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Overexpression of Retinoic Acid Receptor β Induces Growth Arrest and Apoptosis in Oral Cancer Cell Lines

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Expression of retinoic acid receptor β (RARβ) is reported to be absent or down-regulated in oral squamous cell carcinomas. Recently, we found that the growth-inhibitory effect of 9-*cis*-retinoic acid (9CRA) on oral squamous cell carcinoma may depend on the expression levels of endogenous RARβ. In order to clarify the role of RARβ in growth and differentiation, we transfected RARβ expression vector into oral squamous carcinoma cell lines, HSC-4 and Ho-1-N-1. Both RARβ-transfected cell lines displayed growth inhibition. Moreover, RARβ-transfected clones underwent morphological changes, and RARβ-transfected HSC-4 clones underwent apoptosis even in the absence of 9CRA treatment. In contrast, RARβ-transfected Ho-1-N-1 clones exhibited cell cycle arrest without undergoing apoptosis initially; however, apoptosis was induced in these cells after 6 days of 9CRA treatment. RARα and RARγ expression was reduced at both the protein and mRNA levels in RARβ transfectants, whereas the expression of retinoid X receptor α (RXRα) was not altered. RARβ transfectants exhibited alterations in the levels of cell cycle-associated proteins, histone acetyltransferase (HAT) and apoptosis-associated proteins. After 6 days of 9CRA treatment, RARβ transfectants overexpressed Waf1/Cip1/Sdi1/p21, Kip1/p27, chk1, p300/CBP, BAX, Bak, Apaf 1, caspase 3 and caspase 9. Conversely, E2F1, cdc25B and HDAC1 were down-regulated in these transfectants. In addition, histone H4 acetylation was induced in RARβ transfectants. These findings suggest that histone acetylation mediated by histone acetyltransferase and p300/CBP may play a role in the growth arrest and apoptosis induced by RARβ transfection in oral squamous cell carcinoma.

Key words: Retinoic acid receptor-β — Transfection — Growth arrest — Apoptosis

Most of the effects of retinoids on gene expression are mediated by two types of nuclear retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXR), acting as transcription factors. Heterodimers of the RARs and RXR bind to a specific DNA sequence, the retinoic acid response element (RARE).[1–3] This element is located in the promoter region of genes, including the RARβ gene, that retinoids regulate. On the other hand, lack of RARβ expression has been demonstrated by northern analysis in several solid tumor types including lung carcinoma[4] and squamous cell carcinoma of the head and neck.[5] This raises the possibility that RARβ is a general regulator of cellular proliferation and that its selective inactivation allows cells to bypass one of the major pathways for growth control. RARβ was reported to inhibit metastasis and growth of epidermoid lung cancer cell lines in a nude mouse model,[6] and to induce terminal differentiation of squamous cell carcinoma cell lines in the absence of cyclin-dependent kinase inhibitor expression.[7]

Our previous studies[8] have indicated that the growth-inhibitory effect of 9-*cis*-retinoic acid (9CRA) on oral squamous cell carcinoma may depend on expression levels of RARβ and that 9CRA has a cytostatic effect through the cell cycle-regulatory machinery, suggesting that 9CRA also will play a pivotal role in the growth inhibition of human oral squamous cell carcinoma. These effects of 9CRA on cell growth-related gene products in oral cancer cells could prolong G0-G1 arrest. It is likely that 9CRA mediates growth arrest, but not apoptosis in oral cancer cell lines. These findings are similar to recent reports on the role of RARβ in inhibition of growth of some cancer cell lines.[9, 10] However, Ho-1-N-1 did not show any variation in cellular distribution in any of the cell cycle phases. The expression level of RARβ in Ho-1-N-1 cell line was very low in comparison with other cell lines. These observations suggest that RARβ plays a pivotal role in 9CRA signal transduction in oral cancer cells, and acts as a transcription factor when activated by its ligands.

In the present study, we transfected RARβ expression vector into oral cancer cell lines, HSC-4 and Ho-1-N-1, in order to clarify the molecular role of RARβ in the growth-inhibitory effect of retinoic acid.

MATERIALS AND METHODS

Cell culture Two human oral cancer cell lines (HSC-4 and Ho-1-N-1) were used in this study, because the

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expression levels of RARβ were very low in these cell lines, and moreover Ho-1-N-1 cells were resistant to 9CRA.[8] HSC-4 cell line was established from a well-differentiated squamous cell carcinoma of the tongue.[11] Ho-1-N-1 was established from a well-differentiated squamous cell carcinoma of the cheek.[12] HSC-4 is reported to have an insertional mutation at codon 248 of the *p53* tumor suppressor gene.[13] We detected an abnormal shift in p53 exon 7 of Ho-1-N-1 by PCR-SSCP analysis (Hayashi *et al.*, unpublished data). These cell lines were provided by the Japanese Cancer Research Resources Bank (JCRB). They were routinely maintained in RPMI 1640 (Nissui Co., Tokyo) containing 10% fetal bovine serum (FBS; Whittaker M. A. Bioproducts Inc., Walkersville, MD), 1 m*M* glutamine (Nissui), 100 U/ml penicillin G (Meiji Seika Kaisha, Ltd., Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika Kaisha), in an atmosphere consisting of 5% CO_2 in air at 37°C in a humidified incubator.

