and telmisartan suppresses obesity-related systemic oxidative stress.

4. Discussion

There is accumulating evidence to indicate that abnormalities in the RAS play a critical role in several types of carcinogenesis; therefore, agents targeting the RAS might augment cancer therapies [9,10]. The results of the present study clearly indicated that the RAS inhibitors captopril and telmisartan effectively suppress the development of colonic preneoplastic lesions, ACF and BCAC, in male *db/db* obese mice. This is the first report that shows the preventive effect of an ACE inhibitor and ARB on the development of chemically-induced colorectal carcinogenesis in any mouse

Table 2
Effects of captopril and telmisartan on AOM-induced ACF and BCAC formation in the experimental mice.

Group No.	Treatment	No. of mice	Length of colon (cm)	Total No. of ACFs/colon	Total No. of BCACs/cm^2
1	Saline	5	11.1 ± 0.8 [a]	0	0
2	Saline + captopril	5	11.9 ± 0.9	0	0
3	Saline + telmisartan	5	11.7 ± 0.8	0	0
4	AOM alone	10	10.6 ± 0.8	134.0 ± 24.5	3.4 ± 1.8
5	AOM + captopril	10	10.6 ± 1.3	76.9 ± 24.3[b]	0.8 ± 0.9[b]
6	AOM + telmisartan	10	10.8 ± 0.7	81.8 ± 14.0[b]	1.0 ± 1.0[b]

[a] Mean ± SD.
[b] Significantly different from Group 4 ($p < 0.01$).

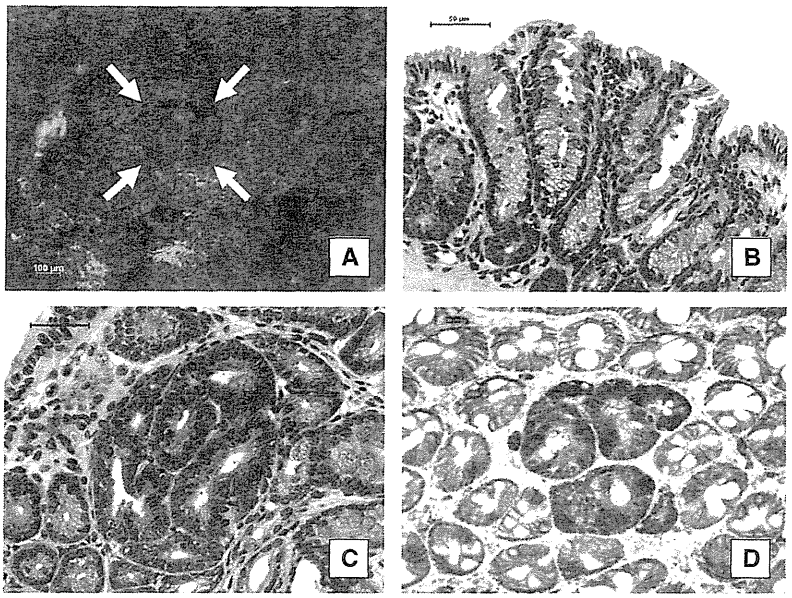

Fig. 1. Histopathology and β-catenin-immunohistochemistry of ACF and BCAC in AOM-exposed *db/db* mice (Group 4). Arrows indicate ACF (A) stained by methylene blue on the colonic mucosa. Representative photographs of ACF (B) and BCAC (C) stained with H&E. Basophilic cytoplasm and hyperchromatic nuclei are observed in the atypical cryptal cells in BCAC (C). Immunohistochemistry of β-catenin protein in BCAC (D). The localization of the accumulated β-catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells. Scale bars, 100 μm (A) and 50 μm (B–D).

