Retinoic Acid 4-Hydroxylase-Mediated Catabolism of All-*trans* Retinoic Acid and the Cell Proliferation in Head and Neck Squamous Cell Carcinoma

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All-*trans* retinoic acid (RA) can be catabolized to polar metabolites by microsomal P450s (P450). The aim of this study was to confirm if retinoic acid 4-hydroxylase (CYP26) is a P450 induced by RA and to investigate the role of cellular RA binding proteins (CRABPs), using a slow catabolizer, AMC-HN-4, and a rapid catabolizer, AMC-HN-6. Also, we analyzed the effect of RA catabolism on cell proliferation of head and neck squamous cell carcinoma (HNSCC) in vitro and in vivo. Both cell lines weakly expressed CYP26 and CRABPs, but RA induced CYP26 only in AMC-HN-6. The sensitivity to RA was variable by the amount of CYP26, and the rapid catabolism by CYP26 made AMC-HN-6 resistant to RA in vitro. In addition, The RA had a stronger effect on the inhibition of tumor growth of AMC-HN-4 than that of AMC-HN-6 in vivo. Conclusively, the CYP26 activity might be one essential factor for the RA sensitivity, but in cells showing induction of CYP26, the RA sensitivity is inversely related to the rate of RA catabolism.

RETINOIDS, INCLUDING vitamin A and its synthetic analogues, are known to suppress carcinogenesis in various epithelial tissues such as skin, bladder, lung, prostate, oral cavity, and mammary glands.[1] Also retinoids inhibit cell proliferation[2] and differentiation[3] of squamous cell carcinomas (SCC). In addition, retinoids can induce apoptosis in SCCs[4] and prevent second primary cancers.[5] Thus, retinoids have been used as a chemopreventive drug, but there are some limitations for the clinical trial due to the interpatient variability, side-effects, and the reversibility of the retinoid effects.[6]

Microsomal P450s (P450) play an important role in retinoic acid metabolism and maintenance of vitamin A homeostasis. All-*trans* retinoic acid (RA), which is known as an active metabolite of vitamin A, can be catabolized to polar metabolites by human retinoic acid 4-hydroxylase (CYP26).[7] The cellular retinoic acid binding proteins (CRABP) I and II have been known to regulate intracellular RA concentration, transport, and metabolism and to share a high degree of homology.[8] Also, Boylan and Gudas[9] reported that the level of CRABP I determined the rate of RA catabolism in F9 teratocarcinoma stem cells.

The rapid catabolism may be associated with resistance to the retinoid therapy[10] and with an increased risk of lung cancer.[11] On the other hand, Takatsuka et al[12] suggested the high activity of polar metabolites for cell growth inhibition, indicating that the cell growth was inhibited more significantly in cells that catabolized RA rapidly. Also, Sonneveld et al[7] reported that the activity of CYP closely corelated with the RA sensitivity.[7] However, Van der Leede et al[13] showed that the biological activity of the polar metabolites was equal to or lower than that of RA, and RA, itself, was the most active retinoid, and Isogai et al[14] found that cell growth inhibition did not correlate to RA metabolism.

Kim et al[15] reported that P450 could be induced by RA in some AMC-HN cell lines, in which RA was rapidly catabolized into polar metabolites. The aim of this study was to confirm if CYP26 was a P450 induced by RA and to investigate the role of CRABPs in RA catabolism using a slow catabolizer, AMC-HN-4, and a rapid catabolizer, AMC-HN-6. Also, we analyzed the effect of RA catabolism on the proliferation of head and neck squamous cell carcinoma (HNSCC) in vitro and in vivo.

