Endothelin-1 stimulates oral fibroblasts to promote oral cancer invasion

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ABSTRACT

Aims: The aims of this study were to examine the role of endothelin-1 (ET-1), a pleiotropic peptide found at elevated levels in a number of malignancies and which has been shown to influence oral cancer cell behaviour via paracrine signalling pathways, on the phenotype of oral fibroblasts.

Main methods: The effect of ET-1 on proliferation and migration of human primary oral fibroblasts was assessed using MTS and scratch assays, respectively. The ability of ET-1 to affect fibroblast contractility was analysed using type-I collagen gels. Changes in gene expression in oral fibroblasts exposed to ET-1 were examined using quantitative PCR. The invasiveness of oral cancer cells in the presence of conditioned media collected from ET-1 treated fibroblasts was determined using 2D Matrigel assays.

Key findings: Here we provide evidence that ET-1 increases the migration of oral fibroblasts and induces a more contractile phenotype which is not associated with changes in gene expression indicative of myofibroblast transdifferentiation. In addition we provide evidence that conditioned medium of ET-1-stimulated oral fibroblasts promotes invasion of OSCC cells in vitro.

Significance: In oral squamous cell carcinoma, a frequently fatal and increasingly common epithelial malignancy of the oral cavity, ET-1 is known to contribute to pro-migratory paracrine signalling between stromal fibroblasts and cancer cells. The ability of ET-1 to modulate the phenotype of human oral stromal fibroblasts, however, has not previously been reported. The findings presented here suggest that targeting the stromal endothelin system may be a viable and novel therapeutic strategy for invasive oral cancer.

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Introduction

Endothelin 1 (ET-1) is a pleiotropic 21 amino acid peptide produced by the action of endothelin converting enzyme-1 on the precursor peptide, big endothelin-1 (Kawanabe and Nauli, 2010). It mediates its effects by binding to one of, or both, G-protein coupled receptors, ET_AR and ET_BR. ET-1 and its receptors are widely expressed and aberrant production or activity is associated with a wide range of pathologies, including cancer (Nelson et al., 2003). Elevated levels of ET-1 have been observed in ovarian, prostate, colon and oral cancer, where it is thought to promote a pro-tumourigenic phenotype, acting both on the epithelial cells and the surrounding stroma (Lambert et al., 2008; Rosano et al., 2005).

The significance of the tumour stroma in carcinogenesis and progression of disease is increasingly recognised, and represents an attractive target for emerging therapeutic approaches (Pietras and Ostman, 2010). The predominant cell type in the stroma, fibroblasts, frequently undergo a number of changes in response to signals released by malignant epithelial cells including increased proliferation, migration and acquisition of a contractile, myofibroblastic phenotype (Kalluri and Zeisberg, 2006). This myofibroblastic transdifferentiation is associated with changes in the gene expression profile, including increased expression of α-smooth muscle actin and members of the matrix metalloproteinase family. Fibroblasts displaying these characteristics (sometimes termed 'cancer-associated fibroblasts') are commonly prominent in the reactive stroma surrounding the invasive front of tumours and are often associated with a poor prognosis (Kalluri and Zeisberg, 2006). In oral squamous cell carcinoma (OSCC), the presence of myofibroblasts in the tumour stroma was recently identified as the strongest negative prognostic indicator, and myofibroblasts were shown to promote cancer cell invasion (Marsh et al., 2011).

ET-1 is known to promote myofibroblast transdifferentiation in a number of pathologies including lung fibrosis (Teder and Noble, 2000) and cardiac fibroblast remodelling following myocardial infarction (Nambi et al., 2001). In the OSCC tumour stroma, TGF-β has been identified as a major mediator of myofibroblast transdifferentiation (Kellermann et al., 2008), but nothing is known of the role of ET-1 in this process, or the implications of this for the neighbouring cancer cell phenotype.