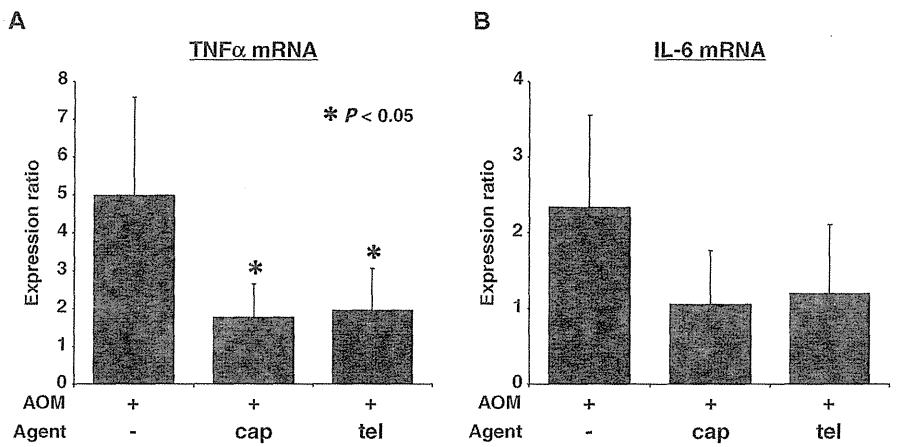

Fig. 2. The effects of captopril and telmisartan on the expression levels of TNF-α and IL-6 mRNAs in the colonic mucosa of AOM-treated *db/db* mice. cDNA was synthesized from scraped colonic mucosa, and real-time RT-PCR was performed using specific primers for TNF-α (A) and IL-6 (B). The expression levels of these genes were normalized to that of the β-actin gene. Data represent mean ± SD ($n = 8$). *$p < 0.05$ vs. AOM-treated control group.

model. The finding seemed to be significant in clinical medicine because these drugs are widely used for patients with hypertension who frequently are obese. Furthermore, high blood pressure is involved in the increased risk of development of CRC and colonic adenomas [26–28], thus indicating that obese and hypertensive patients might be regarded as a high-risk group for CRC development. On the other hand, a recent retrospective study shows that use of an ACE inhibitor is significantly associated with reduction in the incidence and size of colorectal adenomas, the precancerous lesions for CRC [29]. This report [29] along with the results of the present study suggests that inhibition of RAS might be an effective strategy for the prevention of colorectal tumorigenesis, especially in obese individuals.

A key feature of obesity is increased inflammation in the adipose tissue, which might be involved in cancer promotion and progression [3]. Angiotensin-II is considered a pro-inflammatory mediator because activation of its receptor induces a number of molecules that participate in inflammatory responses [8–10]. For instance, mice with elevated adipocyte angiotensinogen expression have increased the expression of TNF-α, IL-6, and IL-1β in the adipose tissue [30]. Treatment with ARB decreased plasma levels of TNF-α and IL-6 in patients with congestive heart failure [31]. In the present study, either captopril or telmisartan decreased the mRNA levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 in the white adipose tissue of AOM-treated *db/db* mice. Therefore, the chemopreventive effect of an ACE inhibitor and ARB on obesity-related colorectal carcinogenesis is most likely associated with the attenuation of systemic inflammation. In addition, the inhibition of the expression levels of TNF-α mRNA in the colonic mucosa might also contribute to this beneficial effect because this cytokine promotes