MATERIALS AND METHODS

Cell Maintenance

HNSCC cell lines, AMC-HN-4 and −6, were established at our laboratory from the patients with HNSCC.[16] The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 1% nonessential amino acid, 2 mmol/L L-glutamine, and 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Grand Island, NY). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

The RA (1 μmol/L) treatment lasted for 3 days. Total RNA was isolated by Trizol reagent (Gibco). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the following primers for *CYP26*, *CRABP I*, and *CRABP II* gene. The sense primer of *CYP26* gene was 5′-TCC TCG CAC AAG CAG CGA AAG AAG GTG ATT-3′, and the antisense primer was 5′-ATG TGG GTA GAG TCC TAG GTA AGT-3′. Thirty-five cycles of PCR were performed under the following conditions: 94°C, 30 seconds; 60°C, 1 minute; 72°C, 1 mintue; and the final extension at 72°C for 10 minutes. The sense primer of *CRABP I* gene was 5′-CGG CAC CTG GAA GAT GCG CA-3′, and the antisense primer was 5′-CCA CGT CAT CGG CGC CAA ACTTG-3′. Forty cycles of PCR were performed under the following conditions: 94°C, 45 seconds; 65°C, 45 seconds; 72°C, 90 seconds; and the final extension at 72°C for 10 minutes. The sense primer of the *CRABP II* gene was 5′-CCC AAC TTC TCT GGC AAC TGG A-3′, and the antisense primer was 5′-CTC TCG GAC GTA GAC CCT GGT-3′. Forty cycles of PCR were performed under the

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following conditions: 94°C, 45 seconds; 65°C, 1 minute; 72°C, 90 seconds; and the final extension at 72°C for 10 minutes. Primers for human β-actin were used as a control.

Cell Proliferation Assay

The cells were plated onto 96-well dishes without RA. There were two 96-well plates, one in which the cell number/well was 10^3 and another plate, in which it was 10^4. Among these 96 wells, 48 were treated with RA and the other 48 were wells for control. Twenty-four hours later, 1 μmol/L of RA (Sigma Chemical, St Louis, MO) was added. Dimethyl sulfoxide (DMSO) was used as the solvent. RA was melted in 10 μL DMSO and made 100 mL of 1 μmol/L solution with the MEM media. Finally, the volume of media filled to each well was 100 μL, and DMSO was approximately 0.1 μL. After 3 days, the culture media were replaced with the same conditioned media, and the total period of drug treatment was 6 days. Cell proliferation was measured using CellTiter 96 AQ_{ueous} Non-Reactive Cell Proliferation Assay (Promega Corp, Madison, WI). In brief, 20 μL of MTS/PMS solution (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate) was added to each well and incubated at 37°C in a humidified atmosphere with 5% CO_2 for an additional 2 hours. The absorbance at 490 nm was recorded with an enzyme-linked immunosorbent assay (ELISA) plate reader. The cell proliferation (%) was calculated using the formula described below. All experiments were repeated in triplicate.

$$\text{Cell Proliferation} = \frac{\text{Test group absorbance} - \text{Background absorbance}}{\text{Control group absorbance} - \text{Background absorbance}} \times 100$$

The number of absorbance was the mean of 8 wells.

Tumor Xenograft Study

The AMC-HN-4 and -6 cells were harvested and resuspended in culture media at a concentration of 1×10^7 cells/mL. The RA was administered orally with an intragastric feeding tube in 0.1 mL of sesame oil (Sigma) at a dose of 20 mg/kg before implantation of the tumor.[3] On the same day, 5×10^6 cells were injected subcutaneously into both axial regions of female athymic nude mice at 6 to 8 weeks of age (Asan Institute for Life Science, Seoul, Korea). The RA was administered PO daily for 6 days/week. The control groups received sesame oil alone in the same way. Each group comprised 4 animals each bearing 2 tumors. Tumor volume (length × width × height) was measured with digimatic caliper (Mitutoyo Corp, Kawasaki, Japan). Animals were killed to collect specimens 30 days after tumor implantation.

