Senescence-Derived Extracellular Molecules as Modulators of Oral Cancer Development: A Mini-Review

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Abstract

Oral cancers are predominantly oral squamous cell carcinomas (OSCCs) derived from keratinocytes, and there is now very detailed knowledge of the genetics and molecular biology of the epithelial tumourigenic component of these cancers, including the identification of cancer stem or tumour-initiating cells. Several key genetic alterations have been identified including the near ubiquitous loss of the CDKN2A/p16INK4A and p53 pathways and telomerase activation, together with frequent inactivation of the NOTCH1 canonical pathway either by somatic genetic alterations or by the presence of human papilloma virus. There is also evidence that OSCCs arise from a ‘field’ of altered cells and that malignant conversion takes place pre-dominantly at the microscopic level. However, in the last decade, it has been realised that tumour development and progression are influenced by the cells of the microenvironment with cross-talk between the epithelial (tumour) and mesenchymal components. OSCCs, especially those that have bypassed cellular senescence, produce an array of proteins and metabolites that induce cellular senescence in the normal surrounding cells; indeed, senescence is a common property of cancer-associated fibroblasts (CAFs). Cellular senescence is defined as an irreversible cell cycle arrest and is associated with the release of molecules known as the senescence-associated secretory phenotype that can selectively promote the growth of pre-neoplastic keratinocytes (osteopontin) and cancer invasion (transforming growth factor β, matrix metalloproteinases, interleukin 6 and lactate). In addition, both old and new work has shown that keratinocytes harbouring NOTCH loss-of-function mutations that lead to defective keratinocyte differentiation and loss of squamous epithelial barrier function may act as a tumour-promoting stimulus for initiated cells harbouring RAS pathway mutations by activating a wound response in the tumour mesenchyme. Thus, not all keratinocytes in the tumour tissue may be tumourigenic and may instead act as promoters of tumour growth and progression analogous to the much-studied CAFs which co-evolve with the genetically altered tumourigenic cells. This new data is discussed in relation to attempts to develop novel non-invasive diagnostics and therapeutics for oral cancer.

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Key Words

Oral cancer · Squamous cell carcinoma · Senescence-derived extracellular molecules · Human papilloma virus
Introduction

Considerable advances have been made in the last decade in the understanding of the molecular and genetic changes associated with oral squamous cell carcinomas (OSCCs) [1, 2] and, to a lesser extent, their pre-malignant lesions (OPMLs) or dysplasias [3]. Integration of this extensive data has resulted in genetic events that stratify OSCCs into four different classes [1] and also predict poor prognosis [1]. The key genetic alterations include the near ubiquitious loss of the CDKN2A/p16\(^{INK4A}\) and p53 pathways and telomerase activation together with frequent inactivation of the NOTCH1 canonical pathway either by somatic genetic alterations or by the presence of human papilloma virus (HPV); all of these alterations are associated with the bypass of senescence in epithelia [4, 5]. However, despite this plethora of data, there has been minimal improvement in the treatment of OSCC in the last 30 years, and there are no obvious drug targets other than protein kinases [2] which are highly susceptible to both intrinsic and acquired drug resistance [6]. Thus, the development of early markers of disease [7] and/or targeting the OSCC environment may represent alternative strategies to the conventional approaches that have so far failed to deliver improvements in the management of this disease. Indeed, there is already evidence that the degree of fibroblast activation in the cancer-associated fibroblasts (CAFs) of the OSCC stroma is also associated with poor prognosis [8]. The role of CAFs in OSCCs is reviewed elsewhere [Prime et al., in preparation], but in this article, we focus specifically on how the senescence programme in both the epithelium and mesenchyme influences OPML and OSCC development and progression and highlight potential future opportunities for early diagnosis.