Transfection of RARβ expression vector Stable transfection of RARβ expression vector and the empty vector (LNSX) was carried out by the lipofection method (Life Technologies, Inc., Rockville, MD) (Fig. 1). RARβ expression vector was constructed by inserting the cDNA of a modified human RARβ into the *Hin*dIII site of the retroviral vector LNSX. The 5′ untranslated region of wild-type human RARβ cDNA was deleted, and the original Kozak sequence was replaced with a modified optimal one to enhance the translation efficiency. Cells were plated at a density of $0.5-1\times10^5$ cells/100-mm dish, and were grown for 24 h. The cells were transfected for 6 h with 6.0 μg of vector using 60 μl of Lipofectin in 6.0 ml of RPMI 1640 media without 10% FBS and antibiotics. The cells were recovered in non-selective media for 48 h, following selection by adding 300–800 μg/ml G418 (GIBCO BRL, Grand Island, NY) to the culture media. Finally individual G418-resistant clones were isolated and expanded.

Treatment with 9CRA A stock solution of 9CRA (Wako Pure Chemical Industries, Osaka) at the concentration of 10 m*M* was prepared in 100% ethanol. The stock solution was added directly to the culture media to make a final concentration of 1×10^{-6} *M*. The same amount of 100% ethanol was added to the RPMI 1640 media in the control experiment.

For cell growth experiments, cells were seeded at the density of $1-2\times10^4$ cells/22-mm well on Tissue Culture Clusters (Costar, Cambridge, MA). Then, the cells were grown in the media in the presence or absence of 9CRA for 0–6 days. The culture media were changed every 2 days. Cell number was counted in triplicate on an improved Neubauer hemocytometer after harvesting cells by gentle trypsinization. Cell viability was assessed by 0.4% trypan blue (Gibco BRL) dye exclusion.

Immunofluorescence For detection of endogenous RARβ, synchronous or asynchronous RARβ-transfected

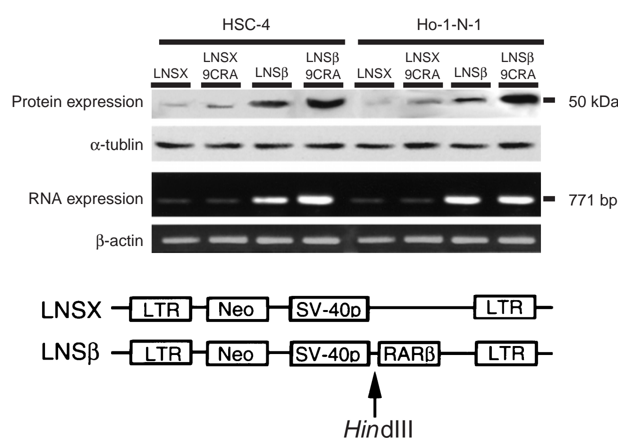

Fig. 1. Expression of RARβ protein and mRNA in transfected oral cancer cell lines. Western blotting using RARβ-specific polyclonal antiserum was performed as described in "Materials and Methods." α-Tubulin was used as an internal control. The 50 kDa RARβ protein was observed in the LNSβ-transfected cells, but not in the cells with control vector. To analyze mRNA expression of RARβ, RT-PCR was performed using 1 μg of total RNA as described in "Materials and Methods." β-Actin was used as an internal control. The 771 bp RARβ mRNA was observed in the LNSβ-transfected cells, but not in the control vector. The expression of RARβ was not altered by 9CRA treatment in control vector (LNSX) transfectants.

HSC-4 and Ho-1-N-1 cells were placed on glass coverslips, and immunofluorescence was detected as described above but with the following modifications. After cells were fixed and permeabilized, they were incubated in blocking solution (5% goat serum, 0.2% fish skin gelatin (Sigma Chemical Co., St. Louis, MO), 0.2% Tween 20) for 60 min. Cells were then incubated with rabbit anti-RARβ polyclonal antibody (diluted 1:20 in blocking solution) for 180 min, washed twice with PBS, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse antibody for 30 min. After several washes with PBS, cells were mounted on glass slides and observed under a fluorescence microscope.

Cell cycle analysis Cell cycle distribution was determined by DNA content analysis after propidium iodide staining as described previously by Yokozaki *et al.* (1992).[14] RARβ-transfected HSC-4 and Ho-1-N-1 cells were each cultured in the presence or absence of 1×10^{-6} *M* 9CRA for 6 days. Cells were harvested and fixed in 70% ethanol and stored at 4°C before analysis. Nuclear DNA was stained with 50 mg/ml propidium iodide (Sigma) in PBS solution under subdued light for 30 min at room temperature. To avoid double-stranded RNA staining, 1 mg/ml RNase (RNase A type I-A; Sigma) was added. The DNA content of cells was analyzed by a FAC-

Scan flow cytometer (Becton-Dickinson, San Jose, CA) coupled with Hewlett-Packard computer and results were analyzed by Consort 30 DNA programs. For each sample, 10 000 events were stored. The fractions of the cells in G0-G1, S, and G2-M phases were analyzed by DNA programs.

