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TGF-β isoforms fail to modulate inositol phosphates and cAMP in normal and tumour-derived human oral keratinocytes

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Abstract

This study examined inositol phosphate and cAMP regulation by TGF-β1, -β2 and -β3 in normal and tumour-derived human oral keratinocytes. Previous findings indicated that the cell lines expressed TGF-β cell surface receptors and had a range of response to exogenous TGF-β1, -β2 and -β3 from being refractory to the ligand to marked inhibition. Basal levels of inositol phosphates broadly reflected the differentiation status of the cells as demonstrated by involucrin expression, but did not correlate with responsiveness to TGF-β1, as measured previously by thymidine incorporation. Treatment of cells with bradykinin or serum caused up-regulation of inositol phosphate levels; by contrast, TGF-β1, -β2 and -β3 failed to modulate inositol phosphates. In two tumour-derived cell lines, the TGF-β isoforms had no effect on cAMP levels, despite a significant increase in cAMP using a potent agonist of adenylate cyclase (forskolin). Furthermore, the cAMP analogue, dibutyryl cAMP, failed to mimic the inhibitory or refractory responses of TGF-β in these cell lines. The results demonstrate that in normal and tumour-derived human oral keratinocytes, TGF-β signal transduction is not mediated by inositol phosphates or cAMP.

Keywords: Inositol phosphate; cyclic AMP; Keratinocyte; Transforming growth factor-β; Isoform

1. Introduction

The human TGF-β family of growth factors (TGF-β1, -β2 and -β3) are highly conserved, ubiquitous peptides that exhibit a remarkable diversity of biological action [1]. TGF-β signal transduction involves serine-threonine kinase activity on types I and II TGF-β cell surface receptors [2,3] and, whilst the nuclear response to TGF-β is under intense scrutiny at the present time, very little is known about events distal to ligand-receptor interaction and, in particular, what second messenger pathways are activated by TGF-β.

There is some evidence to indicate that TGF-β activates p21 *ras* [4] and this, in turn, is thought to lead to mitogen-activated protein kinase (MAPK) activation [5]. *Ras*-independent TGF-β-mediated responses have also been identified [5]. Further, gene expression elicited by TGF-β treatment is sensitive to pertussis toxin, implying a role for G-proteins [6–10], adenylate cyclase [11] and, possibly, phospholipases [12,13]. G-protein activation is also closely linked to the regulation of cAMP; TGF-β1 counteracts the effects of cAMP analogues whilst having no effect on ligand-mediated increases in cAMP concentration [14,15]. In addition, protein kinase C (PKC) is translocated from the cytosol to the cell membrane following treatment of cells with TGF-β1 [16] but whether this is associated with ligand-induced PKC activation via changes in phosphoinositide metabolism is currently unknown.

Studies to examine TGF-β-induced second messenger pathways have used cells of different species, cells of both epithelial and fibroblast origin which are inhibited and stimulated by TGF-β, respectively, and genetically-manipulated cell lines. There are few data comparing the effects of the different TGF-β isoforms (-β1, -β2 and -β3). We have available a number of human tumour-derived oral keratinocyte cell lines, together with normal oral keratinocytes, in which the response to TGF-β isoforms is variable and in which types I, II and III TGF-β receptors are expressed [17]. If specific second messenger pathways

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are involved in TGF-β signal transduction, it might be anticipated that these parameters would change in cells having a range of response to the ligand.

The purpose of this study, therefore, was to examine inositol phosphate metabolism and cAMP production in normal and tumour-derived human oral keratinocytes following treatment with TGF-β1, -β2 and -β3.

2. Materials and methods

2.1. Cell culture

Normal human keratinocytes and untreated primary oral squamous cell carcinomas were cultured as described previously [18]. Briefly, cells were maintained in culture media (DMEM/Ham's F12 Nut mix, 1:1; Gibco) supplemented with 10% (v/v) foetal calf serum (FCS), 0.6 μg/ml L-glutamine, 0.5 mg/ml $NaHCO_3$ and 0.5 μg/ml hydrocortisone at 37°C in 95% air/5% CO_2. Normal keratinocytes were cultured in the presence of 3T3 fibroblast support; fibroblasts were selectively removed prior to inositol phosphate and cAMP assays.