High-Performance Liquid Chromatography Analysis of Plasma and Culture Media

To analyze the change of plasma RA concentration in the tumor xenograft study, blood was collected 1 hour after oral ingestion of RA from another 5 nude mice weekly. A quantitative analysis of RA in plasma was performed by high-performance liquid chromatography (HPLC) as described by Bugge et al.[17] Typically, 150 μL of a 1:1 (vol/vol%) mixture of acetonitrile and isobutyl alcohol was added to 150 μL of blood sample and vortexed for 1 minute. After the addition of 100 μL of saturated K_2HPO_4 solution and mixing for 1 minute, the mixed samples were microcentrifuged for 3 minutes at room temperature. The organic upper layer (150 μL) content was analyzed for RA by Inertsil ODS-3 (25 cm × 4.6 mm) column (GL Science Inc, Tokyo, Japan) with Cosmosil $5C_{18}$-AR microguard (3 cm × 4.6 mm; Nacalai Tesque Inc, Kyoto, Japan) using a liner gradient method. The mobile phase A was acetonitril:0.02 mol/L ammonium acetate:acetic acid (1:1:0.01, vol/vol), and the mobile phase B was acetonitril:0.02 mol/L ammonium acetate:acetic acid (19:1:0.008, vol/vol). At the initial conditions of 100%, A was maintained for 10 minutes, and linear gradiented to 100% B over 30 minutes. These final conditions were maintained for 5 minutes prior to a 5-minute reequilibration to the initial conditions. Retinoids were detected by ultraviolet (UV) absorption at 365 nm (attenuation = 2). The quantitative analysis of RA was calculated by the standard curve of RA by the HPLC technique (10 ng/mL sensitivity).

To analyze the change of RA concentration of the culture media in the cell proliferation assay, the culture media were collected daily for 6 days, and culture conditions were the same as for the proliferation assay. Quantitative analysis of RA in culture media was directly analyzed by the Inertsil ODS-3 (25 cm × 4.6 mm) column with Cosmosil $5C_{18}$-AR microguard (3 cm × 4.6 mm) without preparation.

Data Analysis

Statistical analyses were performed using the Wilcoxon rank sum test to compare the amount of inhibition of cellular proliferation and the unpaired Student's *t* test with 2-tailed comparison to compare the growth of tumor in the xenograft model.

RESULTS

Expression of CYP26 and CRABPs

The AMC-HN-4 and –6 cells themselves expressed CYP26 slightly without RA treatment. RA markedly increased the expression of CYP26 in AMC-HN-6, but resulted in no change of the CYP26 expression in AMC-HN-4. AMC-HN-4 and AMC-HN-6 weakly expressed CRABP I, although the intensity was a little stronger in AMC-HN-6, and RA decreased expression of CRABP I in both cell lines. Interestingly, RA markedly increased CRABP II expression in AMC-HN-4 cells, but had little effect on CRABP II expression in AMC-HN-6 cells (Fig 1). These results indicate that CYP26 was induced by RA only in AMC-HN-6, and the RA-dependent increase in CRABP II might have some role in the cellular sensitivity to RA.

Cell Proliferation and RA Concentration In Vitro

RA inhibited cell proliferation in both the cell lines, but the sensitivity to RA was variable, depending on the CYP26 in-

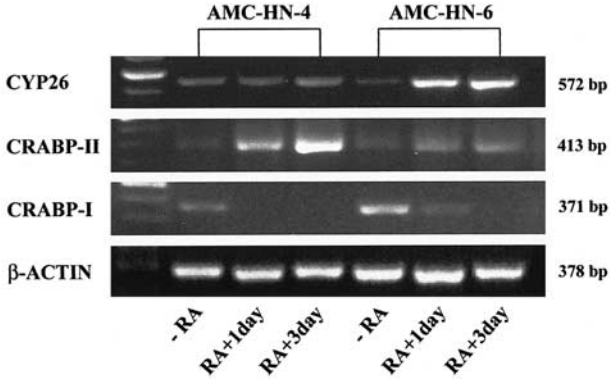

Fig 1. The expression of CYP26 and CRABPs. AMC-HN-4 and -6 expressed CYP26 and CRABPs slightly without RA treatment. RA markedly increased the expression of CYP26 only in AMC-HN-6. RA decreased the expression of CRABP I in both cell lines and increased the expression of CRABP II in AMC-HN-4.