Here, we addressed the hypothesis that ET-1 stimulates paracrine signalling between oral fibroblasts and malignant epithelial cells by

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modifying the fibroblast phenotype. We show that ET-1 stimulation of fibroblasts increases their migration and confers on them a more contractile phenotype. This is associated with an increased ability to stimulate oral cancer cell invasion but is not concomitant with the acquisition of markers of myofibroblastic transdifferentiation, suggesting ET-1 may synergise with other factors to generate the reactive, myofibroblast rich stroma associated with aggressive oral malignancies.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-glutamine and trypsin/EDTA were all purchased from Bio Whitaker (Wokingham, UK). Chemicals routinely used were purchased from Sigma-Aldrich (UK), unless otherwise stated, and were of the highest analytical grade.

Cell culture

The human OSCC-derived cell line SCC4 and primary oral fibroblasts (collected with South Sheffield NHS Trust ethics committee approval and used within passage 3–10, as described in Hearnden et al. (2009)) were routinely cultured in DMEM supplemented with 2 mM L-glutamine and 10% (v/v) FBS. All cells were grown in antibiotic-free media at 37 °C and 5% (v/v) CO_2.

Cell treatments

Oral fibroblasts and OSCC cells were serum starved for 24 h prior to experimentation. Cells were treated with either ET-1 (10 nM), TGF-β (20 ng/ml) and thrombin (0.5 U/ml) and incubated for the times indicated at 37 °C and 5% (v/v) CO_2. Where indicated, fibroblasts were pre-treated with an ET_AR antagonist, an ET_BR antagonist (BQ-123 and BQ-788, respectively, both 1 μM) individually or in combination, for 30 min before addition of ET-1.

Invasion assay

SCC4 at 90% confluency were serum starved for 24 h prior to experimentation, trypsinised and resuspended in DMEM containing 0.1% (w/v) BSA at 1×10^5 cells/ml. A 200 μl cell suspension was added to the top of a transwell chamber containing 200 μl of growth factor depleted Matrigel (BD Biosciences) and 500 μl of DMEM containing conditioned media from fibroblasts (treated as described in individual figure legends) was added to the lower chamber. After 40 h, cells were swabbed away from the inside of the invasion chamber and cells adhering to the underside of the chamber fixed for 10 min in 100% (v/v) methanol. Migrated cells were stained with 0.1% (w/v) crystal violet and counted by light microscopy. Three fields of view from each insert were counted.

Proliferation assay

Oral fibroblasts were seeded at 2000 cells/well and allowed to incubate overnight. Cells were washed in phosphate buffered saline (PBS) before addition of ET-1 and/or ET-1 receptor antagonists (added 30 min prior to addition of ET-1). MTS reagent (Sigma) was added and fluorescence recorded at 492 nm using a fluorescent spectrophotometer (Tecan) following incubation at 37 °C, 5% (v/v) CO_2 for 48 h.

Migration assay

Oral fibroblasts were seeded at 100,000 cells/well and allowed to incubate overnight. Cells were then serum starved for 24 h. A scratch was made in each well using a 200 μl pipette tip. Media was removed, and cells were washed in PBS before the addition of ET-1, serum and/ or antagonists of ET_AR and ET_BR (added 30 min before the addition of ET-1). Mitomycin C (Sigma) at 1 μg/ml was added to each well to prevent proliferation. Each well was photographed at two points along the scratch at 0 h and 24 h. The distance between each edge of the scratch was measured.

Gel contraction assay

Oral fibroblasts (250,000 cells/well) were mixed with rat tail collagen (7.5 mg/ml) in DMEM and pH adjusted to 7 using NaOH. The cell:collagen mixture was added to 24 well plates and incubated for 24 h. The gels were then loosened from the edges of the well and were incubated with serum free medium containing thrombin, ET-1 and/or ET-1 receptor antagonists (added 30 min before addition of ET-1) for 30 min. Collagen lattices were photographed and the distance contracted by the gels measured.

