Abstract. The insulin-like growth factors (IGFs) are a family of mitogenic proteins involved in the regulation of cell growth and differentiation. The presence and role of the IGF system in oral mucosal epithelium is not clear but could influence our understanding of the pathogenesis of oral cancer. We characterised the expression and function of IGF-1, IGF-2 and IGF receptor in human oral squamous carcinoma cell lines and normal oral epithelial cells as well as normal oral and squamous cell carcinoma tissues. Using reverse transcription followed by PCR, IGF-1 mRNA was only detected in normal cells, whereas IGF-2 and IGF-1R mRNA transcripts were highly expressed in tumour cell lines and tissues. Similar observations were seen by Western blot analysis and immunohistochemistry. Exogenous IGF-2, but not IGF-1, caused significant increases in DNA synthesis in the cell lines. IGF-2 also increased cell proliferation which was significantly attenuated in the presence of an IGF-2 neutralizing antibody or one which blocked IGF-1R. Taken together, these studies suggest that autocrine production of IGF-2, together with over-expression of IGF-1R, may be important components controlling the proliferation of oral carcinoma cells.

Introduction

The insulin-like growth factor (IGF) family of proteins is thought to play an important role in regulating cell proliferation, differentiation and apoptosis and as such there is considerable accumulating evidence indicating that it may also be important in promoting carcinogenesis. This is deemed to occur by increasing the risk of cellular turnover and thus increasing the susceptibility of cells to malignant transformation (1-4). The IGF family consists of polypeptide ligands IGF-1 and -2 which are synthesized mainly in the liver. IGF-1 and -2 are present in the circulation in combination with binding proteins (IGFBPs) of which 6 have been identified, IGFBP-1 to IGFBP-6 (5-8). Both IGF-1 and -2 exert their effects by binding to a transmembrane tyrosine kinase receptor, IGF-1R, which is structurally and functionally related to the insulin receptor (IR) (9).

Several studies, both experimental and clinical, have demonstrated that the IGF-1R is over-expressed in tumours compared to normal tissues (3,10,11). Furthermore, epidemiological prospective studies have identified high plasma levels of IGF-1 as a potential risk factor for several malignancies (12-15). In addition, IGF-2, whose expression is normally strictly controlled by parental imprinting, is upregulated and functions as an important stimulant of the IGF-1R in cancer (16,17). Thus, upregulation of IGF-1R and its ligands are probably important events for the malignant cell growth.

The role of IGF in the development of oral carcinomas is less clear. One study failed to establish a predictive link for serum levels of IGF-1 in carcinomas of the tongue (17). In contrast, recent work has demonstrated a clear association between high circulating levels of IGF-1 and high or low levels of IGFBP-3 with the development of second primary tumour in the head and neck (18). Work from our group has recently shown that oral cancer seems to be associated with reduced levels of serum IGF-1 and IGFBP-3, with elevated levels of IGFBP-1 and -2 (19). To know how IGFs act on oral epithelium could have an important impact in understanding the pathogenesis of oral cancer. Whilst there have been studies describing the presence of the IGF system in tooth growth and development (20-22), we are not aware of any studies on oral mucosal epithelial cells.

The aim of this study was to characterise IGF-1, IGF-2, and IGF-1R by investigating their expression and function in human oral epithelial cancer cell lines and normal cells maintained in short-term culture.
Materials and methods

Reagents. All cell lines used were derived from oral squamous cell carcinomas. SCC-4 and SCC-25 were both derived from tongue epithelia, FaDu from the pharynx and TR146 from the buccal mucosa. Cell culture media (DMEM for SCC-4, -25, TR146 and FaDu cells, and KGM Bullet Kit for NOK cells) were purchased from Cambrex Bio-Science (Wokingham, UK). Reagents for reverse transcription and PCR were purchased from Invitrogen (Paisley, UK). Antibodies to human IGF-1 (AF-291-NA), IGF-2 (MAB292) and IGF-1R (MAB391) were purchased from R&D Europe Systems (Oxon, UK) as were IGF-1 and IGF-2 recombinant proteins (291-G1 and 202-G2, respectively). Primer pairs for the detection of IGF-1, -2 or IGF-1R were obtained from Invitrogen based on Neuvians et al (23). All other reagents were the best available grade and purchased from Sigma-Aldrich (Poole, Dorset, UK).

