Retinoic acid is reported to retard many kinds of cancer growth including oral cancers. Early in vivo studies using hamster model to investigate retinoids in inhibition of carcinogenesis showed potential but inconclusive result. The mechanism of this growth inhibition effect is thought that the nuclear receptors of retinoids act as ligand-activated transcription factors to mediate the effect of retinoids on gene expression and to alter the growth and differentiation of normal and tumor cells. The down-stream biological function affected by the retinoids’ nuclear receptors might probe a bly alter the cell cycle events to regulate the growth and differentiation of tumors cells. Clinically, retinoids are found to reverse premalignant lesions and in inhibit the de vel op ment of sec ond pri mary can cers. Retinoids not only regulate oral ep i the lial cells keratinization and dif fer en ti ation, but also have a sig nif i cant ef fect in pre vent ing recurrence of oral ma lignancy. How ever, the ex act ef fect whereby retinoids ex erted on in vi tro tu mor cell growth has to be fully eluci dated. The current study was designed for better un der stand ing the cellular response of hu man oral can cer cell lines to retinoic acid. By testing the cellular response of seven hu man oral can cer lines, we used cell cul ture ex per i ments. Direct cell num ber count ing method was uti lized to eval u ate cell lu lar re sponse of these hu man oral can cer cells at the pre sence or ab sence of all-trans RA at 1 mM.

**Results.** Through 7-day observation, the cell population of SCC9, SCC15 and SCC25 of RA-treated groups decreased when compared with the non RA-treated groups. These three cell lines were further verified using [3H] thymidine incorporation DNA synthesis assay. KB, SCC4, OC1, OC2 and OEC-M1 cell lines did not show growth in inhibition at the presence of RA at 1 mM.

**Conclusions.** The molecular event of how SCC9, SCC15 and SCC25 are inhibited by RA and how KB, OC1, OC2 and OEC-M1 are resistant to RA can be further explored on the basis of this study.

**Key Words**
growth inhibition; hu man oral can cer; retinoic acid; [3H] thymidine incorporation DNA synthesis assay

**Background.** The purpose of this study is to identify the cellular response of retinoic acid-treated human oral cancer cell lines.

**Methods.** Seven human oral cancer cell lines KB, SCC4, SCC9, SCC15, SCC25, OEC-M1, OC1 and OC2 were used for cell culture experiments. Direct cell number counting method was utilized to evaluate cellular response of these human oral cancer cells at the presence or absence of all-trans RA at 1 mM.

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**Methods**

**Cell culture**

Seven human oral cancer cell lines KB, SCC4, SCC9, SCC15, SCC25, OC1, OC2 and OEC-M1 were used for analysis. Direct cell number counting method was utilized to evaluate the response of these cancer cells in the presence or absence of all-trans retinoic acid (C20H28O2) at 1 μM.\(^8,9\) Hu et al in 1991 re ported that 1 μM of RA in hibits its cell growth of SCC25 and also in cultures RAR-β expression in vitro.\(^8\) KB, SCC4, SCC9, SCC15, SCC25 were obtained from ATCC or Wong’s lab. In Harvard School of Dental Medicine. KB, es tab lished in 1955 by H. Eagle,\(^11\) was derived from an epidermoid cancer cell line of the buccal mucosa with HeLa cell markers. We cul tivated the cell with ATCC me dium: min imal es sen tial me dium with 2 mM L-glutamine and Eagle’s BBS ad justed to con tain 1.5 g/L so di um bi car bon ate and 1% fetal bovine serum, 10%. SCC4 was obtained from an epidermoid cancer cell line of the tongue that was exposed to radiation and also induced RAR-β expression in vitro.\(^8,9\) Hu et al in 1991 re ported that 1 μM of all-trans RA at 28 °C was dis solved in 100% ethanol alcohol at stock con centra tion of 1 μM and stored at -20 °C for use. After cells spread ing out for one day, the ex per i men tal group of cells were treated with 1 μM of all-trans RA at day 1, 3, 5, and 7 to gether with chang ing of me dia. The con trol groups of cells were cul tured within 1.5 ml of me dia hav ing the same vol ume of EtOH ve hi cle sim i lar to ex per i men tal group at a final con cen tra tion of 0.01%. There fore, one plate, hav ing 3 well of RA treat ment can cer cells and 3 wells con tained non-treated cancer cells. The plating con cen tra tion was 3000 cells within each well to as sure the cells with out be com ing con flu ent for a 7-day ex per i ment. The all-trans retinoic acid (RA, C20H28O2) was dis solved in 100% ethanol alcohol at stock con centra tion of 1 μM and stored at -20 °C for use. After cells spread ing out for one day, the ex per i men tal group of cancer cells were treated with 1 μM of all-trans RA at day 1, 3, 5, and 7 to gether with chang ing of me dia. The con trol groups of cells were cul tured within 1.5 ml of me dia hav ing the same vol ume of EtOH ve hi cle sim i lar to ex per i men tal group at a final con cen tra tion of 0.01%. Therefore, one plate, hav ing 3 well of RA treated can cer cells and 3 non-treated can cer cells, was sent for ob ser va tion ever dy day for all the can cer cell lines.