Real-time PCR

Total RNA was isolated from oral fibroblasts using the RNeasy mini kit (Qiagen). RNA from each sample was quantified using a NanoDrop spectrophotometer (Thermo). High capacity cDNA reverse transcription kit (Applied Biosystems) was used for synthesis of cDNA according to the manufacturer's instructions. cDNA was subsequently analysed by SYBR green qPCR using the 7900HT Fast thermocycler (Applied Biosystems).

Primers used for SYBR green quantification were as follows: U6 forward 5′ CTCGCTTCGGCAGCACA 3′, U6 reverse 5′ AACGTTCACGAA TTTGCGT 3′; alpha-SMA forward 5′ GAAGAAGAGGACAGCACTG 3′, alpha-SMA reverse 5′ TCCCATTCCCACCATCAC 3′. Taqman chemistry was used to detect MMP-2 and beta-2-microglobulin using primers/ probes obtained from Applied Biosystems. All values were normalised to U6 (SYBR) or beta-2-microglobulin (Taqman) expression levels.

Immunoblotting

Cells were washed twice with PBS and protein extracted using triple detergent lysis buffer (0.1 M Tris–HCl pH 7.4, 0.15 M NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) Nonidet P-40) containing Complete Mini Protease Inhibitor Cocktail (Roche) and Benzonase (Sigma; used according to manufacturer's instructions). Protein concentration was measured using BCA Protein Assay Kit (Thermo). Total protein extracts (50 μg) were separated by 3–8% (v/v) SDS-PAGE and transferred to nitrocellulose membrane. Following blocking of non-specific protein binding, membranes were incubated with antibodies directed to α-SMA (1:1000, Sigma) or β-actin (1:4000, Sigma). Horseradish peroxidase-conjugated secondary antibodies (Sigma) were diluted 1:2000. All antibodies were diluted in 5% (w/v) dried milk and 3% (w/v) bovine serum albumin in Tris-buffered saline containing 0.5% (v/v) Tween 20. Immunoreactive proteins were visualised by enhanced chemiluminescence (ECL, Pierce). Densitometry was performed using Adobe Photoshop.

Statistical analyses

Data are expressed as the mean ± SEM. Normal distribution of data was assessed using the Shapiro–Wilk test; statistical analyses were made between two groups using the non-parametric Mann Whitney *U* test or parametric Student *t*-test, as appropriate and indicated in figure legends. A value of $p < 0.05$ was considered significant.

Results

Fibroblasts treated with ET-1 increase invasion of oral cancer cells

Having previously shown that ET-1 is capable of promoting paracrine signalling between oral fibroblasts and OSCC cells (Hinsley et
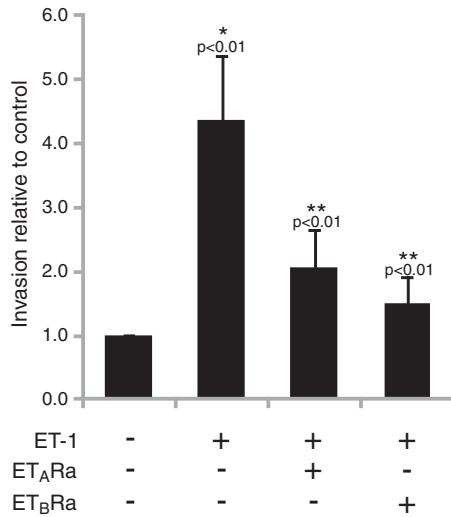
Fig. 1. Endothelin-1 stimulates pro-invasive stromal-epithelial interactions. Conditioned medium (CM) was collected from oral fibroblasts pretreated with an ET_AR antagonist (ET_ARa) or an ET_BR antagonist (ET_BRa) or vehicle control for 30 min before the addition of ET-1 for 4 h. CM was added to the lower side of a Matrigel invasion chamber, and SCC4 cells seeded into the chamber, as described in Materials and Methods. After 40 h, invaded cells were stained and counted, as described. Results are plotted relative to untreated control wells, ±SEM of three separate fields of view from three independent experiments. $^*p < 0.05$ compared to untreated, and $^{**}p < 0.05$ compared to ET-1 treated, as assessed by Student t-test.