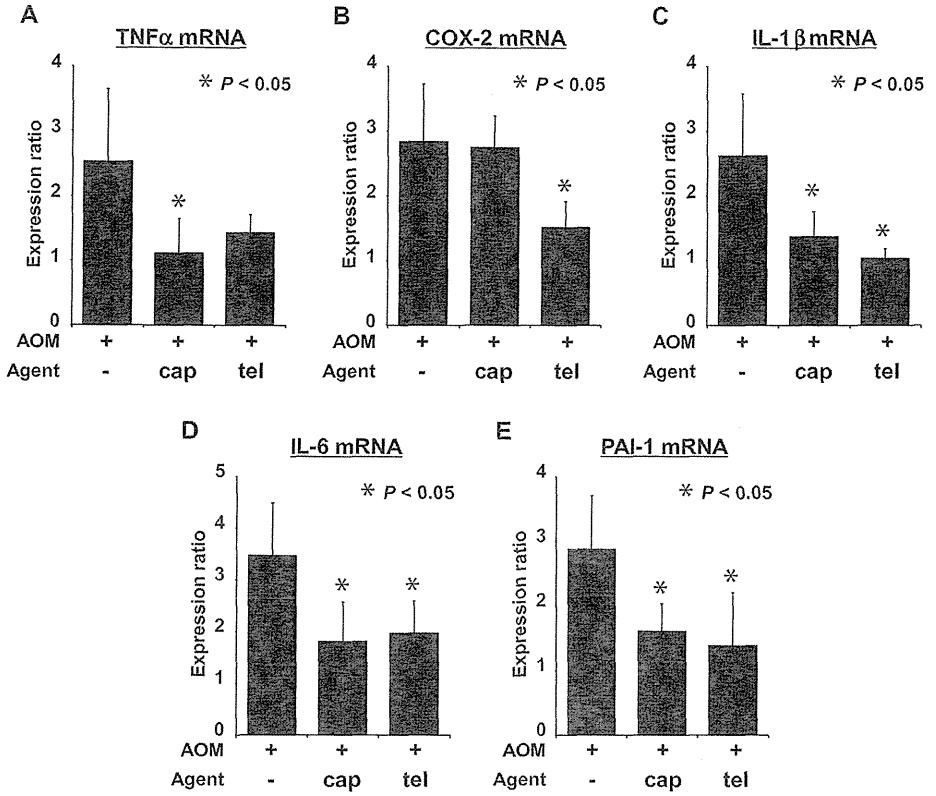

Fig. 3. The effects of captopril and telmisartan on the expression levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 mRNAs in the white adipose tissues of AOM-treated *db/db* mice. cDNA was synthesized from the white adipose tissues of the retroperitoneum, and real-time RT-PCR was performed using specific primers for TNF-α (A), COX-2 (B), IL-1β (C), IL-6 (D), and PAI-1 (E). The expression levels of these genes were normalized to that of the β-actin gene. Data represent mean ± SD ($n = 8$). *$p < 0.05$ vs. AOM-treated control group.

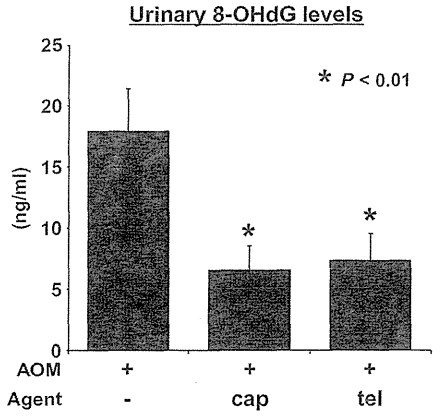

Fig. 4. The effects of captopril and telmisartan on urinary 8-OHdG levels in AOM-treated *db/db* mice. At sacrifice, urine samples were collected from the experimental mice, and the levels of urinary 8-OHdG were measured by ELISA. Data represent mean ± SD ($n = 8$). *$p < 0.01$ vs. AOM-treated control group.

inflammation-related colorectal carcinogenesis, and thus, are critical targets for CRC chemoprevention [5,7,32].

Increased oxidative stress, which is associated with obesity due to metabolic and inflammatory changes [6], promotes damage to cell structures including DNA; this plays a key role in cancer development [33]. Certain types of chemopreventive agents, such as polyphenolic compounds, inhibit colorectal carcinogenesis by exerting anti-oxidant effects [34,35]. Activation of the RAS by enhanced levels of angiotensin-II leads to an increase in oxidative stress [36], but this is significantly reduced by treatment with RAS inhibitors [37,38]. In prostate cancer cells, candesartan, an ARB, also significantly reduces angiotensin-II-upregulated oxidative stress [39]. In the present study, both captopril and telmisartan decreased the levels of urinary 8-OHdG, which is a useful marker of DNA damage induced by oxidative stress, and this might be associated with inhibition of colorectal carcinogenesis. These findings, together with the results of recent studies [36–39], suggest that increased oxidative stress might be a critical target of RAS inhibitors for suppression of CRC.