DNA fragmentation Ten micrograms of genomic DNA extracted as described from the transfectants was electrophoresed in 1.5% agarose gels in 0.5× Tris-borate-EDTA (TBE) running buffer and visualized by ethidium bromide staining.

Protein extraction and western blot analysis The cells were lysed in a buffer containing 50 m*M* Tris-HCl (pH 7.4), 125 m*M* NaCl, 0.1 % (v/v) Nonidet P-40 (NP-40; Sigma), 5 m*M* EDTA, 0.1 *M* NaF, 10 μg/ml leupeptin (Sigma), 0.1 μg/ml trypsin inhibitor (Sigma), 0.1 μg/ml aprotinin (Sigma) and 50 μg/ml phenylmethylsulfonyl fluoride (Wako). The protein concentration was determined by Bradford protein assay (Bio-Rad, Richmond, CA) using bovine serum albumin (Sigma) as a standard. Western blotting was carried out as described previously by Kameda *et al.* (1990).[15] Fifty or one hundred micrograms of protein was solubilized in Laemmli's sample buffer by boiling and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto nitrocellulose filters (Schleicher & Schuell, Dasse, Germany). The filters were incubated firstly with an appropriate primary antibody and then with peroxidase-conjugated secondary antibody. The immune complex was visualized using the ECL western blot detection system (Amersham, Aylesbury, UK).

Antibodies Anti-RARα (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) rabbit polyclonal antibody (PAb) recognizes a denaturation-resistant epitope between amino acids 443–461 mapping at the carboxy terminus of RARα of human origin. Anti-RARβ (C-19; Santa Cruz Biotechnology) rabbit PAb recognizes an epitope between amino acids 430–447 mapping at the carboxy terminus of human RARβ. Anti-RARγ (C-19; Santa Cruz Biotechnology) rabbit PAb recognizes an epitope between amino acids 436–454 mapping at the carboxyl terminus of human RARγ. Anti-RXRα (D-20; Santa Cruz Biotechnology) rabbit PAb recognizes an epitope between amino acids 328–346 mapping at the carboxyl terminus of human RXRα. Anti-p21$^{Waf1/Cip1}$ (6B6, PharMingen, San Diego, CA) monoclonal antibody (MAb), anti-p27^{Kip1} (Transduction Laboratories, Lexington, KY) MAb, anti-caspase 3 (Transduction Laboratories), anti-pRb (Transduction Laboratories) PAb, anti-p53 (Oncogene Research Products, Cambridge, MA) MAb, anti-BAX (PharMingen) MAb, anti-bcl-2 (PharMingen) MAb, anti-caspase 9 (PharMingen) MAb, anti-Bak (Oncogene Research Products) MAb, anti-acetylated histone H4 (Upstate Biotechnology) PAb, anti-E2F1 (Santa Cruz Biotechnology) PAb, anti-chk1 (Santa Cruz Biotechnology) PAb, anti-bcl-XL (Santa Cruz Biotechnology) PAb, anti-Apaf1 (Santa Cruz Biotechnology) PAb, anti-HDAC1 (Santa Cruz Biotechnology) PAb, anti-p300 (Santa Cruz Biotechnology) Pab and anti-CBP (Santa Cruz Biotechnology) MAb were used, respectively, as immunogens. Anti-α-tubulin (Zymed Laboratories, San Francisco, CA) MAb was used for normalization of western blot analysis.

RT-PCR Expression of RARα, RARβ and RARγ was analyzed by a semi-quantitative RT-PCR method. Total RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Total RNA (1 μg) was converted to cDNA using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). For semi-quantitative analysis of RARα and RARγ, the PCR conditions were set at 3 min at 94°C initially, then 30 s at 94°C for denaturation, 30 s at 65°C for annealing and 30 s at 72°C for extension for 34 cycles. For RARβ, the PCR conditions were set at 1 min at 95°C initially, then 40 s at 94°C for denaturation, 30 s at 60°C for annealing and 1 min at 72°C for extension for 45 cycles. For RXRα, the PCR conditions were set at 3.5 min at 94°C initially, then 1 min at 94°C for denaturation, 1 min at 58°C for annealing and 1 min at 72°C for extension for 32 cycles. For β-actin, RT-PCR conditions were 94°C for 10 min initially, then 24 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 4 min, using Ampli Taq Gold (Perkin Elmer, Norwalk, CT). The buffer contained 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, 50 m*M* KCl and 200 μ*M* of each deoxynucleotide triphosphate (dNTP). The primers used for amplification were: RARα specific primer set (sense 5′-ACCCCCTACCCCGCATCTACAAG-3′ (nt 460–484)), RARγ specific primer set (sense 5′-TTCGAGATGCT-GAGCCCTAGCTTCC-3′ (nt 529–553), RAR common anti-sense 5′-CATGCCCACTTCAAAGCACTTCTGC-3′ as forward and 5′-GTCTAACCCTAACTGAGAAG-3′ as backward), RARβ specific primer set (sense 5′-AGGAGACTTCGAAGCAAG-3′ (nt 822–839)), antisense 5′-GTCAAGGGTTCATGTCCTTC-3′ (nt 1593–1574) and RXRα specific primer set (sense 5′-GAGGCAAACA-GACGCCAAG-3′ (nt 932–951)), antisense 5′-TGTCCC-TGCCCTTTCTGGAT-3′ (nt 1633–1652). The resulting amplification products were analyzed by 1% agarose gel electrophoresis with ethidium bromide and examined under UV light. β-Actin-specific PCR products from the same RNA samples were amplified and served as internal controls.[16]