al., 2012), we first sought to examine the effect of this signalling pathway on OSCC cell invasion. Conditioned media collected from oral fibroblasts treated with ET-1 significantly stimulated invasion of SCC4 cells through Matrigel, a murine tumour-derived extracellular matrix substitute (Fig. 1). This stimulation of invasion was blocked by an ET_AR antagonist and an ET_BR antagonist, suggesting ET-1 is able to act through both receptors, which we have previously shown to be expressed by oral fibroblasts (Hinsley et al., 2012). ET-1 had no significant effect on SCC4 invasion in the absence of fibroblasts (data not shown).

ET_AR and ET_BR influence proliferation of oral fibroblasts

Having established that ET-1 is able to stimulate fibroblasts to release pro-invasive factors, we next examined the effect of ET-1 on the
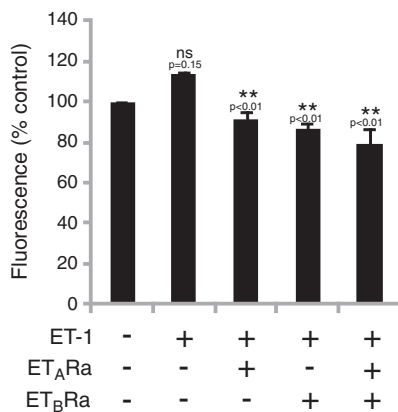

Fig. 2. ET-1 does not significantly alter proliferation of oral fibroblasts. Oral fibroblasts were pretreated with ET_AR and/or ET_BR antagonists for 30 min before the addition of ET-1. MTS was added and fluorescence measured at 48 h, as described in Materials and Methods. Results are plotted relative to untreated control wells, ±SEM of three independent experiments, each carried out in triplicate. $^*p < 0.05$ compared to untreated, ns $p > 0.05$ compared to untreated, and $^{**}p < 0.05$ compared to ET-1 treated, as assessed by Mann Whitney U-test.

growth of oral fibroblasts. Treatment with ET-1 caused a trend of increased proliferation of oral fibroblasts, but this did not reach significance (Fig. 2). Both ET_AR and ET_BR antagonists, however, significantly reduced proliferation compared to ET-1 treatment (Fig. 2), suggesting ET-1 signalling may play a role in oral fibroblast proliferation.

ET-1 promotes migration of oral fibroblasts

As activated fibroblasts commonly display a more migratory phenotype, we next analysed the effect of ET-1 on the ability of oral
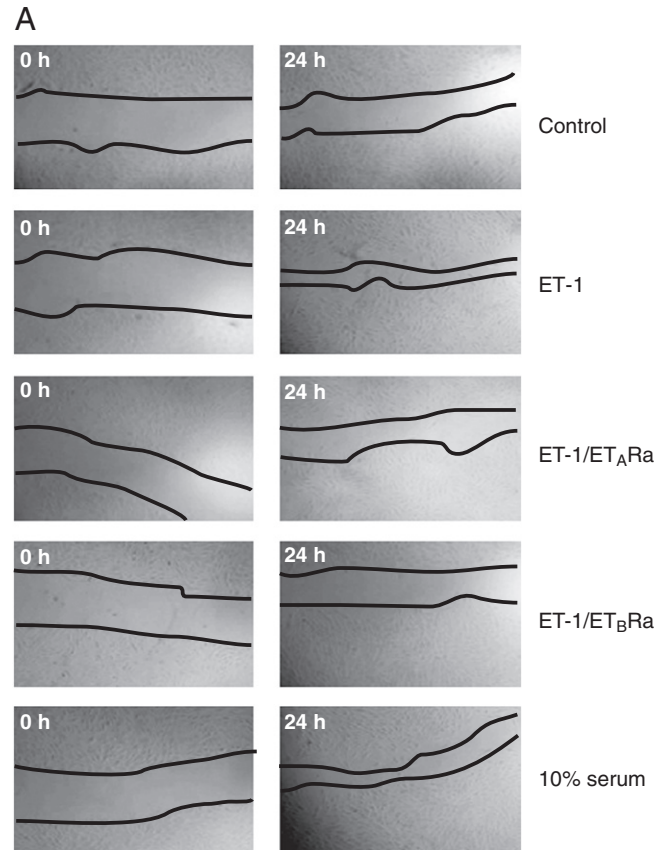