Recent studies have revealed that insulin resistance and hyperinsulinemia, which are closely related to obesity, are some of the key factors in the development of obesity-related CRC, and thus, may be critical targets for the prevention of this malignancy [1,2,22,23]. In addition, activation of the RAS has been implicated in the etiology of obesity and insulin resistance [8,40]. Therefore, in the present study, it was expected that captopril and telmisartan might improve insulin resistance. However, contrary to our expectations, there was no clear evidence indicating an improvement in insulin resistance by these agents (data not shown). Therefore, at least in the present study, insulin resistance might not be a critical target of ACE inhibitors and ARBs to prevent colorectal tumorigenesis in obese mice.

The present experimental study was performed using *db/db* obese mice, which exhibit increased RAS activation and oxidative stress [19]. These mice are also highly susceptible to the colonic carcinogen AOM compared to the wild (+/+) and heterozygous

db/+ mice, neither of which exhibit obesity [41,42]. However, a question whether RAS inhibitors can prevent CRC development under non-obese condition has not yet been determined. Therefore, further studies that can clarify the effects of RAS inhibitors on the development of CRC under physiological RAS condition are required to confirm the possibility that these agents can be widely used as chemopreventive drugs for CRC.

In summary, prevention of CRC by targeting chronic inflammation and oxidative stress, which is caused by obesity and is related to RAS activation, might be a promising chemopreventive strategy for obese people, who are at an increased risk of developing CRC. Therefore, the agents targeting RAS, including ACE inhibitors and ARBs, appear to be potentially effective candidates for this purpose because these drugs attenuate inflammation while reducing oxidative stress.

Conflicts of interest

The authors declare that no conflicts of interest exist.