**Direct cell counting**

The cell growth ex per i ments on all these cell lines with or without RA treatment were primarily per formed by directly counting the number every day from day 1 to day 7. All the cells were trip sinized, di luted into sam pling so lu tion, which was dropped onto glass slides with pipet. Counting was per formed un der mi cro scope. The cell num bers within each plate, hav ing 3 wells of RA treated cancer cells and 3 wells of non-RA treated cancer cells, were ob tained using two-tail, un-
paired student t-test.

**[^3]H** thymidine incorporation DNA synthesis assay

The cancer cell lines which showed growth in inhibition were further verified using the[^3]H thymidine incorporation DNA synthesis assay for scintillation counting on day 1, 3, 5 and 7 using the same cultured method as previously described. The media was changed in the morning of the counting day.[^3]H thymidine was added and diluted to a final concentration of 1 µCi/ml within each well. After incubating 6 hours to allow for [^3]H thymidine incorporation, the cultured cells were processed for scintillation counting. The cells were trypsinized with 3 changes of ice cold 1X PBS (Phosphate-buffered saline) and then fixed in 1 ml of 5% TCA (Trichloroacetic acid) at 4 °C for 30 minutes. This was followed by one more wash of 5% TCA, two washes of methanol, and air dry for 10 minutes. Finally, 1 ml of 0.02 N NaOH solution was added into each well to lyse the cells. 500 µl of the cell lysate was transferred into scintillation vial containing 5 ml of Bio-Safe II™ aqueous counting solution (Research Product International, Prospect, IL, USA). The data obtained were further statistically analyzed using two-tail, unpaired student t-test.

Results

All seven human oral squamous cell carcinoma cell lines were exposed to RA treatment at 1 µM. Significant cell growth in inhibition by RA was detected from direct cell number counting in SCC9 (p = 0.024), SCC15 (p = 0.014) and SCC25 (p = 0.027). Continuous growth in inhibition was observed until the end of experiment. (Fig. 1A, Fig. 1B and Fig. 1C). The OEC-M1 also showed a trend of growth in inhibition by RA but of no statistical significance (p = 0.056) (Fig. 1D). However, no response of the other three cell lines of OC1, OC2 and KB to RA was observed. To confirm the result by direct cell number counting, the cell growth experiment for SCC9, SCC15 and SCC25 cell lines were repeated and further verified using[^3]H thymidine incorporation DNA synthesis assay. Cell growth in inhibition was clearly observed in RA-treated SCC9, SCC15 and SCC25 through a 7-day observation.
tion (Fig. 2A, 2B and 2C). The result of this experiment showed that three human oral cancer cell lines, SCC9, SCC15 and SCC25, were RA-responsive cell lines, while OC1, OC2 and KB were resistant.

Discussion

To explore the mechanism of the potential “tumor suppressor” function of retinoids in oral cancer, we primarily screened 7 human oral cancer cell lines to choose the candidate clones for future experiment. A well-established in vitro cell culture in this experiment can provide a continuous source of characterized human oral cancer cells. By establishing a homogeneous cell culture of RA-treated and non-treated human oral cancer cell lines, we successfully selected the candidate clones showing RA-treated cell growth inhibition. SCC9, SCC15 and SCC25 showed consistent growth inhibition by RA at the concentration of 1 µM in this experiment. To obtain the maximum effect of growth inhibition representing RA-suppressed phenotype of human oral cancer cells, different concentrations of RA were tested in the preliminary experiment. Our unpublished data also showed RA-treated growth inhibition on SCC15 and SCC25 from 10^{-7} to 1 µM. From the standpoint of drug-induced inhibitory effect, a higher concentration of RA might result in more growth inhibition followed by more ethanol vehicle used in culture. This might lead to unward cytotoxicity of EtOH to the oral cancer cells. However, previous reports showed that 1 µM of RA in hibits cell growth of SCC25, induces RAR-β expression in vitro, and induces differentiation of HL-60 cell line. For demonstrating RA-suppressed phenotype of human oral cancer cell lines in this cell growth experiment, we hypothesized that a gene regulatory pathway exists to allow RA acting on the inhibited cell lines via its nuclear receptors to induce or inhibit the expression of the responsive genes. Therefore, previous reports seemingly support our hypothesis using 1 µM of RA in this experiment.

In considering the differential cell growth existing between RA-treated and non-treated cells from day-1 to day-3, and a 60-80% subconfluent cell colonies formed in the non-treated control at day-5 within the 6-wells’ culture plate, the 7-day observation should be suitable for this experiment. Therefore, cell cultures of SCC9, SCC15 and SCC25 were initially chosen as the candidate clones to demonstrate RA-induced suppressed phenotypes of human oral cancer cells. To verify this result, cell growth experiment using [3H] thymidine DNA synthesis as a sensitive assay also demonstrated altered cell proliferation in SCC9, SCC15 and SCC25 that was repeatable. Statistical
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