Inappropriate Retinoic Acid Receptor-β Expression in Oral Dysplasias: Correlation with Acquisition of the Immortal Phenotype

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Abstract

Retinoic acids are useful in the treatment of premalignant oral lesions and in preventing the occurrence of second primary cancers after resection of the initial primary oral cancer, but long-term prognosis is still poor, presumably due to malignant cells escaping retinoid control. Previous work has shown that loss of expression of retinoic acid receptor β is one of the most consistent molecular changes during oral cancer progression in vivo. In this report we demonstrate, using a novel panel of primary cultures of oral lesions, that loss of retinoic acid receptor β expression at the dysplasia stage occurs during the transition from senescent to immortal phenotype but may occur independently to the loss of CDKN2A/p16 expression.

Introduction

Retinoic acids have been shown to inhibit the conversion of premalignant oral lesions to malignant cancers and reduce the occurrence of second primary cancers after resection of primary oral cancers (1, 2). However, long-term survival is not affected, presumably either because malignant cells become insensitive to retinoids during progression or because retinoid-resistant mutants arise during malignant cell proliferation. It is, therefore, important to elucidate how changes in retinoid responsiveness occur during oral cancer progression and identify the molecular mechanisms involved. The biological effects of retinoids are mediated through a complex array of cytoplasmic and nuclear receptors. Vitamin A and synthetic retinoids are transported and metabolized within the cell by two families of cytoplasmic receptors, the CRBPs and the CRABPs, CRBP-I/II and CRABP-I/II, respectively, and their biological effects are mediated by two families of nuclear receptors/transcription factors each containing three members, the RARs (RAR-α, RAR-β, and RAR-γ) and the RXR family (RXR-α, RXR-β, and RXR-γ). RAR-β is highly expressed in non-keratinizing epithelium such as the buccal mucosa and lateral tongue (3) but is not expressed very frequently in late-stage tumors in vivo. In this study, RAR-β expression has been shown to be reduced in premalignant oral lesions, e.g., in about 60% of oral dysplasias (4, 6). RAR-β expression is also lost in lung, breast, cervical, and ovarian cancer cells (7–10). Moreover, re-introduction of RAR-β into epidermal lung cancer cells reduces their tumorigenicity (11). Overall, RAR-β has been implicated in the control of squamous differentiation by retinoic acid in oral cancers, whereas RAR-γ may control growth inhibition by retinoic acid (summarized in Ref. 12). In this study, we have investigated the expression of nuclear and cytoplasmic retinoid receptors in a panel of recently isolated primary cultures of biopsies of normal oral mucosa cells and oral lesions at various stages of progression and demonstrated a correlation between loss of expression of RAR-β and the cyclin/CDk4 and CDk6 inhibitor, CDKN2A/p16, during the transition from senescent to immortal phenotype at the dysplasia stage.

Materials and Methods

Cells and Culture Conditions. The methods for derivation and characterization of the primary cultures of human oral cell cultures, dysplasias, or tumors has been described previously (13). Biopsies were trypsinized and cultured until a growing population of cells was obtained in a 9-cm plate and then passaged once to give a stock culture that was frozen. All cells were maintained on irradiated 3T3 feeders, either in FAD+ medium (1:3 Ham’s F-12/DMEM with 10% FCS, insulin, EGF, transferrin, cholera toxin, hydrocortisone, and adenine) in the case of normal cells or 10H medium (DMEM plus 10% FCS without added growth factors except hydrocortisone) in the case of tumor cells. None grew well in serum-free keratinocyte growth medium at calcium levels in the range of 0.03–0.125 mM. Normal cultures and the senescent dysplasias were used within the first two passages from frozen stocks, before their growth rate deteriorated significantly. 3T3 cells were maintained in 10C medium (DMEM plus 10% donor calf serum). The 3T3 feeder layer was removed by treatment with 0.02% EDTA prior to RNA and protein extraction. All biopsies were categorized according to the Tumor-Node-Metastasis system of staging.