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References

[1] E. Giovannucci, D. Michaud, The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas, Gastroenterology 132 (2007) 2208–2225.
[2] E.E. Frezza, M.S. Wachtel, M. Chiriva-Internati, Influence of obesity on the risk of developing colon cancer, Gut 55 (2006) 285–291.
[3] M.J. Gunter, M.F. Leitzmann, Obesity and colorectal cancer: epidemiology, mechanisms and candidate genes, J. Nutr. Biochem. 17 (2006) 145–156.
[4] G.S. Hotamisligil, N.S. Shargill, B.M. Spiegelman, Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance, Science 259 (1993) 87–91.
[5] P. Szlosarek, K.A. Charles, F.R. Balkwill, Tumour necrosis factor-alpha as a tumour promoter, Eur. J. Cancer 42 (2006) 745–750.
[6] S. Furukawa, T. Fujita, M. Shimabukuro, M. Iwaki, Y. Yamada, Y. Nakajima, O. Nakayama, M. Makishima, M. Matsuda, I. Shimomura, Increased oxidative stress in obesity and its impact on metabolic syndrome, J. Clin. Invest. 114 (2004) 1752–1761.
[7] Y. Yasuda, M. Shimizu, Y. Shirakami, H. Sakai, M. Kubota, K. Hata, Y. Hirose, H. Tsurumi, T. Tanaka, H. Moriwaki, Pitavastatin inhibits azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-db/db obese mice, Cancer Sci. 101 (2010) 1701–1707.
[8] A.D. de Kloet, E.G. Krause, S.C. Woods, The renin angiotensin system and the metabolic syndrome, Physiol. Behav. 100 (2010) 525–534.
[9] A.J. George, W.G. Thomas, R.D. Hannan, The renin–angiotensin system and cancer: old dog, new tricks, Nat. Rev. Cancer 10 (2010) 745–759.
[10] E.I. Ager, J. Neo, C. Christophi, The renin–angiotensin system and malignancy, Carcinogenesis 29 (2008) 1675–1684.
[11] G.R. Smith, S. Missailidis, Cancer, inflammation and the AT1 and AT2 receptors, J. Inflamm. 1 (2004) 3.
[12] A.M. Sharma, The value of current interventions for obesity, Nat. Clin. Pract. Cardiovasc. Med. 5 (Suppl. 1) (2008) S3–S9.
[13] S.G. Chrysant, G.S. Chrysant, C. Chrysant, M. Shiraz, The treatment of cardiovascular disease continuum: focus on prevention and RAS blockade, Curr. Clin. Pharmacol. 5 (2010) 89–95.
[14] A.F. Lever, D.J. Hole, C.R. Gillis, I.R. McCallum, G.T. McInnes, P.L. MacKinnon, P.A. Meredith, L.S. Murray, J.L. Reid, J.W. Robertson, Do inhibitors of angiotensin-I-converting enzyme protect against risk of cancer?, Lancet 352 (1998) 179–184.
[15] L. Lang, ACE inhibitors may reduce esophageal cancer incidence, Gastroenterology 131 (2006) 343–344.
[16] J.B. Christian, K.L. Lapane, A.L. Hume, C.B. Eaton, M.A. Weinstock, Association of ACE inhibitors and angiotensin receptor blockers with keratinocyte cancer prevention in the randomized VATTC trial, J. Natl. Cancer Inst. 100 (2008) 1223–1232.
[17] C. Rocken, K. Neumann, S. Carl-McGrath, H. Lage, M.P. Ebert, J. Dierkes, C.A. Jacobi, S. Kalmuk, P. Neuhaus, U. Neumann, The gene polymorphism of the angiotensin I-converting enzyme correlates with tumor size and patient survival in colorectal cancer patients, Neoplasia 9 (2007) 716–722.
[18] J.H. Neo, C. Malcontenti-Wilson, V. Muralidharan, C. Christophi, Effect of ACE inhibitors and angiotensin II receptor antagonists in a mouse model of colorectal cancer liver metastases, J. Gastroenterol. Hepatol. 22 (2007) 577–584.
[19] G.H. Tesch, A.K. Lim, Recent insights into diabetic renal injury from the db/db mouse model of type 2 diabetic nephropathy, Am. J. Physiol. Renal Physiol. 300 (2011) F301–F310.
[20] R.P. Bird, C.K. Good, The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer, Toxicol. Lett. 112–113 (2000) 395–402.
[21] Y. Yamada, H. Mori, Pre-cancerous lesions for colorectal cancers in rodents: a new concept, Carcinogenesis 24 (2003) 1015–1019.
[22] M. Shimizu, Y. Shirakami, H. Sakai, S. Adachi, K. Hata, Y. Hirose, H. Tsurumi, T. Tanaka, H. Moriwaki, EGCG suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-db/db mice, Cancer Prev. Res. 1 (2008) 298–304.
[23] M. Shimizu, Y. Shirakami, J. Iwasa, M. Shiraki, Y. Yasuda, K. Hata, Y. Hirose, H. Tsurumi, T. Tanaka, H. Moriwaki, Supplementation with branched-chain amino acids inhibits azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-db/db mice, Clin. Cancer Res. 15 (2009) 3068–3075.