Cell maintenance and treatments. None of the cells used required 3T3 feeder cells for growth. SCC-4 and -25 cells were maintained in T75 cm² tissue culture flasks with DMEM supplemented with 10% fetal bovine serum (FBS), 400 ng/ml hydrocortisone and routine antibiotics in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. Medium was replaced and replaced every 2-3 days. FaDu and TR146 cells were maintained as described above but in medium without hydrocortisone. Normal oral keratinocytes (NOK) were acquired from the departmental archive of frozen cells (CDOS, Queen Mary University, London). They were taken from normal oral mucosa at the time of third molar surgery in 1995 and frozen at the time of first passage. NOK were maintained in KGM supplemented with the manufacturer's Bullet Kit which consisted of hydrocortisone, insulin, epidermal growth factor (EGF), bovine pituitary extract (Cambrex) and 1 mg/ml recombinant IGF-2 protein or 10 μg/ml recombinant IGF-2 protein for further 4 h. These experiments were repeated three times.

Cell proliferation was determined using the MTT assay. Serum-free medium (200 μl) was pipetted into the wells and cells were incubated for 4 h with 20 μl of 5 mg/ml MTT solution (w/v in sterile PBS). Medium was then carefully aspirated and the formazan product was dissolved by adding 500 μl DMSO to each well. Plates were shaken gently on a plate shaker for 15 min. Each sample (200 μl) was placed in a 96-well plate using DMSO as assay blanks. Absorbance was read at 570 nm using a microplate reader.

RNA extraction from formalin-fixed, paraffin-embedded tissue. Formalin fixed, paraffin-embedded tissue of 10 oral tumours and 10 normal oral mucosa tissues were obtained with Research Ethics Committee (REC) approval (St. James' Hospital and Federated Dublin Voluntary Hospitals Joint REC, reference 020512/8602). RNA was extracted from tissues following published methods (24). To 20 micron sections of tissue 100 μl of 0.5% Tween-20 was added, agitated and heated to 90°C for 10 min in a Thermal Cycler (Jencons Ltd., East Sussex, UK). Samples were then digested with 2 μl of 10 mg/ml Proteinase K for 3 h at 55°C with gentle agitation every 60 min. Each digest mixture was then heated to 99°C for 10 min with 100 μl 5% Chelex-100 (Bio-Rad) in Tris-EDTA. Samples were gently shaken and immediately centrifuged at 12,000 rpm for 15 min and then placed on ice to harden the wax. The wax was lifted out of the tubes and the sample heated to 45°C and 100 μl chloroform added. Tubes were gently flicked to mix contents before further centrifugation. The top phase (about 150 μl) was removed and stored at -70°C until required. Extracted RNA (5 μl) was subsequently used for RT-PCR.

RT-PCR. Total cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction using Ultraspec solution (AMS Biotechnology, Abingdon, UK) following manufacturer's instructions. Reverse transcription of the RNA and subsequent PCR was carried as described previously (25). For PCR the following oligonucleotide primers were used: IGF-1 sense 5’-ccagctctcttcatcttg-3’ and anti-sense 5’-gcgctctcctctcctccctc-3’ (product size 260 bp); IGF-2 sense 5’-gcgctctcctctcctccctc-3’, antisense 5’-aaga cttgcccaaggggtat-3’ (product size 203 bp); IGF-1R sense 5’-tataaggtgctgctg-3’ and antisense 5’-ttccagcggctctc-3’ (product size 314 bp) and GAPDH sense: 5’-ccacagctccatc-3’ and antisense: 5’-tcacccagcttgtct-3’ (product size 420 bp). The reaction cycles of PCR were 40 cycles denaturation for 30 sec at 94°C, primer annealing for 1 min at 65°C (54°C for GAPDH) and primer extension for 1 min at 72°C. PCR products were separated by electrophoresis in a 0.8% agarose gel stained with ethidium bromide. Bands were visualized under ultraviolet light.