Normal and tumour-derived keratinocytes express variable proportions of types I, II and III TGF-β cell surface receptors, demonstrate a range of response to exogenous TGF-β1, from being refractory to the ligand (H314) to marked inhibition, and express variable amounts of involucrin (Table 1) [17,19].

2.2. [^{3}H]Inositol phosphate assay

Cells were seeded into 35-mm culture dishes (2×10^5 cells/dish) and grown for 5–7 days in culture medium containing 10% (v/v) FCS until 70–80% confluent. 24 h prior to the assay, cells were labelled with [^{3}H]*myo*-inositol (10 μCi/ml; 17 Ci/mM; Amersham) in inositol-free, serum-free culture medium (assay medium; Gibco). Following labelling, the cells were washed ($\times$2) in DMEM and equilibrated at 37°C for 30 min in fresh assay medium containing 10 mM LiCl before the addition of either recombinant TGF-β1, TGF-β2, TGF-β3 (1 ng/ml), serum (10% v/v) or bradykinin (10nM). The assay was terminated after 30 min by the replacement of the assay medium with ice-cold perchloric acid (18%, w/v) and then the addition of 100 μl EDTA (100 mM, pH 8). Cells were fixed on ice for 30 min prior to the addition of 1 ml tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane (1:1v/v). The mixture was transferred to capped, 2-ml polypropylene tubes and centrifuged at $12\,000 \times g$ for 2 min. The aqueous phases were loaded onto a Dowex 1-X8 (formate form) ion exchange column (750 μl bed volume; Biorad) and after washing (5×5 ml) with water, the total inositol phosphates (IP1, IP2, IP3, IP4) and glycerophosphoinositides (GroPIns) were collected by sequential elution with 4×2 ml washes of the following solutions; (GroPins: 5 mM sodium tetraborate, 60 mM sodium formate; IP1: 200 mM ammonium formate, 100 mM formic acid; IP2: 400 mM ammonium formate, 100 mM formic acid; IP3: 700 mM ammonium formate, 100 mM formic acid; IP4: 1 M ammonium formate, 100 mM formic acid). An equal volume (8 ml) of Quicksafe A scintillant (Zinsser Analytic) was added to the fractions which were then counted by liquid scintillation [20]. Preliminary studies indicated that following a 30-min incubation with serum, only IP1 levels were elevated. The IP1-IP4 data therefore, were pooled to give total inositol phosphate production over a 30-min period.

2.3. Cyclic AMP production

Cells were seeded into 35-mm culture dishes (2×10^5 cells/dish) and grown for 5–7 days in culture medium containing 10% (v/v) FCS until 70–80% confluent. Culture media was replaced with 1 ml Biggers BGJ medium (Fitton-Jackson modification; Sigma) supplemented with $NaHCO_3$ (0.5 mg/ml) and equilibrated at 37°C for 30 min. 10 μl 3-isobutyl-1-methylxanthine (IBMX; 100 mM in ethanol) was then added and the dishes left for a further 5 min. TGF-β1, TGF-β2, TGF-β3 (10 ng/ml; R&D) or forskolin (1 μM; Sigma) was added to the medium and the cells incubated at 37°C for 5 min. The reaction was terminated by the addition of 1 ml ice-cold acidified ethanol (200 mM HCl in 100% ethanol) and the plates left overnight at −20°C. The supernatants were lyophilized and the samples stored at −20°C.

Cyclic AMP production was determined by a competitive binding assay [21]. Standard concentrations of unlabelled cAMP (0.125–16 pmol; Sigma) and lyophilized samples were prepared in 50 μl assay buffer (50 mM Tris-HCl, 4 mM EDTA; pH 7.5). 50 ml [^{3}H]cAMP (0.5 μCi/ml; Amersham) and 100 μl diluted cAMP binding protein [21] were added to the samples and standards and incubated on ice for 2 h. 100 μl of charcoal mixture (0.5% w/v BSA; 4% w/v Norit GSX charcoal) was then added and the solutions briefly vortexed. Samples and standards were centrifuged at $12\,000 \times g$ for 2 min. 200-μl aliquots of supernatant were collected and counted in 4 ml Optiphase 'Safe' scintillant (LKB) in a β-scintillation counter.