[24] H. Sakai, Y. Yamada, M. Shimizu, K. Saito, H. Moriwaki, A. Hara, Genetic ablation of Tnfalpha demonstrates no detectable suppressive effect on inflammation-related mouse colon tumorigenesis, Chem. Biol. Interact. 184 (2010) 423–430.
[25] S. Rose-John, K. Mitsuyama, S. Matsumoto, W.M. Thaiss, J. Scheller, Interleukin-6 trans-signaling and colonic cancer associated with inflammatory bowel disease, Curr. Pharm. Des. 15 (2009) 2095–2103.
[26] R.L. Ahmed, K.H. Schmitz, K.E. Anderson, W.D. Rosamond, A.R. Folsom, The metabolic syndrome and risk of incident colorectal cancer, Cancer 107 (2006) 28–36.
[27] L.A. Colangelo, S.M. Gapstur, P.H. Gann, A.R. Dyer, K. Liu, Colorectal cancer mortality and factors related to the insulin resistance syndrome, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 385–391.
[28] Y.Y. Wang, S.Y. Lin, W.A. Lai, P.H. Liu, W.H. Sheu, Association between adenomas of rectosigmoid colon and metabolic syndrome features in a Chinese population, J. Gastroenterol. Hepatol. 20 (2005) 1410–1415.
[29] R. Kedika, M. Patel, H.N. Pena Sahdala, A. Mahgoub, D. Cipher, A.A. Siddiqui, Long-term use of angiotensin converting enzyme inhibitors is associated with decreased incidence of advanced adenomatous colon polyps, J. Clin. Gastroenterol. 45 (2011) e12–e16.
[30] L. Yvan-Charvet, F. Massiera, N. Lamande, G. Ailhaud, M. Teboul, N. Moustaid-Moussa, J.M. Gasc, A. Quignard-Boulange, Deficiency of angiotensin type 2 receptor rescues obesity but not hypertension induced by overexpression of angiotensinogen in adipose tissue, Endocrinology 150 (2009) 1421–1428.
[31] T. Tsutamoto, A. Wada, K. Maeda, N. Mabuchi, M. Hayashi, T. Tsutsui, M. Ohnishi, M. Sawaki, M. Fujii, T. Matsumoto, M. Kinoshita, Angiotensin II type 1 receptor antagonist decreases plasma levels of tumor necrosis factor alpha, interleukin-6 and soluble adhesion molecules in patients with chronic heart failure, J. Am. Coll. Cardiol. 35 (2000) 714–721.
[32] Y. Shirakami, M. Shimizu, H. Tsurumi, Y. Hara, T. Tanaka, H. Moriwaki, EGCG and Polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM and DSS, Mol. Med. Report 1 (2008) 355–361.
[33] M. Valko, M. Izakovic, M. Mazur, C.J. Rhodes, J. Telser, Role of oxygen radicals in DNA damage and cancer incidence, Mol. Cell. Biochem. 266 (2004) 37–56.
[34] P. Dolara, C. Luceri, C. De Filippo, A.P. Femia, L. Giovannelli, G. Caderni, C. Cecchini, S. Silvi, C. Orpianesi, A. Cresci, Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats, Mutat. Res. 591 (2005) 237–246.
[35] G.K. Harris, A. Gupta, R.G. Nines, L.A. Kresty, S.G. Habib, W.L. Frankel, K. LaPerle, D.D. Gallaher, S.J. Schwartz, G.D. Stoner, Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′-deoxyguanosine levels in the Fischer 344 rat, Nutr. Cancer 40 (2001) 125–133.
[36] A.M. Garrido, K.K. Griendling, NADPH oxidases and angiotensin II receptor signaling, Mol. Cell. Endocrinol. 302 (2009) 148–158.
[37] T. Nakamura, N. Fujiwara, E. Sato, Y. Ueda, T. Sugaya, H. Koide, Renoprotective effects of various angiotensin II receptor blockers in patients with early-stage diabetic nephropathy, Kidney Blood Press. Res. 33 (2010) 213–220.
[38] Y. Dincer, N. Sekercioglu, M. Pekpak, K.N. Gunes, T. Akcay, Assessment of DNA oxidation and antioxidant activity in hypertensive patients with chronic kidney disease, Ren. Fail. 30 (2008) 1006–1011.
[39] H. Uemura, H. Ishiguro, Y. Ishiguro, K. Hoshino, S. Takahashi, Y. Kubota, Angiotensin II induces oxidative stress in prostate cancer, Mol. Cancer Res. 6 (2008) 250–258.
[40] Y. Wei, J.R. Sowers, S.E. Clark, W. Li, C.M. Ferrario, C.S. Stump, Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-kappaB activation via NADPH oxidase, Am. J. Physiol. Endocrinol. Metab. 294 (2008) E345–E351.
[41] Y. Hirose, K. Hata, T. Kuno, K. Yoshida, K. Sakata, Y. Yamada, T. Tanaka, B.S. Reddy, H. Mori, Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/Ks-db/db mice, Carcinogenesis 25 (2004) 821–825.
[42] R. Suzuki, H. Kohno, Y. Yasui, K. Hata, S. Sugie, S. Miyamoto, K. Sugawara, T. Sumida, Y. Hirose, T. Tanaka, Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male db/db mice, Int. J. Cancer 120 (2007) 252–258.