Measurement of IGF-1 and -2 secretion. IGF-1 and IGF-2 secretion in conditioned media of untreated cells were quantitated by ELISA following manufacturer's instructions (Oxford Bio-Innovation Ltd., Oxon, UK). Cells were seeded at 1x10⁶ cells/well in 6-well cell culture plates for 24 h to
72 h. Conditioned media were collected 24, 48 and 72 h, stored at -20˚C and later analyzed for IGF-1 and -2.

**Immunohistochemistry.** Archival formalin-fixed paraffin-embedded tissue samples of 15 human oral squamous cell carcinomas were used for immunohistochemistry (REC reference as above). Sections (5 μm) were deparaffinised and rehydrated in xylene followed by graded alcohols. Sections were treated as described previously (26) except antigen retrieval was carried out by microwaving for 9 min in 0.01 M citrate buffer. Antibodies used were from Abcam (Cambridge, UK): rabbit polyclonal to human IGF-1 (ab15320) used undiluted, rabbit polyclonal to human IGF-2 (ab9674) used at 1:100; rabbit polyclonal to human IGF-1R (ab30657) used at 1:20. The secondary antibody and subsequent detection was carried out at room temperature using the LSAB + System-HRP kit following manufacturer’s instructions (Dako Ltd., Ely, UK).

**Western blot analysis.** After treatment cells were lysed and proteins extracted, electrophoresed, transferred to a PVDF membrane and blotted as described previously (26). Antibodies used are listed in the reagents section and used at 1:1000 dilution. Blots were analysed by densitometry (Kodak 1D 3.5 software, version 1; UVP International, Cambridge, UK).

**Statistical analysis.** The data were analyzed using the SPSS 7.5 Windows Students version software. For all the measurements, one-way ANOVA followed by Student’s Newman Keuls (SNK) test was used to assess the statistical significance. Experiments were performed in triplicate.

**Results**

**IGF-1, IGF-2 and IGF-1R are highly expressed in oral cancer cell lines.** IGF-1, -2 or IGF-1R expression was measured by RT-PCR and were found to be differentially expressed in the cell lines used. The cells were deprived of serum/growth factors 24 h before experiments. Fig. 1 illustrates that only the normal oral epithelial cells, NOK, expressed a transcript for IGF-1. None of the oral squamous carcinoma cell lines used in this study expressed IGF-1 mRNA regardless of PCR cycle number or increasing the amount of starting RNA in the reverse transcription reaction (data not shown). In contrast, all cells expressed IGF-2 mRNA, as can be seen in Fig. 1, with lower message levels in NOK, FaDu and TR146 cells. Fig. 1 also demonstrates that all cells expressed IGF-1R, with the squamous carcinoma cell lines SCC-4 and -25 having higher levels of expression.

The experiment were repeated for protein levels and analysed by Western blot. The results are very similar to the RT-PCR data described above. The upper panel of Fig. 2 illustrates that NOK cells expressed IGF-1 protein but none of the carcinoma cell lines were detectable. All cells expressed IGF-2 protein, with slightly higher levels in cell lines SCC-4 and -25. Fig. 2 also clearly shows that all cells expressed IGF-1R protein with over-expression in SCC-4 and -25 cells. The lower panel of Fig. 2 is a graphic representation of densitometry data from repeated Western blot experiments.