RNA Extraction and Northern Analysis. Total cellular RNA was extracted using TRizol reagent according to the manufacturer’s recommendations (Life Technologies, Inc., Paisley, Scotland). Northern analysis using 30 μg of total cellular RNA was carried out essentially as described (4). The cDNA probes used were made from plasmids kindly provided as follows: human RAR-α, -β, and -γ and mouse RXR-α, -β, and -γ from Professor P. Chambon (Strasbourg, France). Human CRABP-I and -II was from Professor A. Anstrom (Lund, Sweden). Rat CRBP was from Professor F. Chytil (Nashville, TN). For retinoic acid treatment, duplicate cell cultures at 80% confluence were treated with growth medium containing either 0, 0.1, 1, or 10 μM all-trans-retinoic acid for 3 days before extracting mRNA as described above.

Western Blot Analysis. Cellular protein was extracted as described (14). Total cell lysate (100 μg) was separated on a 12% SDS-polyacrylamide gel and dry blotted for 2 h. All incubations and washes were carried out in TBS with 5% Marval and 0.1% Tween 20. p16 was detected using a COOH-terminal antibody (Santa Cruz Biotechnology). Keratin 14 was detected using an antibody kindly supplied by Prof. B. Lane (Dundee University, Scotland). Immune complexes were visualized by enhanced chemiluminescence. Filters were subsequently stained with Amido black to confirm even transfer and loading.

Results

Human Oral Cell Cultures. In these studies, we have used a panel of primary cultures of human oral biopsies from nonkeratinizing sites (Table 1), which we have characterized with regard to their growth requirements and properties in relation to the stage of tumor progression. The cultures were shown to be epithelial in origin by the
expression of keratin K14. They include two biopsies of normal tissue, six samples of apparently normal tissue adjacent to oral lesions, seven dysplasias (two of which were erythroplakias), and seven stage T2-T4 tumors. In three cases, we obtained cultures of normal adjacent tissue, six samples of apparently normal tissue adjacent to oral lesions, and five normal cultures, including those paired with tumour cultures, proved to be senescent within 4–9 passages. All tumor cultures were confirmed as being neoplastic by biological and cytogenetic criteria as described previously (13). With the exception of one T2 tumor (BICR 66) that was senescent, all tumor cultures were immortal, growing indefinitely in culture. Senescent cultures were shown not to be normal by their resistance to suspension-induced death after placing in Methocel for 24 h (13). Within the group of dysplasias, four cultures proved to be senescent and two immortal; a seventh dysplasia culture (BICR D6) was more complex. RAR-α3 expression was barely detectable in normal culture may be lack of retinoids because RAR-α3 expression was not induced by AtRA (Fig. 1D). However, there are three exceptions where RAR-α was the only one that proved to be immortal in culture. The BICR D4 dysplasia is also particularly informative because early passages of the culture showed detectable RAR-β expression, whereas RAR-β expression was lost in later passages when it became immortal (Fig. 2). Moreover, the only tumor (BICR 66) found to express RAR-β was senescent, in contrast to the other six immortal tumour cultures tested (Figs. 1A and 2). Overall, therefore, all five senescent cultures available expressed RAR-β; in contrast, all nine immortal dysplasia or tumor cultures tested did not. These results, therefore, demonstrate that loss of expression of RAR-β correlates strongly with the transition from senescence to immortality during oral cancer progression. This may explain why other workers have found both very low and high levels of RAR-β in cultures of leukaemias from nonkeratinizing sites (4).

**Expression of CDKN2A/p16.** About 80% of cancers in vivo have been found to have lost normal function of the cell cycle inhibitor CDKN2A/p16, and this has been shown to be an early event in oral cancer progression (15–18). We, therefore, examined our senescent and immortal dysplasia cultures for p16 protein expression. The results show that our two senescent dysplasia cultures (BICR D6 and BICR D4) expressed CDKN2A/p16 at similar levels as did the normal cultures, whereas CDKN2A/p16 protein was undetectable in two immortal dysplasias (BICR D9 and BICR D4), and the third immortal dysplasia BICR D49 expressed only a low level of a truncated (and therefore, most likely, nonfunctional) CDKN2A/p16 protein (Fig. 3). Immortal tumor cultures are also negative for CDKN2A/p16 protein (e.g., BICR F7 and 56), confirming the extensive previous work of our colleagues with tumor cultures (19).

