The Induction of P450-Mediated Oxidation of All-*trans* Retinoic Acid by Retinoids in Head and Neck Squamous Cell Carcinoma Cell Lines

Sang Yoon Kim, In Suk Han, Hwa Kyung Yu, Hye Rim Lee, Jong Woo Chung, Jee-Ho Choi, Sun Hee Kim, Youngro Byun, Thomas E. Carey, and Kwang-Sun Lee

All-*trans* retinoic acid (RA) can be catabolized into polar metabolites by cytochrome P450 (P450) in several tissues including the skin. We examined eight different squamous cell carcinoma (SCC) cell lines to determine their capacity to induce P450-mediated oxidation of RA. Among the eight different cell lines, enhanced catabolism was detected in AMC-HN-1, -2, -5, and -6, whereas it was not found in the cell lines of AMC-HN-3, -4, -7, and -8. It was found that the enhanced catabolism brought on by P450 induction was blocked when RA was added to AMC-HN-6 along with actinomycin D or cyclohexamide. Also, this catabolism was inhibited by ketoconazole. P450-mediated oxidation was detectable within 4 hours of RA treatment, and RA catabolism reached its maximum 16 hours after treatment. P450 was induced by 13-*cis*-RA, 9-*cis*-RA, and retinal; however, retinol could not induce P450. In conclusion, P450 can be induced by retinoids in head and neck SCC (HNSCC) cells and the ability of retinoids to induce P450 can serve as an important factor in determining the biological effect of retinoids.

RETINOIDS, INCLUDING VITAMIN A and its synthetic analogs, can induce differentiation of many tumors such as embryonal carcinoma, leukemia, neuroblastoma, and melanoma.[1] Retinoids are also known to suppress carcinogenesis in various epithelial tissues, such as skin, bladder, lung, prostate, oral cavity, and mammary glands.[2] In addition, retinoids inhibit squamous cell differentiation in cultured normal keratinocytes[3,4] and in some head and neck squamous cell carcinomas (HNSCCs).[5,6] Retinoids can induce apoptosis in SCCs[7] and are found to be effective in preventing second primary cancers in patients treated for HNSCC.[8] Also, the combination of retinoids and interferon has been found to have an effect of differentiation on the treatment of SCCs.[9]

All-*trans* retinoic acid (RA) can be catabolized into polar metabolites such as all-*trans* 4-hydroxy-retinoic acid (4-OH-RA) and all-*trans* 18-hydroxy-retinoic acid (18-OH-RA) by RA-induced microsomal cytochrome P450 (P450). These can be further catabolized to all-*trans* 4-oxo-retinoic acid (4-oxo-RA) in several cell types, such as murine embryonal carcinoma cells, fibroblasts, LLC-PK1 pig kidney cancer cells, human skin, and human breast cancer cells.[10,11] RA metabolism by P450 is one of several mechanisms that can affect the level of retinoids in cells. Therefore, the enhanced catabolism of RA would affect the biological functions of the parent compounds in HNSCCs. The objective of this study was to determine how different HNSCC cell lines respond to retinoids treatment by inducing P450-mediated oxidation. We examined eight different AMC-HN cell lines[12] and found that four kinds of cell lines, AMC-HN-1, -2, -5, and -6, induced P450 by responding to RA, whereas the other four cell lines, AMC-HN-3, -4, -7, and -8, did not.

MATERIALS AND METHODS

Retinoids

RA, all-*trans* retinol (ROL), all-*trans* retinal (RAL), 9-*cis*-retinoic acid (9-*cis*-RA), and 13-*cis*-retinoic acid (13-cis-RA) were obtained from Sigma Chemical (St Louis, MO). All-*trans*-[11, 12-^{3}H(N)]-RA ([^{3}H]-RA) (0.021 μmol/mL, 48.5 Ci/mmol) was obtained from Du Pont New England Nuclear (Boston, MA).