The Telomere Hypothesis and the Canonical Mechanism of Cellular Senescence

In 1961, Hayflick and Moorhead [9] showed that cultured human fibroblasts could not proliferate indefinitely and, following many cell doublings, entered a permanent growth arrest state, referred to as senescence, despite being viable and metabolically active [5, 10]. This process is associated with shortening of telomeres, the 6 base repeats in DNA that protect the ends of chromosomes with each round of replication. When telomeres reach a critical length, DNA double-strand breaks ensue, and DNA damage foci assemble. Subsequently, checkpoint kinases signal to p53 and p21\(^{Waf1}\) and inhibit cyclin E/cdk2 and cyclin D/cdk4 which, in turn, maintains pRB in an unphosphorylated active state; this causes repression of specific genes that are essential for progression into S phase and cell cycle arrest in G1. The cyclin D/cdk4 inhibitor p16\(^{INK4A}\) accumulates slowly, and permanence of cell cycle arrest is eventually achieved by the assembly of chromatin structures called senescence-associated heterochromatic foci that form specifically at E2F target genes where they inhibit transcription of these genes [10]. During this process, other senescence-associated phenotypes are generated including senescence-associated β-galactosidase [11].

Senescence is induced by a wide variety of stimuli other than exhaustive cell replication [5, 10]. Persistent DNA damage is a common theme, and factors that induce these changes include oxidative stress arising from mitochondrial dysfunction, overexpression of oncogenes leading to over-replication of the genome and uncontrolled cell division and anti-cancer treatments such as chemotherapy and irradiation. There is also evidence for senescence mechanisms that do not require DNA damage or a functional p53, especially during development [12, 13] and following therapeutic doses of ionising radiation [14].

Senescence as a Tumour Suppressor Mechanism

In the last 10 years, evidence has accumulated demonstrating that cellular senescence acts in concert with the immune system as an alternative to apoptosis to remove damaged and/or potentially malignant cells [5]. A number of studies have shown that senescent cells and DNA damage accumulate in human PMLs and that these cells are reduced in the corresponding malignancies [5, 10]. Senescence, therefore, does not appear to restrain the development of pre-malignancies but, rather, prevents malignant conversion. One hypothesis to account for these observations is that levels of signalling in oncogenic pathways that are required to induce tumourigenicity, cellular motility and invasion trigger senescence whilst lower levels of signalling that induce only increased proliferation remain below the threshold required to induce senescence [15, 16]. This allows PMLs to form without triggering senescence, although it is also possible that senescent cells in the PMLs form at a rate faster than their clearance by the immune system.

Recent evidence has shown that senescent cells are targeted by both the innate [5] and the adaptive [17] immune system and that their clearance is essential to prevent malignancy [17]. Disabling either the immune sys-
Senescence in Keratinocytes and the Suppression of OSCC Development

The evidence that senescence operates as a malignancy suppressor mechanism in OSCCs has been reviewed relatively recently [5]. In brief, the first identified barrier to OSCC development is the accumulation of p16INK4a encoded by the CDKN2A gene which is associated with an abnormal wound response that is linked to the micro-invasive step of advanced OPMLs [18]. The loss of p16INK4a expression and the methylation of the CDKN2A promoter is a strong indicator of OPML progression to OSCC [19]. However, this is not sufficient to bypass senescence [5] and is followed by telomeric attrition and inactivation of p53 by mutation. Senescence bypass leads to near complete telomere erosion, chromosome fusions and the phenomenon known as ‘crisis’. There follows massive genetic alteration and reactivation of telomerase, both of which lead to indefinite replicative capacity, the down-regulation of cytokines involved in the innate immune system and tumour progression [5]. The fact that the acquisition of an indefinite replicative lifespan but not the acquisition of tumourigenicity is detectable in some OPMLs suggests that the senescence programme is dismantled prior to malignancy [5].