**Discussion**

Previous work has demonstrated that expression of RAR-β is frequently lost in tumors derived from nonkeratinizing oral sites (3) and in cell lines derived from them (4), and this is confirmed in our extensive series of oral tumor cell cultures. By *in situ* studies on histological sections, RAR-β expression was found in less than one-half of premalignant oral lesions (3, 6). Our present studies with primary cell cultures extend this conclusion with the novel finding that the loss of RAR-β expression at the dysplasia stage correlates with the transition from senescence to immortal growth potential. In general, the loss of CDKN2A/p16 expression in dysplasias also correlates with immortality, consistent with other recent work by our colleagues (19). However, there are three exceptions where RAR-β expression does not correlate with CDKN2A/p16 expression. The first exception is that normal oral mucosa cells in vivo express both CDKN2A/p16 and RAR-β, but in culture they express CDKN2A/p16.
Fig. 1. A, expression of mRNAs encoding RAR-α, -β, and -γ. B, expression of mRNAs encoding RXR-β, CRABP-II, and CRBP, in cultures of normal oral mucosa and oral lesions at various stages of progression. Total cellular RNA was analyzed by Northern blotting followed by sequential hybridization with the [32P]dCTP-labeled cDNA probes indicated, or with a 7S RNA probe as a loading control, as described in “Materials and Methods.” C, comparison of the levels of RAR-β mRNA in 30 μg of total cellular RNA obtained from a paired frozen biopsy and cell culture sample of apparently normal tissue adjacent to a tumor (BICR F5). D, RAR-β mRNA expression in response to treatment with 0.1—10 μM AtRA for 3 days. Cultures of normal mucosa (FNB), senescent dysplasia (BICR D6), and an immortal tumor (BICR50) are shown. Analysis at each drug concentration was carried out in duplicate.
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Fig. 2. RAR-β mRNA expression in sebaceous (S) cell cultures from normal mucosa (FN5 and FNT), erythroplasia (BICRE4 and BICRE5), dysplasia (BICRD6 and BICRD8), and tumor (BICRD6). Immortal (I) cell cultures from dysplasias (BICRD9 and BICRD19) and tumors (BICRD5 and BICRD7) are shown. mRNA from both early (Ep) and late (Lp) passages of BICRD4, which was ultimately immortal, is shown. Equal loading was confirmed using a 7S probe.

but not RAR-β, unless treated with retinoic acid. Conversely, the senescent erythroplasia, BICR E5, expresses RAR-β but not CDKN2A/p16 (19). The third exception is that dysplasia BICR D4, which lacks CDKN2A/p16, is immortal in culture; yet early passage cultures have a low but detectable level of RAR-β that declines as the cells undergo the change from epithelial to transformed morphology. Overall, therefore, the correlation of RAR-β expression with senescent phenotype is perhaps better than for CDKN2A/p16. Further work will be required to clarify whether CDKN2A/p16 and RAR-β are functionally involved in senescence, but it seems unlikely from this data that RAR-β directly regulates CDKN2A/p16 expression.

Fig. 3. Comparison of CDKN2A/p16 protein expression in a selection of senescent and immortal cell cultures. Western blot analysis was carried out using 100 μg of total cellular protein/lane. Senescent normal mucosa (FN5), normal adjacent mucosa (NB1), and dysplasias (BICRD6 and BICRD9) and tumors (BICRD5 and BICRD19) are also shown. BICRD4-ep, early passage of the ultimately immortal dysplasia. Positive control was SV40-transformed human foreskin keratinocytes.

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References

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