Cell Culture and Treatment

Eight different AMC-HN cell lines were established at our laboratory from patients with HNSCC.[12] Cells were maintained in Eagle's minimum essential medium supplemented with 1% nonessential amino acid, 2 mmol/L L-glutamine, and 10% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY). Cells were treated in the dark for 12 hours with different concentrations of RA, which ranged from 0 to 2 μmol/L 13-*cis*-RA, 9-*cis*-RA, ROL, and RAL, which were dissolved in dimethyl sulfoxide solvent (DMSO), were also treated with the same concentrations of RA mentioned earlier. Cells were treated in the dark for respective durations of 0, 4, 12, 16, 24, 48, and 96 hours with 1 μmol/L of RA. Cells were also treated with 1 μmol/L of RA and 10 μg/ml of actinomycin D (Sigma) or cyclohexamide (Sigma) for 12 hours. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Control cultures were incubated in the same conditions with an equal volume of DMSO.

Enzyme Assay

Cells (4×10^6) were characterized for enzymatic conversion of 33 nmol/L [^{3}H]-RA to polar metabolites, as previously described.[11] Cells were incubated with [^{3}H]-RA in 600 μL phosphate-buffered saline at 37°C for 60 minutes in the dark. Ketoconazole (1, 10, and 100 μmol/L; Janssen Pharmaceutica, Beerse, Belgium) with labeled RA was added to the cells to determine the ketoconazole effect on RA metabolism. Reactions were terminated by addition of 600 μL of chloroform and methanol (2:1), and the reacted solution was centrifuged for 5 minutes at 5,000 × *g*. The organic phase containing retinoids was collected and dried under the nitrogen atmosphere in the dark. Dried extracts were resuspended in ethanol (25 to 100 μL) and analyzed by thin-layer chromatography (TLC). Cell activity for metabolizing RA was measured from the total radioactivity of polar metabolites.

From the Department of Otolaryngology and Dermatology, Asan Medical Center, University of Ulsan College of Medicine, Seoul; Department of Clinical Pathology, Samsung Medical Center, Seoul; Department of Materials Science and Technology, Kwangju Institute of Science and Technology, Kwangju, Korea; and the Laboratory of Head and Neck Cancer Biology and College of Pharmacy, University of Michigan, Ann Arbor, MI.

Submitted September 5, 1997; accepted February 9, 1998.

Supported by Grant No. HMP-96-G-32 from the 1996 Highly Advanced National Projects on the development of Biomedical Engineering and Technology, Ministry of Health and Welfare, Republic of Korea.

Drs Kim and Han are equal contributors as first author.

Address reprint requests to Sang Yoon Kim, MD, Department of Otolaryngology, Asan Medical Center, University of Ulsan College of Medicine, 388-1, Poongnap-Dong, Songpa-Gu, Seoul 138-736, Korea.

0026-0495/98/4708-0011$03.00/0

TLC

TLC was performed as previously described.[11] In brief, dissolved samples (25 μL in ethanol) and standard retinoids were applied to the TLC plates (LK6D silica gel; Whatman, Hillsboro, OR) in the dark. TLC plates were developed for 90 minutes in a glass tank preequilibrated for 1 hour with 150 mL of developing solvent (hexane:ether:acetic acid, 90:60:1.5 vol/vol/vol). The glass tank contained one sheet of solvent-saturated Whatman no. 1 paper. TLC plates were air-dried for 5 minutes, and then the standard retinoids were marked on the plates. TLC plates were then sprayed with [^{3}H]enhancer (Du Pont), followed by air-drying for 2 hours, and exposed to the X-OMAT film (Kodak, Rochester, NY) for 12 hours at −80°C to localize the retinoids. Radiolabeled RA metabolites on the TLC plates were scraped and their radioactivities measured.

RESULTS

Enhanced Catabolism of Radiolabeled RA

The upper strong band on the TLC plate was RA and the several bands below RA were polar metabolites, which included 4-OH-RA, 18-OH-RA, and 4-oxo-RA[11] (Fig 1). [^{3}H]-RA was converted to polar metabolites in AMC-HN-6 after induction with RA (Fig 1, lanes 3 and 4), but AMC-4 (Fig 1, lanes 1 and 2) failed to metabolize [^{3}H]-RA. Compared with the control, the amount of polar metabolites in AMC-HN-1, -2, -5, and -6 increased after a 60-minute incubation of RA-treated cells with [^{3}H]-RA (Table 1). On the other hand, AMC-HN-3, -4, -7, and -8 showed little increase in the production of polar metabolites.