RESULTS

Transfection of RARβ Western blots and RT-PCR were performed on the transfected cells to determine the levels of RARβ protein and mRNA expression (Fig. 1). The expected single *Mr* 53 000 band was detected in cellular

lysates from HSC-4-LNSβ and Ho-1-N-1-LNSβ cells, but not in those from cells transfected with control vector. The 771 bp RARβ mRNA was observed in the LNS-RARβ-transfected cells, but not in cells transfected with control vector (LNSX). Moreover, 9CRA treatment upregulated the expression of RARβ protein and mRNA in both RARβ transfectants. On the other hand, the expression of RARβ after 9CRA showed no change in HSC-4-LNSX and Ho-1-N-1-LNSX.

Growth-inhibitory effect of RARβ in oral cancer cell lines The growth curves of both the RARβ-transfected clones and control vector (LNSX)-transfected clones in the absence and presence of 1×10^{-6} *M* 9CRA are illustrated in Fig. 2. Although we found in our previous study[8] that Ho-1-N-1 cells were resistant to 9CRA, RARβ-transfected clones and LNSX-transfected clones displayed growth inhibition by 9CRA. Table I shows that the growth inhibition rates relative to LNSX were 73.1% in HSC-4-LNSβ and 29.3% in HSC-4-LNSX after 6 days of 9CRA treatment, while they were 10.8% in Ho-1-N-1-LNSβ clones and 16.9% in Ho-1-N-1-LNSX after 6 days of 9CRA treatment. Moreover, 9CRA exerted enhanced and time-dependent growth inhibition on RARβ transfectants. After 6 days of 9CRA treatment, the growth inhibition rates relative to LNSX were 86.5% in HSC-4-LNSβ cells and 70.0% in Ho-1-N-1-LNSβ cells.

Effect of RARβ transfection on the cell cycle of oral cancer cell lines To determine whether RARβ can induce apoptosis and/or cell cycle arrest, FACScan analysis was performed on the two transfected cell lines (Fig. 3a). Chen *et al.*[17] suggested that cells containing a sub-G1 content of DNA reflect the extent to which apoptosis is occurring. When RARβ was transfected, the cell fraction with a sub-G1 content of DNA was increased in HSC-4-LNSβ cell lines, but not in Ho-1-N-1-LNSβ cells. However, in Ho-1-N-1-LNSβ cells after 6 days of 9CRA treatment, the fraction with a sub-G1 content of DNA was slightly increased. The sub-G1 cell fraction did not increase in control vector (LNSX) transfectants. Moreover, the number of live cells containing S phase amounts of DNA was decreased in RARβ transfectants. S phase cells were decreased from 47 to 20% in HSC-4-LNSβ cells and from 39 to 24% in Ho-1-N-1-RARβ. Moreover, after 6 days of 9CRA treatment, the S phase cells were decreased to 14% and 20% in HSC-4-LNSβ cells and Ho-1-N-1-LNSβ, respectively. In addition, the G0-G1 phase cells were increased from 47 to 64% in HSC-4-LNSβ cells and from 43 to 61% in Ho-1-N-1-LNSβ cells. On the other hand, after 6 days of 9CRA treatment, the G0-G1 phase cells were increased to 68% in HSC-4-LNSβ cells and to 62% in Ho-1-N-1-LNSβ. The G2/M phase cells were increased slightly from 10 to 16% in HSC-4-RARβ-transfected clone and from 15 to 17% in Ho-1-N-1-LNSβ cells. Moreover, after 6 days of 9CRA treatment, the G2/M phase cells were increased to 18% in HSC-4-LNSβ cells and to 18% in Ho-1-N-1-LNSβ.

DNA fragmentation patterns in RARβ-transfected cells are illustrated in Fig. 3b. Although control vector-transfected clones revealed no evidence of DNA fragmentation, a characteristic ladder of DNA fragments was observed in HSC-4-LNSβ cells. Ho-1-N-1-LNSβ cells did not reveal DNA ladder formation. On the other hand, a ladder of DNA fragments on agarose gel electrophoresis was seen in both RARβ-transfected clones in the presence of 9CRA.