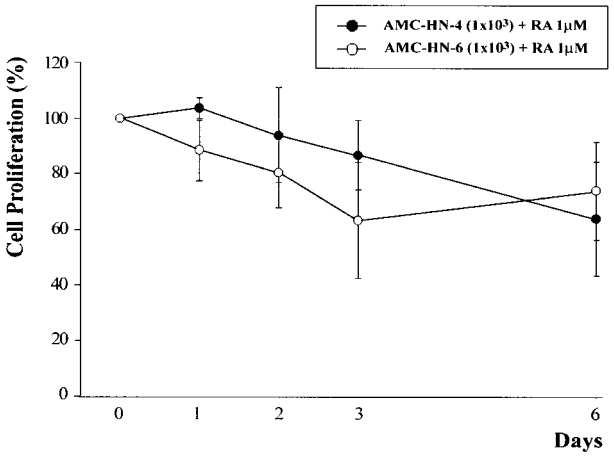

Fig 2. The cell proliferation in the event of culturing 10^3 cells. RA inhibited the cell proliferation in both cell lines. Although the cell proliferation of AMC-HN-6 was slightly increased in a 6-day treatment compared with a 3-day treatment, the inhibitory effect of cell proliferation was nearly the same in both cell lines in the event of culturing for 6 days ($P > .1$). Results are mean ± SD.

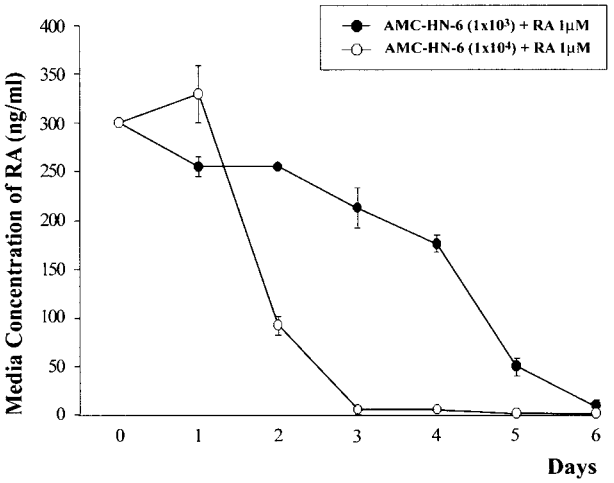

Fig 4. RA concentration (ng/mL) of media in the event of culturing 10^4 cells of AMC-HN-4. RA concentration was rapidly decreased in both conditions. However, the rate of catabolism was more rapid in the event of culturing 10^4 cells, and RA was not detected in media after 3 days. Results are mean ± SD.

duction and the number of cultured cells. The slow catabolizer, AMC-HN-4, did not exhibit a significant difference in the amount of inhibition of cell proliferation by RA according to the cultured cell number (Figs 2 and 3). Six days after treatment with 1 μmol/L RA, the fractions of cellular proliferation of 10^3 and 10^4 AMC-HN-4 cells were 63.9% and 46.6%, respectively ($P > .1$). The RA concentrations of media, measured during 6 days, were always more than 270 ng/mL irrespective of cell number. These results indicate that the slow catabolizer, AMC-HN-4, is sensitive to RA.

However, the rapid catabolizer, AMC-HN-6, showed a different pattern. As shown in Fig 4, the RA concentration in media was rapidly decreased, and the rate of catabolism was different with the cultured cell number. The difference in the cell number indicates a difference in the amount of induced CYP26. In other words, if 10-fold more of CYP26 had been induced in the culture of 10^4 cells, compared with the culture of 10^3 cells, this difference might have affected the rate of RA catabolism to the same extent.

Interestingly, the rate of RA catabolism also affected inhibition of cell proliferation. Although there was no difference in the sensitivity to RA between AMC-HN-4 and AMC-HN-6 in the culture of 10^3 cells (Fig 2) ($P > .1$), 2 cell lines showed a significant difference in the event of a 10-fold increase of the cell number ($P < .1$). In AMC-HN-4, cell proliferation was continuously inhibited until day 6, but AMC-HN-6 was resistant to RA after 3 days, and cell proliferation was slightly increased by a 6-day treatment compared with a 3-day treat-