Inhibition of RA Catabolism by Actinomycin D, Cyclohexamide, or Ketoconazole

To confirm that RA catabolism was induced by newly formed enzymes, cells were either treated with actinomycin D to inhibit gene transcription or with cyclohexamide to inhibit protein synthesis. Figure 2 shows that actinomycin D and cyclohexamide block RA catabolism. Similarly, we studied the inhibition of RA catabolism by ketoconazole, the P450 inhibitor, to show that the newly formed enzyme was actually one of the p450s,

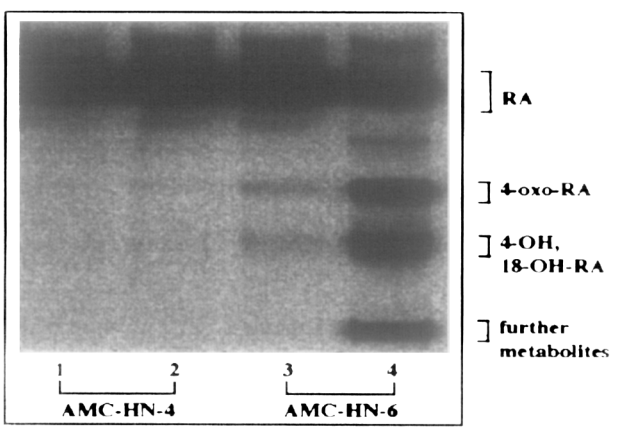

Fig 1. TLC separation profile of RA metabolites in AMC-HN-4 and -6. Cells were treated in the dark for 12 hours with RA (1 μmol/L). Polar metabolites are detected in RA-treated AMC-HN-6 cells, but not in AMC-HN-4 cells. Lane 1, RA-untreated AMC-HN-4 cells; lane 2, RA-treated AMC-HN-4 cells; lane 3, RA-untreated AMC-HN-6 cells; lane 4, RA-treated AMC-HN-6 cells.

Table 1. Induction of P450-Mediated Oxidation by RA in AMC-HN Cell Lines

	Polar Metabolites (%)		RA (%)		Other Retinoids (%)	
Cell Line	RA(+)	Control	RA(+)	Control	RA(+)	Control
AMC-HN-1	46	10	50	89	4	1
AMC-HN-2	31	7	67	90	1	2
AMC-HN-3	21	14	76	83	2	2
AMC-HN-4	7	6	92	91	1	3
AMC-HN-5	41	10	57	83	3	7
AMC-HN-6	63	10	36	89	1	2
AMC-HN-7	6	3	93	95	1	1
AMC-HN-8	7	8	92	91	1	1

NOTE. Cell lines were treated in the dark for 12 hours with RA (1 μmol/L). Numbers under RA(+) indicate percentages of radioactivity in RA-treated cells, and numbers under control are percentages of radioactivity in RA-untreated cells. Numbers are the mean of 2 independent experiments.

which is highly specific to RA. Figure 3 shows that RA metabolism is inhibited by ketoconazole in a concentration-dependent manner, indicating that P450 is induced by RA in AMC-HN-6.

Variation of P450-Mediated Oxidation by RA Treatment Duration

Production of polar metabolites changed during RA treatment of AMC-HN-6, whereas it remained constant in AMC-HN-4 (Fig 4). P450-mediated oxidation was detectable within 4 hours of RA treatment and catabolism reached its maximum after 16 hours of treatment.

P450 Induction by Various Retinoids and Concentrations in AMC-HN-6

P450-mediated oxidation was induced by treatment with 1 μmol/L of retinoids, including RA, 13-*cis*-RA, 9-*cis*-RA, and RAL, but not by ROL in AMC-HN-6. The level of P450-mediated oxidation depended on the concentration of retinoids, as well as the kind of retinoids. Both RA and 13-*cis*-RA induced

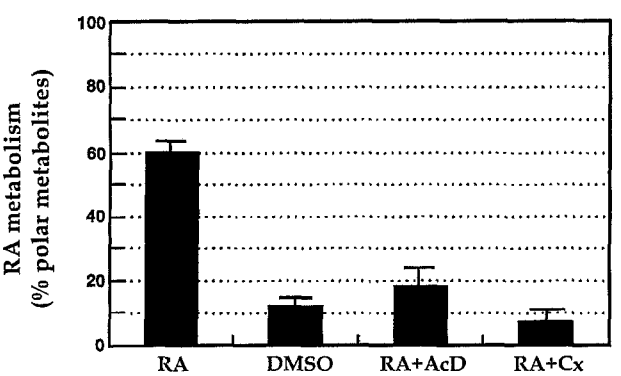

Fig 2. Graph shows that [^{3}H]RA metabolism is inhibited by actinomycin D (AcD) and cyclohexamide (Cx) in the AMC-HN-6 cell line. AMC-HN-6 cells were incubated with RA (1 μmol/L), RA (1 μmol/L) plus AcD (10 μg/mL), and RA (1 μmol/L) plus Cx (10 μg/mL) for 12 hours. Results are means ±SD of 2 independent experiments.