OSCC Keratinocytes Are Heterogeneous

We have identified and characterised genetically stable (GS-OSCC) and genetically unstable (GU-OSCC) subtypes of OSCCs in cell culture [20, 21] and their corresponding pre-malignant counterparts (GS-OPML and GU-OPML [3, 22]; fig. 1a), with the latter being associated with marked loss of heterozygosity and gene copy number alterations, together with loss of genes such as TP53 and p16INK4a [3, 20–22], and the former possessing minimal genetic alterations [20, 21]. The GU-OSCC keratinocytes are tumourigenic in immunosuppressed mice, but it is unclear whether the GS-OSCC keratinocytes are tumourigenic, as these cultures reach senescence before sufficient cells can be obtained for analysis. It was reported that OSCCs consist of mixtures of immortal and senescent cells [23], but it was assumed at the time that the senescing component was derived from normal or dysplastic tissue contaminating the biopsy. However, both the GS-OPML and -OSCC keratinocytes display neoplastic phenotypes [21] such as resistance to suspension-induced terminal differentiation [23] and altered transcriptional profiles [3], which is consistent with an independent progression route to OSCC [3]; preliminary data suggests that one GS-OSCC culture of two tested possesses a NOTCH1 loss-of-function mutation, but such mutations are infrequent in GS- and GU-OPMLs, arguing that they are late events in tumour progression [Thakker, pers. commun.]. NOTCH mutations are not specifically associated with either GU- or GS-OSCCs.

Are GU-OSCC and GS-OSCC Cells Found in vivo?

There are reports of SCC samples that have few or no copy number variations [1, 2, 24], and these tumours are reported to possess RAS, PI3K and CASP8 mutations and to have a much better prognosis [1]. However, we found no evidence of the above mutations in either GS- or GU-OSCC cultures [25, 26], except for one CASP8 mutation in a GU-SCC [Thakker, pers. commun.] with a good clinical outcome. Colon carcinomas have been divided into microsatellite instability and chromosomal instability subtypes [27], but neither GS-OSCCs nor GU-OSCCs show any evidence of microsatellite instability [20, 21] or mismatch repair gene mutations [1, 2], and, interestingly, chromosomally stable colon cancers have been reported that are neither microsatellite instable nor chromosomally instable [27]. It is also possible that SCCs are mixtures of GU-SCCs and GS-SCCs (see above), and the inevitable selectivity of a biopsy (whether it is cultured or not) may add to the difficulty of interpretation. What is clear is that the GS-OSCCs are not merely normal keratinocytes as they are resistant to suspension-induced terminal differentiation [21] and have altered transcriptional profiles [3]. Some studies do support the ‘mixture’ hypothesis; notably, CDKN2A/p16INK4a deletions are detectable in vivo using in situ techniques [28, 29] and are very common in GU-SCC tumour lines [30] but very hard to detect in vivo with PCR or Southern blotting, and although this has been interpreted as contaminating normal tissue [28, 29], it may equally be due to GS-OSCC tumour cells that are known to contain intact and wild-type CDKN2A genes. Furthermore, the frequency of tumours carrying CDKN2A/p16INK4a inactivation is very similar to the fraction of GU-OSCC tumours.
Possible Role of NOTCH1 Dysfunction in the Extracellular Regulation of Tumour Development and Progression

In mouse skin, Notch1 deletion results in the loss of epidermal barrier function and, although not capable of initiating neoplasia, the Notch1-deficient keratinocytes promote both the development of papillomas harbouring RAS gene mutations and their progression to carcinomas by the secretion of transforming growth factor β (TGF-β) 1 and 2 and the induction of an activated stroma with increased vasculature and increased levels of the keratinocyte mitogens fibroblast growth factor-7 (FGF-7) and CXCL12 [31] (fig. 1b). Interestingly, in a subset of human epidermal SCCs, RAS mutations and NOTCH1 loss-of-function mutations are often found in different parts of the same tumour [32], which supports the hypothesis that similar NOTCH1 tumour-promoting mechanisms may...
operate in heterogeneous human tumours. It remains to be established, however, whether GS-OSCCs or -OPMLs can promote the progression of the GU cells, either directly or by inducing fibroblast activation indirectly.