Fig. 3. ET-1 stimulates migration of oral fibroblasts. A scratch was made in oral fibroblasts cultured as a monolayer. Cells were then pretreated with ET_AR and/or ET_BR antagonists for 30 min before the addition of ET-1 or 10% (v/v) serum. The diameter of the scratch was measured before treatment, and after 24 h, photographs taken (A) and the extent of wound closure relative to control calculated (B). The data represent the mean values, ±SEM, of three independent experiments, each carried out in duplicate. $^*p < 0.05$ compared to untreated, $^{**}p < 0.05$ compared to ET-1 treated, as assessed by Mann Whitney U-test.

fibroblasts to migrate into a simulated wound *in vitro*. In the presence of mitomycin C (to prevent proliferation), ET-1 was found to significantly increase the migration of oral fibroblasts, almost to the same extent as that observed in response to serum (Fig. 3A,B). The stimulation of migration by ET-1 was abrogated by antagonists of ET_AR and ET_BR, both alone and in combination.

ET-1 stimulates contraction of oral fibroblasts

ET-1 treatment of oral fibroblasts seeded in collagen resulted in a significant contraction of gels (~30%) compared to untreated controls, and was of a similar magnitude to that provoked by thrombin, a well documented stimulator of fibroblast contraction (Bogatkevich, et al., 2001; Fig. 4A,B). The ET-1-mediated contraction was not significantly blocked by antagonism of ET_AR but was completely ablated by an ET_BR antagonist or with antagonism of both ET_AR and ET_BR (Fig. 4A,B).