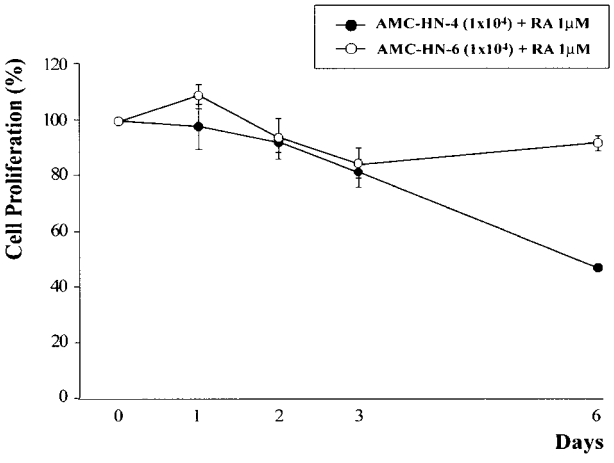

Fig 3. The cell proliferation in the event of culturing 10^4 cells. In AMC-HN-4, cell proliferation was continuously inhibited until the 6th day, but AMC-HN-6 was resistant to RA after 3 days, and cell proliferation was slightly increased in a 6-day treatment compared with a 3-day treatment ($P < .1$). Results are mean ± SD.

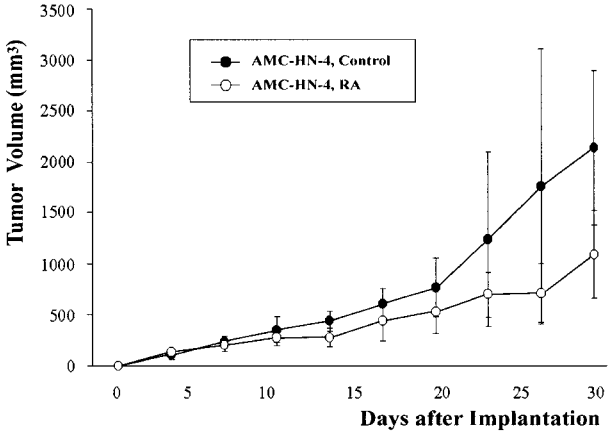

Fig 5. The effect of RA on tumor growth in vivo. Tumor volumes were decreased to 40% by RA after 30 days of AMC-HN-4 implantation ($P < .05$; n = 8). Results are mean ± SD.

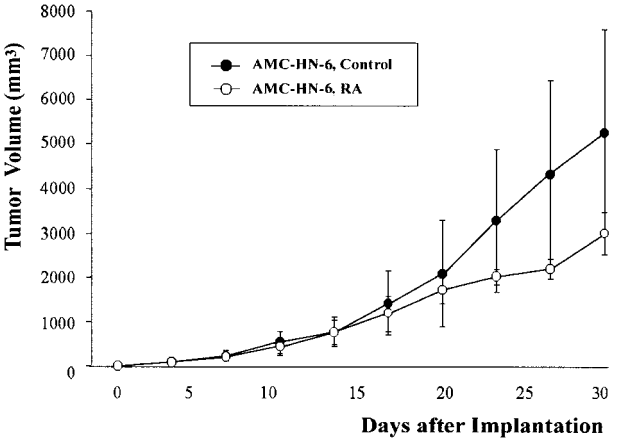

Fig 6. RA decreased tumor volumes by 52% after 30 days of AMC-HN-6 implantation ($P > .05$; n = 8). Results are mean ± SD.

ment (Fig 3). Figure 4 explains why AMC-HN-4 and –6 showed the different sensitivities to RA. In the experiment with 10^4 cells in the culture, RA was not detected in culture media after 3 days. These results indicate that CYP26 rapidly catabolized RA, and RA resistance in AMC-HN-6 was developed due to the rapid disappearance of RA in media.