**The Role of HPV in GU-OSCCs and GS-OSCCs**

None of our keratinocyte GU- or GS-OSCC cultures from Glasgow were HPV positive, probably because none were from the tonsil site. However, many HPV types [16, 18, 31] do inactivate p53 and pRB via the expression of the E6 and E7 proteins, respectively, and there is evidence that the loss of p53 is likely to disable canonical NOTCH signalling through negative regulation of the ROCK1/2 and MRCK kinases [33]. Thus, GU-OSCCs and HPV-positive tonsil SCCs will likely have disabled all of these key driver pathways. However, in oncogenic HPV types that do not inactivate p53, such as the cutaneous β HPV types, the E6 proteins of these viruses repress canonical NOTCH signalling downstream of the cleaved NOTCH receptor by interacting with the Mastermind-like transcriptional co-activators [34].

**Fibroblast Senescence and CAFs as Promoters of OPML and OSCC Development**

Whilst senescence acts as a valuable tumour suppressor when activated in the epithelial compartment, the senescence of fibroblasts in the surrounding microenvironment is tumour promoting (fig. 1a). The mechanism by which senescent cells in the microenvironment stimulate epithelial tumour progression has been the focus of intense scrutiny in recent years. Significantly, senescent cells secrete a wide variety of pro-tumourigenic proteins collectively referred to as the senescence-associated secretory phenotype (SASP; [35, 36]). The SASP is made up of soluble signalling factors (growth factors, chemokines and interleukins), secreted proteases (matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator/tissue-type plasminogen activator) and extracellular matrix components (collagen and fibronectin) that have the capacity to regulate inflammation, tumour cell behaviour, the tumour microenvironment and angiogenesis (fig. 1a). Amongst the factors that constitute the SASP, osteopontin has been shown to be essential for the selective induction of pre-neoplastic keratinocyte proliferation at the expense of normal keratinocytes [37, 38], whilst IL-6 and IL-8 [35], MMPs [39–41] and VEGF [35] have all been associated with epithelial tumour progression in different systems (fig. 1a). Invariably, this is associated with an epithelial-mesenchymal transition (EMT) [35] which, in turn, has been linked to cancer stem cell potential [42].

Fibroblast senescence associated with OSCC [43] is thought to be a very early event in the carcinogenic process [41] and has been shown to be induced by the primary risk factors associated with OSCC including tobacco [44, 45], alcohol [46] and betel nut alkaloids [Rehman et al., unpubl. data]. With respect to OSCC, CAF senescence is a characteristic of the genetically unstable and aggressive tumours (GU-OSCCs) compared to their more stable counterparts (GS-OSCCs). Our current understanding suggests that malignant keratinocytes from GU-OSCCs produce high levels of ROS [43] that induce fibroblast activation and senescence [43] (fig. 1a). By contrast, GS-OSCCs and GS-OMPLs retain an intact senescence programme and do not produce high levels of ROS but instead over-express a multitude of cytokines, such as IL-8, IL-6, CXCL6, CXCL5, CXCL1 and CCL20 [5], but notably not IL-1β which has recently been shown to drive the spread of senescence from cell to cell [47]. The bypass of senescence in GU-OSCCs leads to a collapse of the expression of the above cytokines, and preliminary data suggests that this is linked to the loss of expression of p53, p16INK4A or both [Ng et al., unpubl. data]. In summary, the inflammasome component of the OSCC SASP appears to be upregulated in GS-OSCCs and replaced by an upregulation of ROS in the GU-OSCCs; this is consistent with the possible existence of tumours and pre-cancerous fields that are mixtures of both GU and GS types of keratinocytes as suggested many years ago [23].

More recent data show that TGF-β is over-expressed by senescent oral CAFs and acts in concert with MMP-2 to down-regulate a broad spectrum of cell adhesion molecules, induce EMT (fig. 1a) and promote epithelial invasion in vitro [39; Cirillo, pers. commun.].

Interestingly, CAFs [43, 48] and fibroblasts from the premalignant condition oral submucous fibrosis [41] show a reduced replicative lifespan in vivo compared to normal fibroblasts. Further, many of the above CAF-associated molecular changes, such as IL-1β [47], TGF-β family members including Activin A [47], FoxM1 [49] and several matrix MMPs, including MMP-2 [39], have also been identified in normal senescent fibroblasts, but the exact role that senescence plays in the CAF phenotype has not been definitively tested. Extensive work by Coppe et al. [35] has shown that the loss of p53 actually augments at least a subset of the SASP, including the interleukins,
but the complete ablation of the senescence programme by the dual knockout of p53 and the INK4A locus in mice observed by Kang et al. [17] and Xue et al. [50] suppresses the inflammatory response; this apparent controversy requires further investigation.