2.4. [^{3}H]Thymidine incorporation following treatment with dibutyryl cAMP

Assays of [^{3}H]thymidine incorporation following treatment of cells with mitogens have been reported previously [22]; similar techniques were used to examine the effect of dibutyryl cAMP. Cells were seeded into 24-well plates (5×10^4 cells/well) and grown for 5–7 days in culture medium containing 10% (v/v) FCS until 70–80% confluent. Culture media was replaced with serum-free culture medium (assay medium) with or without the addition of dibutyryl cAMP (1 mM). The cells were then incubated for 24 h prior to assay.

2.5. Statistics

Mean and standard deviation values were compared using the two-sample t-test with a probability (P) of < 0.05 taken as being statistically significant.

3. Results

3.1. [^{3}H]-labelled inositol phosphate release

Fig. 1A shows the regulation of total inositol phosphate levels following treatment of normal and tumour-derived

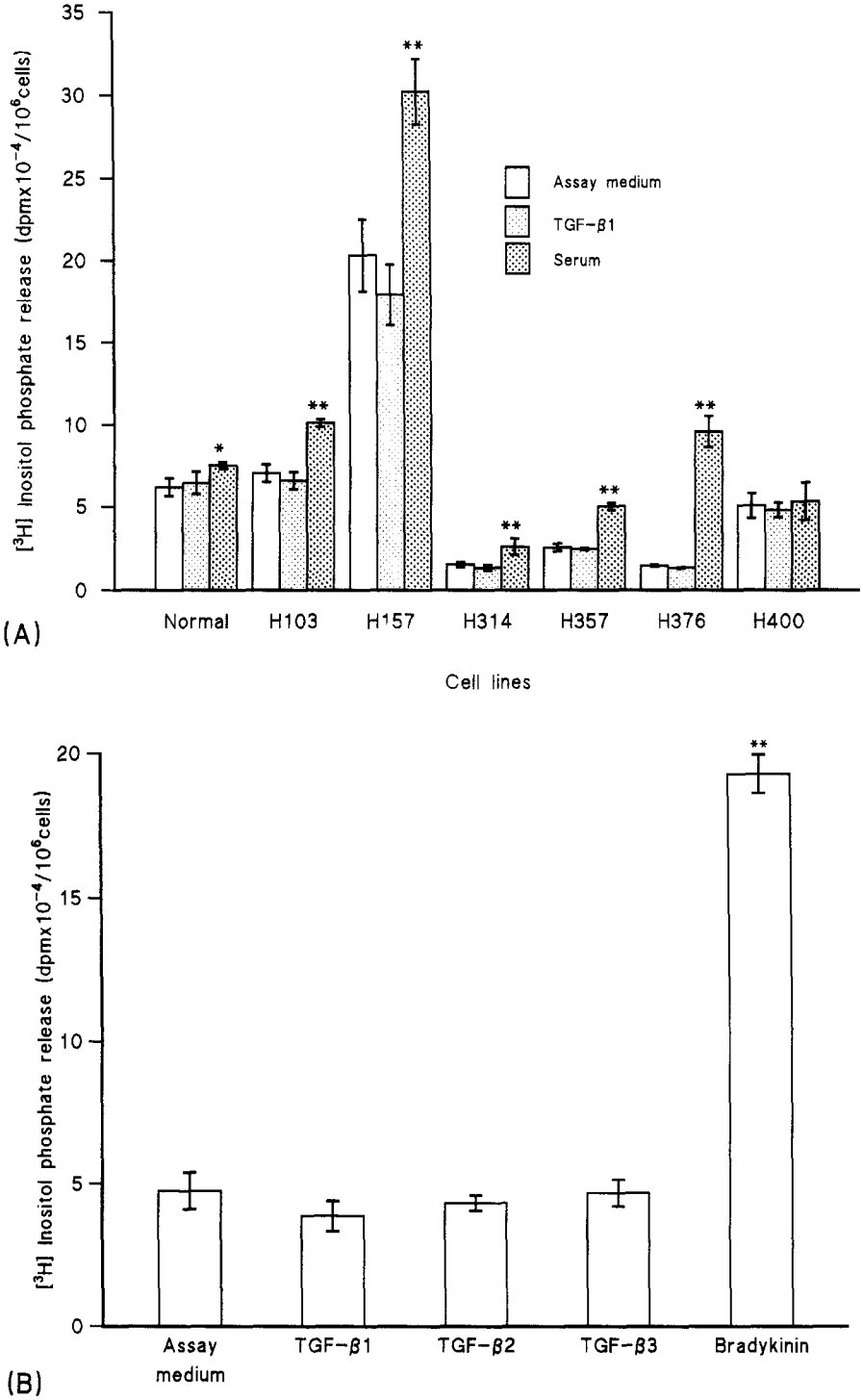

Fig. 1. [^{3}H]Inositol phosphate production. (A) Normal and tumour-derived human oral keratinocytes were incubated with TGF-β1 (1 ng/ml) and a positive control (10% v/v serum). (B) H357 was incubated with TGF-β1, -β2, -β3 (1 ng/ml) and a positive control (10 nM bradykinin). [^{3}H]inositol