Morphological changes observed in RARβ-transfected clones In addition to the above-noted growth arrest, we observed that RARβ-transfected clones of HSC-4 and Ho-1-N-1 underwent morphological changes (Fig. 4). These changes included nuclear condensation, cytoplasmic vacuolization, and cell shrinkage. These cells with morphologi-

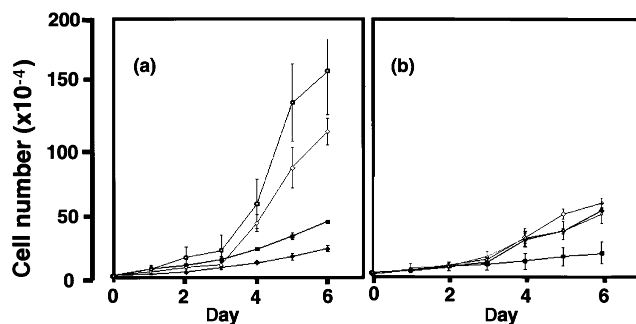

Fig. 2. Effect of 9CRA on RARβ-transfected cancer cells. (a) HSC-4, (b) Ho-1-N-1. Both LNSβ transfectants and LNSX transfectants were grown in the presence of 9CRA at 1×10^{-6} *M* (LNSβ, ◆; LNSX, ◇) or the same amount of ethanol (LNSβ, ■; LNSX, □). At the indicated times, cell counts were performed in duplicate. Data represent the average values±SD (bars). LNSX transfectants after 9CRA treatment and RARβ transfectants displayed growth inhibition. Enhanced growth inhibition was observed with 9CRA treatment in RARβ transfectants.

Table I. Effect of 9CRA on the Growth of Oral Cancer Cell Lines

Cell lines	% Growth inhibition 9CRA (1×10^{-6}*M*) −	+
HSC-4-LNSX		29.3
HSC-4-LNSβ	73.1	86.5
HO-1-N-1-LNSX		16.9
HO-1-N-1-LNSβ	10.8	70.0

Cell counts in triplicate were performed on the 6th day. % Growth inhibition was calculated from the equation $(1-N_t/N_c)\times100$ where N_t and N_c represent the number of cell in treated and control cultures, respectively.

cal changes demonstrated positive immunoreaction to RARβ polyclonal antibody. On the other hand, these changes were not seen in the cells transfected with control vector.

Expression of RARs and RXR in RARβ transfectants The expression of RARα and RARγ was reduced at the protein level as well as at the mRNA level in RARβ transfectants. On the other hand, the expression of RXRα was not altered in RARβ transfectants (Fig. 5).

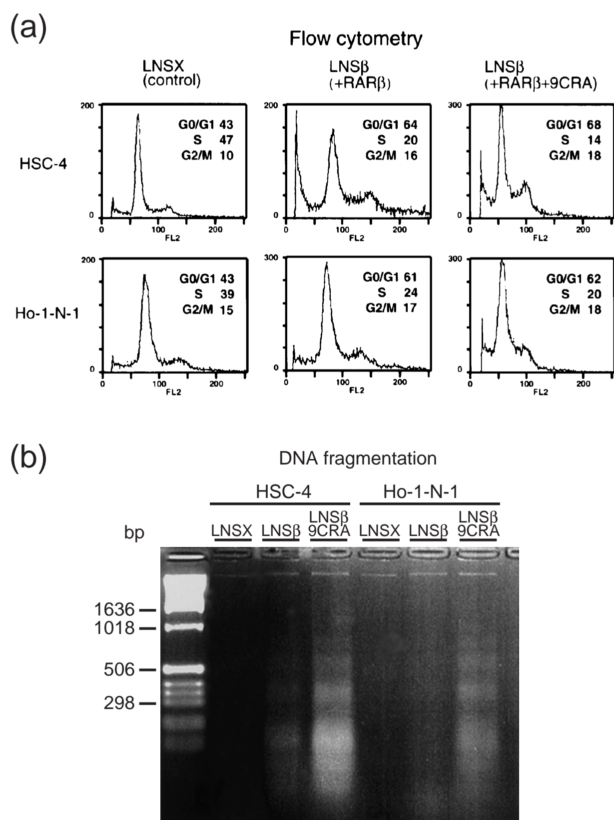

Fig. 3. Flow cytometry and DNA fragmentation analysis for the detection of apoptosis induction by RARβ in oral cancer cells. (a) Cell cycle distribution of control cells and RARβ transfectants after no treatment and after 6 days of 9CRA treatment was determined with a FACScan flow cytometer using PI staining and Consort 30 DNA programs as described in "Materials and Methods." Data represent the average values of two independent experiments. The fraction of sub-G1 content of DNA increased in both HSC-4-LNSβ cells and in Ho-1-N-1-LNSβ cells treated with 9CRA. In addition, the cells in G0-G1 phase increased in RARβ transfectants. (b) DNA fragmentation in RARβ transfectants. Five micrograms of DNA was fractionated in a 2% agarose gel and strained with ethidium bromide. A DNA ladder was observed in HSC-4-LNSβ cells. Ho-1-N-1-LNSβ cells showed a DNA ladder after 9CRA (1×10^{-6} *M*) treatment. The lane on the left represents DNA size markers.