The Human Fibroblast Extracellular Senescence Metabolome

Very recently, we completed a detailed unbiased screen of the extracellular senescence metabolome (ESM) [51] and identified alterations in several metabolites including increased levels of alanine, citrate, molecules involved in oxidative stress, a sterol, mono-hydroxy lipids, tryptophan metabolism, phospholipid and nucleotide catabolism, as well as reduced levels of dipeptides containing branched-chain amino acids. Interestingly, over half of these molecules showed a similar pattern of alteration in human ageing [51], suggesting that senescent cells may be detectable in vivo. However, to date, only few of these have been revealed in studies of cancer body fluid metabolomics [51, 52], perhaps because of the great variation in cancer patient metabolism and other confounding factors that are currently not being taken into account.

Furthermore, most clinical studies on the serum metabolome have so far been seriously underpowered [52]. The recent acquisition of a serum metabolome database (the HUSERMET project) derived from over 1,000 healthy subjects [52] should enable previous studies to be corrected for parameters such as age, body mass index, blood pressure, drugs and smoking and future studies designed to take account of these variables.

Another issue is whether any of the ESM molecules transfer phenotypes from the senescent cells (or CAFs) to other non-senescent cells analogous to the SASP [47]. Martinez-Outschoorn et al. [53] have provided persuasive evidence that lactate and ketones from glycolytic fibroblasts induced to undergo senescence and autophagy can provide an energy source for cancer cells in mixed cultures and thus enhance tumourigenicity and invasion. There is no evidence, however, that these metabolites can act independently of other molecules present in the SASP.

In our own work, we did not find that either lactate or ketones were reproducibly elevated in senescent fibroblasts in the absence of co-cultured tumour cells, although lactate was elevated in the ESM of some fibroblast lines [James et al., unpubl. data]. By far the most reliable senescence marker in the ESM was citrate [51], and whilst this metabolite cannot induce alterations in growth or senescence on its own [James and Parkinson, unpubl. data], it has been reported to enhance the invasive behaviour of prostate cancer cells and is secreted in large amounts by the normal prostate epithelial cells to provide an energy source for sperm in semen [54, 55]. Extracellular citrate is also induced by irreparable DNA damage [51], accumulates with advanced age in humans [52] and is reduced in some mouse models of longevity [51]. Thus, extracellular energy metabolites such as citrate and lactate may increase the risk of cancer in old age.

The OPML Extracellular Metabolome

In an attempt to identify non-invasive markers of OPML that are likely to progress, we have conducted an unbiased metabolomic screen of GS- and GU-OPML extracellular metabolites [Parkinson, unpubl. data]. Whilst this study is still ongoing, GS-OPMLs that retain an intact senescence programme accumulate many metabolites that are also associated with the fibroblast ESM [51], whereas these changes are muted in GU-OPMLs. The findings are analogous to the regulation of cytokines in these two classes of OPML keratinocytes as discussed above. No one extracellular metabolite accumulates in GU-OPML media, the serum or saliva, and, to date, metabolomic studies of OPMLs have not revealed consistent changes associated with OPML progression [52]. However, this may be due to the limitations of the platforms that are available and appropriate standards, the relatively small sample sizes and the failure to correct for known variables such as donor age [52]. Therefore, much further work is required if metabolomics is to be successfully applied to the early diagnosis of OSCC.

Conclusions and Future Directions

In conclusion, although a large amount is known about the OSCC keratinocyte genome and transcriptome, and we will shortly have similar knowledge of the epigenome and metabolome, the future challenge will be to understand how the different cell types within the tumour mass interact with each other and co-evolve. It is anticipated that this will lead to improvements in the early detection, prevention and therapy of OSCC.
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