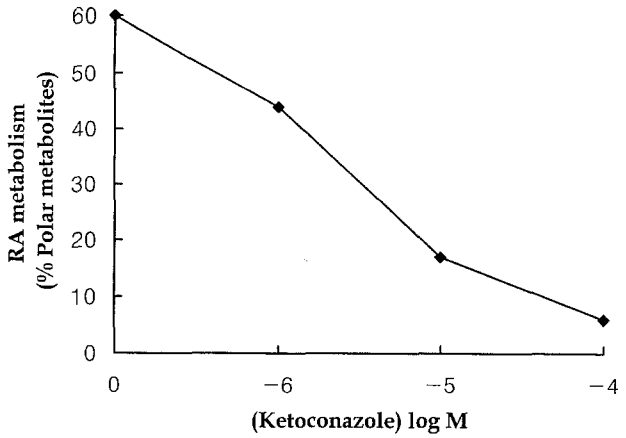

Fig 3. Graph shows that ketoconazole inhibits the metabolism of [^{3}H]RA in a concentration-dependent manner. AMC-HN-6 cells were treated with RA (1 μmol/L) for 12 hours and incubated with 33 nmol/L [^{3}H]RA and various concentrations of ketoconazole (1, 10, 100 μmol/L) in 600 μL of PBS at 37°C for 60 minutes in the dark. Results are the mean of 2 independent experiments.

P450 in a low concentration (0.1 μmol/L), whereas P450 induction was detected in concentrations greater than 1 μmol/L in the cases of 9-*cis*-RA or RAL (Fig 5).

DISCUSSION

Previous studies in T47D human breast cancer cells have shown that the increased RA catabolism was detectable within 4 hours after RA pretreatment, and RA catabolism reached its maximum after 12 hours of pretreatment.[11] This indicates that induction of P450 appears to be regulated at the level of transcriptional control.[11] Similarly, this study found that the increased RA catabolism in AMC-HN (-1, -2, -5, -6) cell lines is induced within 12 hours, and RA catabolic reaction is blocked by actinomycin D or cyclohexamide. Also, RA catabolism is inhibited by the P450 inhibitor, ketoconazole. These findings

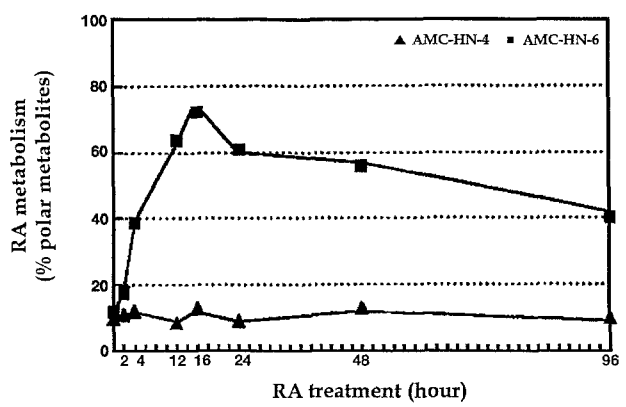

Fig 4. Variation of P450-mediated oxidation by RA treatment duration. Cells were treated for respective durations of 0, 4, 12, 16, 24, 48, and 96 hours with RA (1 μmol/L). P450-mediated oxidation is detectable within 4 hours of RA treatment and reaches maximum 16 hours after RA treatment in AMC-HN-6 cell lines, but, in AMC-HN-4 cell lines, RA catabolism is not induced. Results are the mean of 2 independent experiments.