Expression of cell cycle-associated molecules, RAR coactivators and apoptosis-associated molecules in RARβ transfectants We then examined the expression of cell cycle-associated molecules in RARβ-transfected cell lines

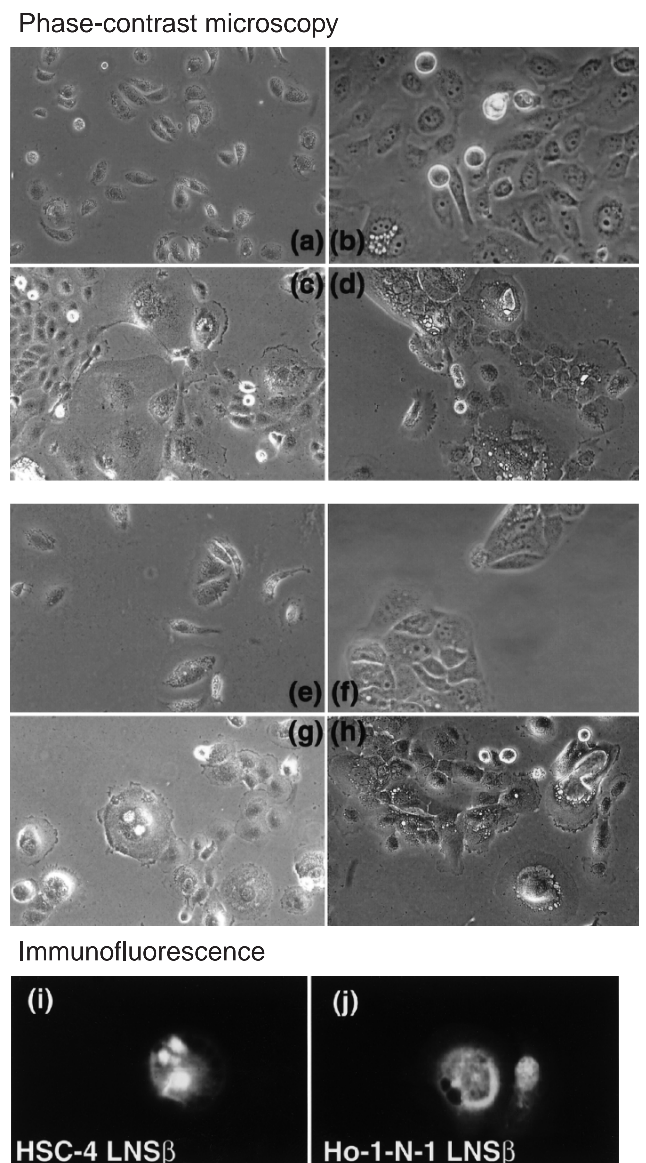

Fig. 4. Morphological changes of RARβ-transfected oral cancer cells. (a) HSC-4-LNSX, (b) HSC-4-LNSX+9CRA (1×10^{-6} *M*), (c) HSC-4-LNSβ, (d) HSC-4-LNSβ+9CRA (1×10^{-6} *M*), (e) Ho-1-N-1-LNSX, (f) Ho-1-N-1-LNSβ and (g) Ho-1-N-1-LNSβ +9CRA (1×10^{-6} *M*). RARβ-transfected oral cancer cells showed morphological changes including nuclear condensation, cytoplasmic vacuolization and cell shrinkage (e, f). These changes were more prominent after 9CRA treatment (d, g). The cells with these morphological changes showed a positive immunoreaction to RARβ (i, j).

(Fig. 6). The expression of p53 protein was reduced in HSC-4-LNSβ cells, but not in Ho-1-N-1-LNSβ cells. In both RARβ-transfected cell lines, the expression of p21/Waf1/Cip1/Sdi1 protein was transiently induced. The expression of p27/Kip1 protein was induced in HSC-4-LNSβ cells, but not in Ho-1-N-1-LNSβ cells. RARβ dramatically reduced the expression of E2F1. On the other hand, a reduction of the phosphorylation of pRb was observed. The expression of chk1 was slightly induced, and the expression of cdc25B was reduced in transfectants. However, the protein expression levels of cyclin D1, cyclin E and CDK-activating kinase (cyclin H and cdk7) were at constitutive values and were not changed in 9CRA-treated RARβ transfectants (data not shown). Moreover, mRNA expression levels of p21/Waf1/Cip1/Sdi1 and p27/Kip1 were at constitutive values in RARβ transfectants exposed to 9CRA treatment (data not shown).

Next, we determined whether RARβ can regulate transcriptional co-activators[18–20] including p300, CREB-binding protein (CBP), histone acetyltransferases (HATs), or the co-regulator histone deacetylase 1 (HDAC1).[21] We found that p300 was significantly induced in transfectants. CBP was induced in HSC-4-LNSβ cells, but not in Ho-1-N-1-LNSβ cells. In addition, CBP, as well as p300, was induced in RARβ transfectants by 9CRA treatment. Conversely, HDAC1 was slightly reduced in both of the RARβ transfectants. Interestingly, the expression of acetylated histone H4 was induced in Ho-1-N-1-LNSβ cells, whereas it was only slightly induced in HSC-4-LNSβ cells.