**Expression of IGF-1, IGF-2 and IGF-1R in oral cancer tissues.** We studied the expression of IGF-1, -2 and IGF-1R in oral cancer by immunocytochemistry and semi-quantitative RT-PCR. In normal tissue, we found there was weak immunoreactivity for IGF-1, IGF-2 or IGF-1R (data not shown) by immunohistochemistry. However, in oral squamous cell carcinoma, of which Fig. 3 is illustrative, there was increased immunoreactive IGF-1 and IGF-2 and IGF-1R in particular. We then extracted RNA from a number of archival tissues of histologically normal and OSCC tissue and performed RT-PCR. Fig. 4 illustrates that there was expression of IGF-1, IGF-2 or IGF-1R message in normal tissues but this was increased at least 2-fold in OSCC.
Effect of exogenous IGFs on cell proliferation. In another series of experiments the secretion of IGF-1 or -2 from unstimulated cells was measured. Fig. 5a illustrates that only the normal oral cells produced significant levels of IGF-1 which increased with time over the 72-h period measurements were taken. Maximum secretion occurred at 48 h with 30±2.5 ng/ml IGF-1 secreted. SCC-4, -25, FaDu or TR146 cells did not secrete IGF-1 significantly at any of the time points measured. Fig. 5b shows that all cells secreted IGF-2 with basal levels around 17.5±2.4 ng/ml. The SCC cell lines produced the most IGF-2, with peak secretion at 48 h and the normal oral epithelial cells, FaDu and TR146 cells producing up to 3-fold more IGF-2 compared to time point zero.

DNA synthesis was studied by [3H]-thymidine incorporation experiments. Fig. 6 illustrates that IGF-1 caused some increases in DNA synthesis in OSCC cells with the greatest levels in SCC-4 and SCC-25 cells. IGF-2 caused a significant increase in all the oral tumour cell lines used. Normal epithelial cells did not respond to either IGF-1 or -2. Adding serum caused significant incorporation of [3H]-thymidine in all cells used.

It was evident (Fig. 5) that the oral cancer cell lines synthesised and secreted significantly more IGF-2 endo-
tissue to be compared with. A study by Tsai and co-workers is not clear since there was no previous data in oral epithelial oral cancer cells is an interesting observation but its meaning cell lines used in this study. This lack of IGF-1 expression in expressed in normal cells but not in any of the oral cancer oral cancer. We found IGF-1 mRNA and protein was only derived in order to determine the role of the IGF system in oralepithelial cells, both normal and squamous carcinoma-

structures of the oral cavity (20,28,29). In this study, we measured in human saliva (27) and the IGF-1 signaling axis number of clinical studies. Free IGF-1 and -2 have been caused significant increases in [3H]-thymidine incorporation. 
demonstrate the abundance of IGF-2 and IGF-1R in human epithelium have been sadly lacking, though there have been a discussions have been reported of over-expression of IGFs and receptors in primary tumors and transformed cell lines (12-15,32).

Like IGF-1, IGF-2 has a similar role in development and acts as an autocrine growth factor in a variety of cell types. To our knowledge, this is the first study to demonstrate that oral epithelial cell lines express IGF-2 transcript and protein, and that all cells secreted modest amounts of IGF-2 over time, in the absence of stimuli. Over-expression of IGF-2 appears to be strongly associated with neoplastic transformation of some types of epithelial cells and cancers (33-36). We found that the squamous carcinoma cell lines consistently expressed higher levels of IGF-2 transcripts and protein. These cells also responded to exogenous IGF-2, but not IGF-1, by significantly increasing DNA synthesis. This was further confirmed by studying cell proliferation. Fig. 6 illustrates that addition of exogenous IGF-2 significantly increased cell proliferation of cells, the SCC in particular. A large component of the increase was due to IGF-2 and mediated via IGF-1R specifically, use of neutralizing IGF-2 or IGF-1R blocking antibodies attenuated cell proliferation significantly.

The SCC cell lines are derived from tumors of the oral cavity and have been described as being ‘aggressive’ cells (37,38). A comprehensive study by Gasparoni et al (39) have suggested that these cells are more invasive than other tumor-derived epithelial cells due to increased levels of E-cadherin and involucrin, and morphological changes characteristic of aggressive differentiation. We suggest that because these SCC cells, in particular, express high levels of IGF-2 this may contribute to oral cancer development or growth. In fact, a recent study has identified increased levels of IGF-2 as a biomarker for head and neck squamous cell carcinomas (40).