Effects of RA on Tumor Growth In Vivo and Plasma Concentration of RA

Figure 5 shows the effect of RA on tumor growth of AMC-HN-4. The tumor volume was decreased to 40% by RA 30 days after tumor implantation ($P < .05$; n = 8). Although the inhibition of tumor by RA was less in AMC-HN-6 than in AMC-HN-4, RA decreased tumor volume by 52% 30 days after the AMC-HN-6 implantation ($P > .05$; n = 8) (Fig 6). Although a blood sample was collected 1 hour after the oral ingestion of RA, the plasma concentration of RA was maintained at above 500 ng/mL for 4 weeks. RA is mainly catabolized in the liver, and the catabolism in the implanted tumor cells might not affect the plasma concentration of RA in vivo. Therefore, an in vivo study may reflect the sensitivity to RA more accurately than an in vitro study. These results indicate that RA inhibited tumor growth of AMC-HN-4 more than that of AMC-HN-6, and the rapid catabolism of RA in AMC-HN-6 itself might have decreased the sensitivity to RA in vivo.

DISCUSSION

RA can induce human RA CYP26 in some cells, which is known to be highly specific for RA.[7] Our results showed that RA induced CYP26 in AMC-HN-6, and we confirmed that the CYP26 was also a main P450 involved in rapid catabolism in HNSCCs.

The CRABPs have been known to regulate intracellular RA concentration, transport, and metabolism and to share a high degree of homology.[8] The expression pattern of CRABPs was various in HNSCCs.[2] Boylan and Gudas[9] reported that the level of CRABP I determined the rate of RA catabolism. Giannini et al[2] reported that the RA-resistant SCC cell line, CCL-17, did not express CRABP I. However, CRABP I may not be directly involved in the retinoid receptor-mediated RA-signaling pathway.[18] AMC-HN-6 expressed CRABP I, but RA decreased that expression. Also, a previous experiment demonstrated that RA catabolism was continued for 4 days only in AMC-HN-6.[15] These results suggest that CRABP I may not be essential for RA catabolism by CYP26, at least in AMC-HN-6.

CRABP II, which shows a large increase after introduction of RA, is generally thought to be a protein that mediates intracellular regulatory activities of RA.[19] In 1992, Busch et al[20] reported decreased CRABP I and increased CRABP II expression after topical application of RA on the keratinocytes. Our study showed similar results as the previous reports. However, there is still a lot of controversy about the role of CRABP II in RA activities.

The effect of RA on cell proliferation is various, and one of the causes might be the extent of RA metabolism.[12,13] Although Isogai et al[14] reported no correlation with cell growth inhibition, Takatsuka et al[12] reported that cells, which catabolized RA rapidly, were more sensitive to RA, and the polar metabolites might be more potent. Also, Sonneveld et al[7] showed that the CYP26 activity was closely correlated with RA sensitivity in the breast cancer cells. These reports were opposite to our results. We think that AMC-HN-4 may be in the category of rapid catabolizers by the definition of Takatsuka et al,[12] because AMC-HN-4 has the CYP26 activity and expresses CRABP I, although the rate of catabolism is considerably lower than that of AMC-HN-6. Therefore, the CYP26 activity might be one essential factor for the RA sensitivity of cells. However, as the rate of RA catabolism was too rapid in AMC-HN-6, RA was not detected in culture media after the third day, and the RA concentration of culture media was inversely related to the cell proliferation. In addition, RA decreased the tumor growth of AMC-HN-4 more in the xenograft model than that of AMC-HN-6, and AMC-HN-4 was also more sensitive to RA in vivo. These results suggest that the rate of RA catabolism might be another factor that determines the RA sensitivity in cells showing CYP26 activity. In addition, our results support those of van der Leede et al[13] in that RA was a more active metabolite, and the mechanism that activates the rapid catabolism might prevent the cells from continuous exposure to RA.

We conclude that CYP26 activity might be one essential factor for RA sensitivity, but in cells showing induction of CYP26, the RA sensitivity is inversely related to the rate of RA catabolism.