The transfection of RARβ concomitantly induced BAX and Bak proteins. Furthermore, the expression of bcl-2 was not detected in the RARβ transfected clones. On the other hand, the expression of bcl-X_L protein was reduced in HSC-4-LNSβ cells, but not in Ho-1-N-1-LNSβ cells. However, after 6 days of 9CRA treatment, bcl-X_L was induced by RARβ transfectants. Moreover, the up-regulation of Apaf 1 and caspase 9 occurred in HSC-4-LNSβ

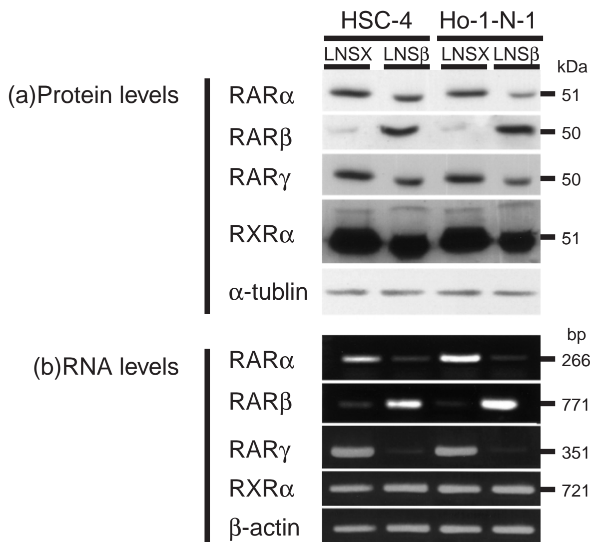

Fig. 5. Expression of RARs and RXR in RARβ-transfected HSC-4 and Ho-1-N-1 cells. Western blotting (a) was performed as described in "Materials and Methods" with specific antibodies to RARs or RXRα. α-Tubulin was used as an internal loading control. RT-PCR (b) was conducted using 1 μg of total RNA as described in "Materials and Methods." β-Actin was used as an internal control. In RARβ transfectants, the expression of RARα and RARγ was reduced at the protein as well as RNA levels. On the other hand, the expression of RXRα was not altered in RARβ transfectants.

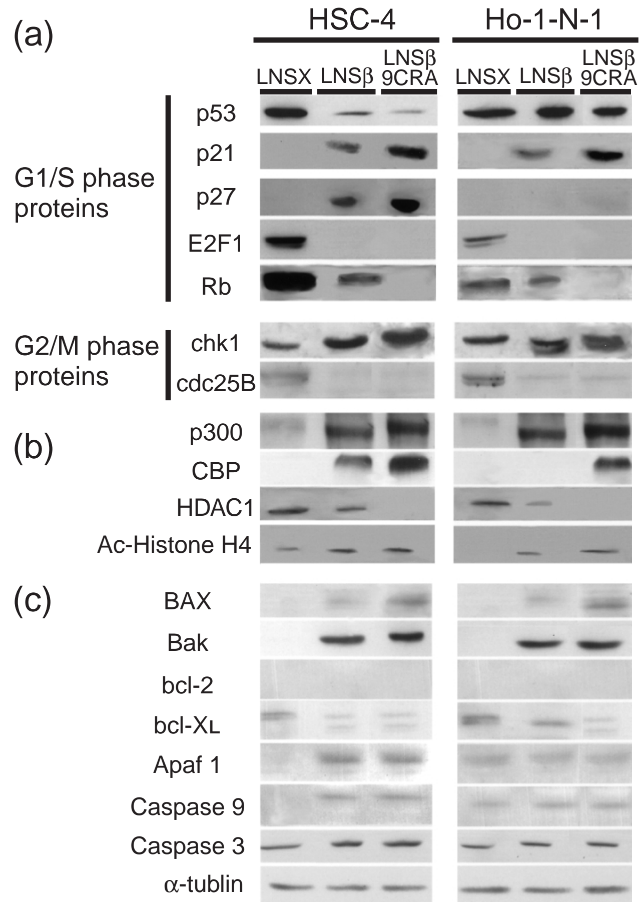

Fig. 6. Expression of cell cycle-associated molecules, RAR co-activators and apoptosis-associated molecules in RARβ-transfected HSC-4 and Ho-1-N-1 cells. Western blot analysis was performed using 50 μg of protein isolated from control cells and RARβ transfectants after no treatment and after 6 days of 9CRA as described in "Materials and Methods." α-Tubulin was used as an internal control. (a) Cell cycle-associated molecules, (b) RAR co-activators and (c) apoptosis-associated molecules.

cells, but not in Ho-1-N-1-LNSβ cells. The expression of caspase 3 was slightly up-regulated in RARβ transfectants. mRNA expression of BAX and bcl-2 was not changed in 9CRA-treated RARβ transfectants (data not shown).

DISCUSSION

The expression levels of proteins that bind to the β retinoic acid response element (RAR/RXR and orphan receptors) and also the differential expression of a number of co-activators were reported to modulate the RA response with both natural and synthetic reporters.[22] The function of retinoid receptors depends on a variety of factors including accessibility to the promoter, the nature of flanking sequences in RAREs, and the presence and levels of co-repressors or co-activators. Because the expression of both receptors and co-factors may be cell type-specific, it is plausible to assume that the overexpression of RARβ may exert different effects in different cell types.[23] Moreover, the biological activity of retinoic acid is thought to be mediated through a number of closely related nuclear receptors that possess discrete DNA and ligand binding domains and act to regulate transcription of specific target genes.[1, 2, 24–26]