The implication of our findings is that the IGF-1R may also play an important role in rescuing cells from apoptosis. Blockade of the IGF-1R has been demonstrated to inhibit tumour cell growth and invasion in some cancers and has been targeted for possible therapeutic intervention (41-44). All cell lines used in this study expressed increased levels of transcripts and protein for IGF-1R compared to normal oral epithelial cells, more so in the SCC lines. The effects mediated by the interaction of IGF-1 and IGF-1R are dependent on the levels of IGF-I receptor expressed on the

Discussion

We found that normal and oral tumour epithelial cells express IGF-2 and IGF-1R mRNA transcripts and protein, whereas only normal epithelial cells expressed IGF-1 mRNA and protein. To our knowledge, this is the first study to demonstrate the abundance of IGF-2 and IGF-1R in human oral epithelial cells. At concentrations known to activate their receptors, IGF-1 had no effect on DNA synthesis, but IGF-2 caused significant increases in [3H]-thymidine incorporation.

Studies looking at the role of IGFs and receptor in oral epithelium have been sadly lacking, though there have been a number of clinical studies. Free IGF-1 and -2 have been measured in human saliva (27) and the IGF-1 signaling axis is known to have a role in the developmental regulation of structures of the oral cavity (20,28,29). In this study, we have investigated the expression of IGFs and IGF-1R in oral epithelial cells, both normal and squamous carcinoma-derived in order to determine the role of the IGF system in oral cancer. We found IGF-1 mRNA and protein was only expressed in normal cells but not in any of the oral cancer cell lines used in this study. This lack of IGF-1 expression in oral cancer cells is an interesting observation but its meaning is not clear since there was no previous data in oral epithelial tissue to be compared with. A study by Tsai and co-workers (22) noted IGF-1 mRNA in fibroblasts cultured from biopsies from patients with oral submucous fibrosis, a pre-cancerous condition. However, studies by Steller and co-workers (30,31) demonstrated that primary cultures of normal and cancerous human cervical epithelial cells did not express IGF-1 mRNA or protein. One of the most important roles of IGF-1 action is that it acts as an anti-apoptotic factor hence the peptide is a cell survival agent (1-4,10,15). It is tempting to speculate that the apparent loss of IGF-1 expression in the oral squamous carcinoma cells may play a role in malignant transformation in cancers of the head and neck. Indeed, previous studies from our group, reported that head and neck cancer patients had reduced serum levels of IGF-1 (and IGFBP-3) (19). It is well documented that IGF, as well as having an important role in normal growth, is also crucial in malignancy. This view is supported by clinical and in vitro studies where many observations have been reported of over-expression of IGFs and receptors in primary tumors and transformed cell lines (12-15,32).

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cell surface. As the number of receptors increases, so does the effect that IGF-I exerts (45). With enough IGF-1 receptor activity, the signaling effects become mitogenic. It is believed that IGF-1 receptor expression is essential for the emergence of malignant transformation (46). The enhancement of IGF-1 receptor activity can be a result of increased receptor number, increased receptor stimulation, or both. It is tempting to speculate that such events are occurring in OSCC. Figs. 1 and 2 clearly illustrate the enhancement of IGF-1 receptor activity can be a result of the emergence of malignant transformation (46). The activity, the signaling effects become mitogenic. It is

Cellular over-expression of IGF-1R means an increased likelihood of a cell’s survival and it makes sense for the squamous carcinoma cell lines to have higher levels of IGF-1R. The findings in this study can be added to the many published observations which have suggested that transformed cells in breast, prostate, cervical and other tumor types, express higher levels of IGF-1R than normal cells. How this occurs on a molecular level in tumour cells is not clear.

Logically, it seems clear that cells can increase their chances of survival by modulating IGF signaling in a number of ways. For example, increasing ligand production, increased expression of IGF-1R or decreased levels of IGFBPs which compete with IGFs to bind to the receptor. This study did not investigate the role of IGFBPs in these cells but this is currently underway. Nevertheless, these experiments lend further evidence to support the importance of IGF-2 and IGF-1R overexpression in tumourigenesis. The inappropriate production of IGF-2 in these oral squamous carcinoma cells represents an important pathway which may be exploited by malignant cells to evade targeted cell elimination.

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References