REFERENCES

1. Moon RC, Mehta RG: Retinoid; inhibition of experimental carcinogenesis, in Dawson MI, Okamura WH (eds): Chemistry and Biology of Synthetic Retinoids. Boca Raton, FL, CRC, 1990, pp 501-518

2. Giannini F, Maestro R, Vukosavljevic T, et al: All-trans, 13-cis and 9-cis retinoic acids induce a fully reversible growth inhibition in HNSCC cell lines: Implications for in vivo retinoic acid use. Int J Cancer 70:194-200, 1997

3. Shalinsky DR, Bischoff ED, Gregory ML, et al: Retinoid-induced suppression of squamous cell differentiation in human oral squamous

cell carcinoma xenografts (line 1483) in athymic nude mice. Cancer Res 55:3183-3191, 1995

4. Sacks PG, Harris D, Chou TC: Modulation of growth and proliferation in squamous cell carcinoma by retinoic acid: A rationale for combination therapy with chemotherapeutic agents. Int J Cancer 61: 409-415, 1995

5. Benner SE, Lippman SM, Hong WK: Retinoid chemoprevention of second primary tumors. Semin Hematol 31:26-30, 1994 (suppl 5)

6. Hong WK, Itri LM: Retinoids and human cancer, in Sporn MB, Roberts AR, Goonman DS (eds): The Retinoids. Biology, Chemistry and Medicine. New York, NY, Raven, 1994, pp 597-631

7. Sonneveld E, Van den Brink CE, Van der Leede BM, et al: Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-trans-RA and can be induced through RA receptors in human breast and colon carcinoma cells. Cell Growth Differ 9:629-637, 1998

8. Eller MS, Oleksiak MF, McQuaid TJ, et al: The molecular cloning and expression of two CRABP cDNAs from human skin. Exp Cell Res 198:328-336, 1992

9. Boylan JF, Gudas LJ: The level of CRABP-I expression influences the amounts and types of all-trans-retinoic acid metabolites in F9 teratocarcinoma stem cells. J Biol Chem 267:21486-21491, 1992

10. Muindi JF, Scher HI, Rigas JR, et al: Elevated plasma lipid peroxide content correlates with rapid plasma clearance of all-trans-retinoic acid in patients with advanced cancer. Cancer Res 54:2125-2128, 1994

11. Rigas JR, Miller VA, Zhang ZF, et al: Metabolic phenotypes of retinoic acid and the risk of lung cancer. Cancer Res 56:2692-2696, 1996

12. Takatsuka J, Takagashi N, De Luca LM: Retinoic acid metabolism and inhibition of cell proliferation: An unexpected liason. Cancer Res 56:675-678, 1996

13. Van der Leede BM, Van den Brink CE, Pijnappel WW, et al: Autoinduction of retinoic acid metabolism to polar derivatives with decreased biological activity in retinoic acid-sensitive, but not in retinoic acid-resistant human breast cancer cells. J Biol Chem 272:17921-17928, 1997

14. Isogai M, Chiantore MV, Haque M, et al: Expression of a dominant-negative retinoic acid receptor construct reduces retinoic acid metabolism and retinoic acid-induced inhibition of NIH-3T3 cell growth. Cancer Res 57:4460-4464, 1997

15. Kim SY, Han IS, Yu HK, et al: The induction of P450-mediated oxidation of all-trans retinoic acid by retinoids in head and neck squamous cell carcinoma cell lines. Metabolism 47:955-958, 1998

16. Kim SY, Chu KC, Lee HR, et al: Establishment and characterization of nine new head and neck cancer cell lines. Acta Otolaryngol 117:775-784, 1997

17. Bugge CJL, Rodriguez LC, Vane FM: Determination of isotretinoin or etretinate and their major metabolites in human blood by reversed-phase high-performance liquid chromatography. J Pharm Biomed Anal 3:269-277, 1985

18. Venepally P, Reddy LG, Sani BP: Analysis of the effects of CRABP I expression on the RA-induced transcription mediated by retinoid receptors. Biochemistry 35:9974-9982, 1996

19. Vo HP, Crowe DL: Transcriptional regulation of retinoic acid responsive genes by cellular retinoic acid binding protein-II modulates RA mediated tumor cell proliferation and invasion. Anticancer Res 18:217-224, 1998

20. Busch C, Siegenthaler G, Vahlquist A, et al: Expression of cellular retinoid-binding proteins during normal and abnormal epidermal differentiation. J Invest Derm 99:795-801, 1994