We have already described the induction of RARβ, chronological reduction of RARα and RARγ and no significant alteration of RXRα in oral cancer cell lines exposed to 9CRA. Further, the expression level of RARβ in 9CRA-resistant Ho-1-N-1 was very low in comparison with those in other sensitive cell lines.[8] These observations suggest that the expression of RARβ may play a pivotal role in 9CRA signal transduction in oral cancer cells. Recently, Wan *et al.*[27] reported that slight growth inhibition and induction of differentiation were found in SqCC/Y1 head and neck squamous cell carcinoma cell line following transfection and expression of exogenous RARβ. In this RARβ-transfected cell line, alteration of RARα and RARγ expression was not observed. In contrast, RARα and RARγ expression was dramatically reduced in HSC-4 and Ho-1-N-1 oral cancer cell lines by the introduction of the same vector in the present study.

Our present data demonstrate the induction of cell cycle arrest in RARβ transfectants. Cell cycle analysis showed that RARβ-dependent arrest occurred in both G0-G1 and G2-M. RARβ induced p21/Waf1/Cip1/Sdi1, p300, HDAC1 and E2F1 in HSC-4-LNSβ and Ho-1-N-1-LNSβ. Reduction of hyperphosphorylated Rb was also observed in these transfectants. Although the activity of p21/Waf1/Cip1/Sdi1 is sufficient to cause G0-G1 cell cycle arrest,[28] it remains to be elucidated whether other genes may also be involved in RARβ-dependent cell cycle arrest. Furthermore, the expression of p53 protein was reduced in HSC-4-LNSβ cells, but not in Ho-1-N-1-LNSβ cells. These findings suggested that RARβ induced p21/Waf1/Cip1/Sdi1 independently of the p53 pathway in these cell lines. Recently, it was reported that expression of p300, but not CBP, was required for the induction of transcriptional up-regulation of p21/Waf1/Cip1/Sdi1 by retinoic acid.[29] We also found dramatic reduction of E2F1 expression in HSC4-LNSβ and Ho-1-N-1-LNSβ. It is known that suppression of E2F1 strongly participates in G0-G1 cell cycle arrest.[30–32] Moreover, HSC-4-LNSβ as well as Ho-1-N-1-LNSβ also exhibited a reduction of hyperphosphorylated Rb. Rb silences specific genes that are active in the S phase of the cell cycle and which are regulated by E2F transcription factors. The alteration of cell cycle regulators found in RARβ-transfected oral cancer cell lines was implicated in G0-G1 arrest associated with the reduction of HDAC1.

The induction of chk1 and reduction of cdc25B in RARβ transfectants suggests that a signaling pathway to induce G2-M arrest involves overexpression of RARβ. The overexpression of chk1 has been reported to induce G2-M arrest by inhibition of cdc25 and thereby prevents cdc2 dephosphorylation.[33]

It remains to be determined whether the alterations of these proteins by RARβ are sufficient to cause cell cycle arrest and whether these cellular genes may also be involved in RARβ-dependent cell cycle arrest.

We found that apoptosis was induced in HSC-4-LNSβ cells. Apoptosis was also induced in Ho-1-N-1-LNSβ cells by 9CRA. Apoptosis is known to be induced by both p53-dependent and p53-independent mechanisms.[34] As HSC-4 as well as Ho-1-N-1 have mutant p53, the apoptosis observed in the RARβ transfectants must have occurred through a p53-independent mechanism. Induction of p27/Kip1 and CBP in HSC-4-LNSβ cells and Ho-1-N-1-LNSβ cells with 9CRA treatment may play a role in the p53-independent apoptotic cell death.

Wyllie[35] suggested that the Bcl-2 family including Bax, a homologous protein that dimerizes with Bcl-2, promotes apoptosis when overproduced. In this study, we demonstrated that the transfection of RARβ concomitantly induced BAX and Bak, and reduced the expression of bcl-X_L in both transfected clones treated with 9CRA. Apaf 1, caspase 9 and caspase 3 were induced in RARβ-transfected HSC-4 cells, but not in Ho-1-N-1-LNSβ cells. Therefore, we hypothesized that increased levels of bcl-X_L, Apaf 1, caspase 9 and caspase 3, which can be induced by RARβ, may also be responsible for mediating apoptosis. BAX, Bak and bcl-2 may not be involved in RARβ-dependent apoptosis. Identification of such RARβ target genes is necessary to understand the mechanism of RARβ-dependent apoptosis.

Although histone acetylation may have a role in transcription, our data demonstrated that acetylated histone H4 was induced in RARβ transfectants. These results suggest that RARβ transfection might alter a local chromatin

environment to enhance RXR/RAR heterodimer action. Blanco *et al.*[36] suggested that the RXR/RAR heterodimer directly recruits the HAT from mammalian cells and that increased expression of HAT leads to enhanced retinoid-responsive transcription. Thus, histone acetylation influences the activity of the heterodimer, which is in line with the observed interaction between the RXR/RAR heterodimer and HAT presented elsewhere.

Histone acetylation may contribute to the enhanced function of RXR/RAR heterodimer, and the growth arrest and apoptosis induced by RARβ transfection may, at least, be mediated by p300/CBP in oral squamous cell carcinoma.

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