Effect of ET-1 on myofibroblast transdifferentiation

It was recently reported that TGF-β mediated transdifferentiation of oral fibroblasts into myofibroblasts increased their ability to promote cancer cell motility (Kellermann et al., 2008; Marsh et al., 2011). In light of our finding that ET-1 is able to promote fibroblast-mediated pro-invasive signalling, we next examined the ability of ET-1 to promote two markers of myofibroblast transdifferentiation, α-smooth muscle actin (α-SMA) and matrix metalloproteinase-2 (MMP-2). TGF-β treatment of oral fibroblasts increased the expression levels of α-SMA transcript and protein (Fig. 5A,B) and MMP-2
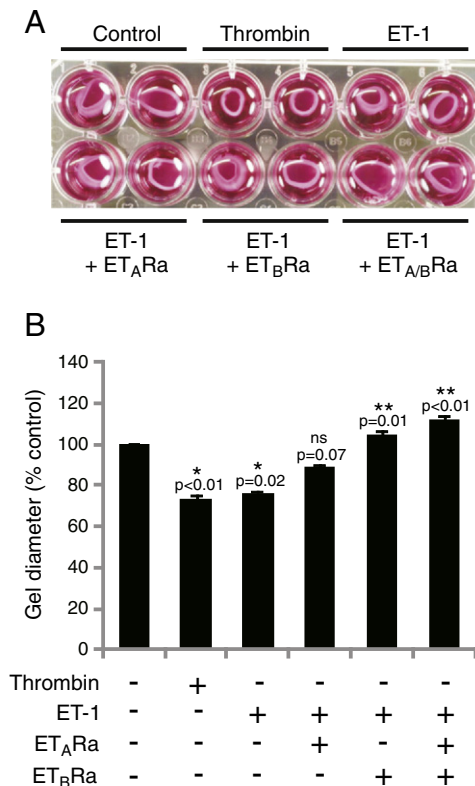
Fig. 4. ET-1 provokes oral fibroblast contraction. Oral fibroblasts were seeded into collagen as described in Materials and Methods. Following overnight incubation in serum free medium, oral fibroblasts were pretreated with ET_AR and/or ET_BR antagonists (BQ-123 and BQ-788, respectively) for 30 min before the addition of ET-1 or thrombin for 30 min. The gels were photographed (A; representative gels shown) and the distance contracted measured (B). Results are plotted relative to untreated control wells, with values representing the mean, ±SEM, of three independent experiments, carried out in duplicate. *$p < 0.05$ compared to untreated, **$p < 0.05$ compared to ET-1 treated, and ns $p > 0.05$ compared to ET-1 treated, as assessed by Mann Whitney *U*-test.
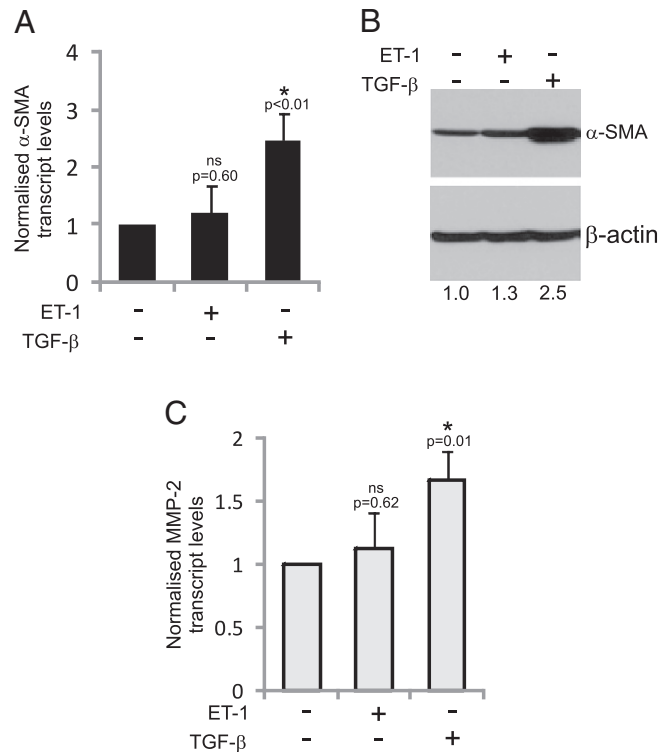
Fig. 5. ET-1 does not stimulate markers of myofibroblast transdifferentiation. Oral fibroblasts were treated with ET-1 or TGF-β for 48 h before cells were harvested and RNA extracted or lysates prepared as described in Materials and Methods. α-SMA (A) and MMP-2 (C) transcript levels were assessed by qPCR; values are shown normalised to U6 expression levels in the same samples. Results are plotted relative to untreated control wells, with values representing the mean, ±SEM, of three independent experiments, each carried out in triplicate. *$p < 0.05$, as assessed by Mann Whitney *U*-test. Cell lysates were separated by SDS-PAGE and immunoblotted for α-SMA and β-actin (as a loading control). A representative blot is shown (B), and the intensity of the band corresponding to α-SMA, determined by densitometry and normalised to β-actin levels in the same sample, is indicated under each lane.

transcript levels (Fig. 5C); ET-1, however, did not significantly increase the expression of either gene (Fig. 5 A–C).