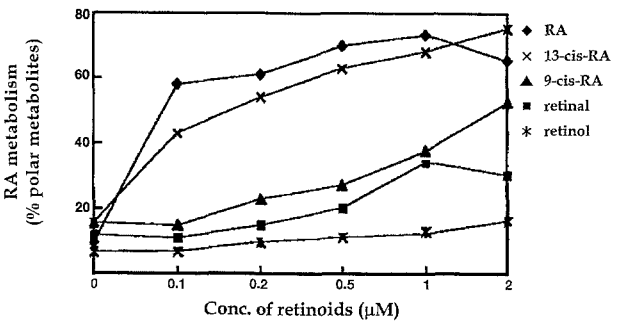

Fig 5. P450 induction by various retinoids in AMC-HN-6. Cells were treated for 12 hours with respective concentrations of retinoids, such as 0, 0.1, 0.2, 0.5, 1, and 2 μmol/L. P450-mediated oxidation is induced after treatment of RA, 13-*cis*-RA, 9-*cis*-RA, and RAL, but not ROL in AMC-HN-6. Results are the mean of 2 independent experiments.

suggest that RA catabolism in AMC-HN (-1, -2, -5, -6) cell lines is enhanced by the newly formed P450. For the other AMC-HN cell lines (-3, -4, -7, -8) that did not induce P450, we can offer several explanations: first, they may not have the specific retinoic acid receptors involved in the P450 induction; second, they may not stimulate the protein kinase C[13]; and third, they may have an inactive P450 gene, or they may lack factors necessary for the P450 mRNA stabilization.[14] We also cannot exclude the possibility that the cell lines can induce P450, but not express cellular retinoic acid–binding proteins involved in the catabolic process of RA.[15]

Microsomal P450s play important roles in RA metabolism and the maintenance of vitamin A homeostasis.[16,17] P450 induction and subsequent catabolism would decrease the level of RA in cells when the cells are treated with retinoids. Thus, the level of RA in P450-inducible cell lines (AMC-HN-1, -2, -5, and -6) may be lower than that in cell lines AMC-HN-3, -4, -7, and -8. RA is known to inhibit squamous cell growth and differentiation in HNSCC cell lines in in vitro studies, whereas some cell lines are found to be insensitive to RA.[7,18,19] These variable responses of HNSCC to RA may be due to the difference in the RA levels in SCCs, which may be modulated by the P450 induction. The function of the polar metabolites is not known completely, and they could have other functions for cell growth and differentiation. Our findings suggest that the AMC-HN cell lines might be good models to study the biological functions of polar metabolites of retinoids and the mechanism involved in the P450 induction.

P450 can be induced by various retinoids, such as 13-*cis*-RA, 9-*cis*-RA, and RAL, with the exception of ROL, and the inducibility of these retinoids is different with each one. P450 induction by 13-*cis*-RA is almost the same as by RA, whereas the inducibility by 9-*cis*-RA and RAL is less than that by RA. The conversion of ROL, RAL, 9-*cis*-RA, and 13-*cis*-RA to RA is regulated by the activity of metabolizing enzymes and the concentration of retinoids. These findings suggest that 13-*cis*-RA is readily converted to RA, whereas 9-*cis*-RA is not readily converted. It is also possible that 9-*cis*-RA is rapidly converted into inactive metabolites, such as 9,13-di-*cis*-RA, thereby preventing RA synthesis. RAL, the precursor of biologi-

cally active retinoids, can be converted to RA by enzymes in the presence of cellular retinol-binding protein I (CRBP-I),[20] and the conversion efficacy of RAL may be less than 13-*cis*-RA. Conversion of ROL to RAL in cytosol is catalyzed slowly compared with conversion of RAL to RA.[20]

We have observed that AMC-HN-1, -2, and -5 have relatively weak cell-to-cell contact and weak cell-to-substrate adhesion. AMC-HN-6 tends to stratify into thick layers of differentiated cells.[12] AMC-HN-1, -2, -5, and -6 express the $\alpha 6\beta 4$ integrin at very low levels compared with AMC-HN-3, -4, -7, and -8.[12] It is interesting that there is a correlation between the growth pattern and P450 induction, although it is not known if it is causal or coincidental.

There have been many studies using retinoids as therapeutic and chemopreventive agents in HNSCCs. Takatsuka et al[21] have shown a correlation between retinoid metabolism and retinoid sensitivity in a variety of epithelial and epithelial cancer cell lines. Rigas et al[22] reported that rapid catabolism of RA was linked to an increased risk of squamous cell cancers of lung. In conclusion, P450 induction could be an important factor in determining the therapeutic effect of retinoids, just as P450 inducibility could be a good indicator in selecting patients.

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