Discussion

Cancers of the head and neck, predominantly oral squamous cell carcinoma, are the sixth most common malignancy worldwide. Survival rates remain stubbornly low, with surgery and radiotherapy the mainstay of treatment options. There exists, therefore, a pressing need for novel therapeutic strategies. It is becoming apparent that the tumour microenvironment may be a viable target for a new generation of therapeutic approaches (Joyce and Pollard, 2009). Cancer cells are surrounded by a stroma comprising extracellular matrix components, soluble proteins and peptides, and a number of different cell types, of which fibroblasts are the most numerous (Kalluri and Zeisberg, 2006). In the tumour microenvironment, fibroblasts frequently have an altered morphology and phenotype, resembling the myofibroblast phenotype found in post-insult tissue remodelling. These myofibroblasts (often termed cancer-associated fibroblasts) display increased proliferative and migratory capacity, and have a contractile phenotype. They exhibit over-expression of a number of genes such as the archetypal myofibroblast marker, α-smooth muscle actin, and matrix metalloproteinases (Kalluri and Zeisberg, 2006). In oral cancer, the presence of myofibroblasts in the tumour stroma is a strongly negative prognostic indicator, and myofibroblasts in culture were recently shown to increase invasion of cancer cells by a paracrine signalling mechanism (Marsh et al., 2011).

Although the source of cancer-associated fibroblasts remains controversial, there is a weight of evidence suggesting that conversion from 'normal' fibroblasts, provoked by factors present in the tumour microenvironment, is a key aetiological mechanism. A number of factors are known to promote transdifferentiation of fibroblasts into myofibroblasts, including TGF-β (Desmouliere et al., 1993) and peptides such as ET-1 (Leask, 2010). ET-1 plays a key role in the generation of myofibroblasts, and resulting fibrosis, in lung and cardiac disease and wound healing (Leask, 2010; Guo et al., 2011), but its role in modulating the behaviour of fibroblasts in the tumour microenvironment is not known. This study shows that ET-1, which is elevated in a number of malignancies including oral cancer, alters the phenotype of oral fibroblasts to provoke elevated invasion of oral cells in a paracrine manner. ET-1 treatment of fibroblasts increased the migration of primary human oral fibroblasts, congruent with previous findings examining the effect of ET-1 on rabbit, guinea pig and rat gingival fibroblasts (Ohsawa et al., 2005; Ohuchi et al., 2009, 2002), suggesting this function may be well conserved. ET-1 stimulated these effects in fibroblasts by acting through both of its receptors, ET_AR and ET_BR, in keeping with our previous findings (Hinsley et al., 2012). The stimulation of a contractile phenotype in oral fibroblasts by ET-1 appeared to be predominantly mediated by ET_BR, with antagonism of ET_AR only having a minor effect. Interestingly, this predilection for different receptors in invoking distinct phenotypic sequelae was also observed in colonic fibroblasts (Knowles et al., 2011). In this case, antagonism of ET_AR was sufficient to abrogate the effects of ET-1 on colonic fibroblast growth and proliferation, but ET_BR antagonism was necessary to block ET-1-mediated contraction.

The mechanism by which ET-1 stimulates phenotypic changes in oral fibroblasts remains unclear. In this study we found that, unlike TGFβ, a well characterised stimulant of myofibroblast transdifferentiation (Sobral et al., 2011), ET-1 did not significantly alter the expression of α-SMA and MMP-2, two 'markers' of myofibroblasts. In other physiological and pathophysiological settings, such as wound healing and lung fibrosis, it is well documented that ET-1 acts in concert with other factors such as TGF-β and angiotensin II to provoke myofibroblast transdifferentiation (Porter and Turner, 2009). Studies are ongoing to address whether such synergism exists in the oral cancer microenvironment, and to further elucidate the pathways by which ET-1 modulates the oral fibroblast phenotype.

Conclusion

This is the first study to elucidate a role for ET-1 in modulating stromal–epithelial interactions in oral cancer by influencing the phenotype of oral fibroblasts. These data may provide a platform from which new and existing pharmacological agents targeting the endothelin system in the tumour microenvironment could be utilised as a novel therapeutic strategy.

Conflict of interest

None.

Acknowledgments

References

Bogatkevich GS, Tourkina E, Silver RM, Ludwicka-Bradley A. Thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the proteolytically activated receptor-1 and a protein kinase C-dependent pathway. J Biol Chem 2001;276(48):45184–92.

Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 1993;122(1):103–11.

Guo F, Carter DE, Leask A. Mechanical tension increases CCN2/CTGF expression and proliferation in gingival fibroblasts via a TGFβ-dependent mechanism. PLoS One 2011;6(5):e19756.

Hearnden V, Lomas H, Macneil S, Thornhill M, Murdoch C, Lewis A, et al. Diffusion studies of nanometer polymersomes across tissue engineered human oral mucosa. Pharm Res 2009;26(7):1718–28.

Hinsley EE, Hunt S, Hunter KD, Whawell SA, Lambert DW. Endothelin-1 stimulates motility of head and neck squamous carcinoma cells by promoting stromal–epithelial interactions. Int J Cancer 2012;130(1):40–7.

Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer 2009;9(4):239–52.

Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006;6(5):392–401.

Kawanabe Y, Nauli SM. Endothelin. Cell Mol Life Sci 2010;68(2):195–203.

Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation. Oral Oncol 2008;44(5):509–17.

Knowles JP, Shi-Wen X, Haque SU, Bhalla A, Dashwood MR, Yang S, et al. Endothelin-1 stimulates colon cancer adjacent fibroblasts. Int J Cancer 2011;130:1264–72.

Lambert LA, Whyteside AR, Turner AJ, Usmani BA. Isoforms of endothelin-converting enzyme-1 (ECE-1) have opposing effects on prostate cancer cell invasion. Br J Cancer 2008;99(7):1114–20.

Leask A. Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. Circ Res 2010;106(11):1675–80.

Marsh D, Suchak K, Moutasim KA, Vallath S, Hopper C, Jerjes W, et al. Stromal features are predictive of disease mortality in oral cancer patients. J Pathol 2011;223(4):470–81.

Nambi P, Clozel M, Feuerstein G. Endothelin and heart failure. Heart Fail Rev 2001;6(4):335–40.

Nelson J, Bagnato A, Battistini B, Nisen P. The endothelin axis: emerging role in cancer. Nat Rev Cancer 2003;3(2):110–6.

Ohsawa M, Ohuchi N, Taniguchi Y, Kizawa Y, Koike K, Iwamoto K, et al. Inhibition of angiotensin II- and endothelin-1-stimulated proliferation by selective MEK inhibitor in cultured rabbit gingival fibroblasts. Fundam Clin Pharmacol 2005;19(6):677–85.

Ohuchi N, Koike K, Sano M, Kusama T, Kizawa Y, Hayashi K, et al. Proliferative effects of angiotensin II and endothelin-1 on guinea pig gingival fibroblast cells in culture. Comp Biochem Physiol C Toxicol Pharmacol 2002;132(4):451–60.

Ohuchi N, Hayashi K, Iwamoto K, Koike K, Kizawa Y, Nukaga M, et al. Thrombin-stimulated proliferation is mediated by endothelin-1 in cultured rat gingival fibroblasts. Fundam Clin Pharmacol 2009;24(4):501–8.

Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. Exp Cell Res 2010;316(8):1324–31.

Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther 2009;123(2):255–78.

Rosano L, Spinella F, Di Castro V, Nicotra MR, Dedhar S, de Herreros AG, et al. Endothelin-1 promotes epithelial-to-mesenchymal transition in human ovarian cancer cells. Cancer Res 2005;65(24):11649–57.

Sobral LM, Zecchin KG, Nascimento de Aquino S, Lopes MA, Graner E, Coletta RD. Isolation and characterization of myofibroblast cell lines from oral squamous cell carcinoma. Oncol Rep 2011;25(4):1013–20.

Teder P, Noble PW. A cytokine reborn? Endothelin-1 in pulmonary inflammation and fibrosis. Am J Respir Cell Mol Biol 2000;23(1):7-10.