phosphate production was expressed per 10^6 cells; cells were counted in parallel culture dishes. Results are the means and standard deviations of triplicate determinations. Statistical correlations were made against the assay medium control. * $P < 0.05$, ** $P < 0.01$.

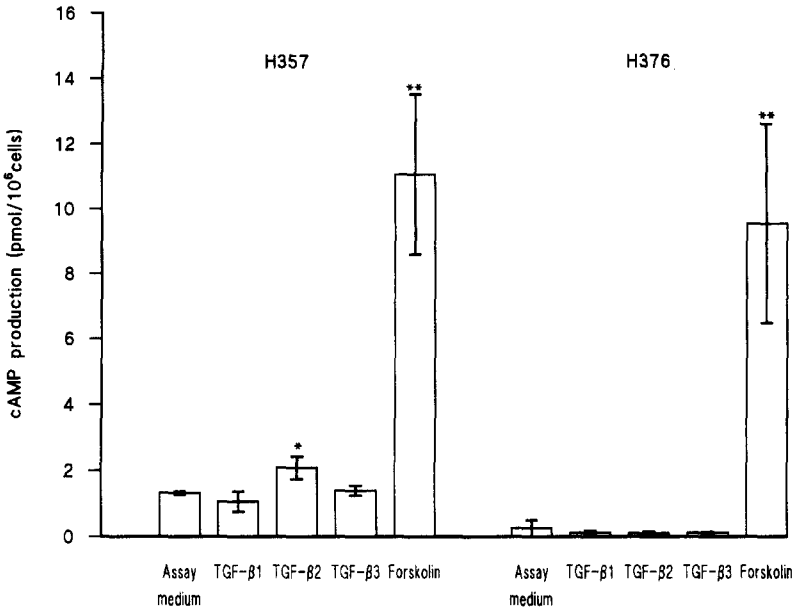

Fig. 2. cAMP release in H357 and H376 following treatment with TGF-β1, -β2, -β3 (1 ng/ml) or forskolin (10 mM). cAMP was expressed per 10^6 cells; cells were counted in parallel culture dishes. Results are the means and standard deviations of triplicate determinations. Statistical correlations were made against the assay medium control. * $P < 0.05$, ** $P < 0.01$.

cell lines with TGF-β1 or foetal calf serum (10%). There was a significant increase in [^{3}H]inositol phosphates after incubation with 10% serum (H357, H376 > H103, H157 > normal, H314 > H400), but there was no significant increase in [^{3}H]inositol phosphate production after TGF-β1 treatment. The baseline turnover of inositol phosphates (H157 > Normal, H103 > H314, H357, H376, H400) broadly parallel the expression of involucrin, but not TGF-β1-mediated thymidine incorporation ($r = -0.404$), these cell lines (Table 1).

As with TGF-β1, TGF-β2 and TGF-β3 failed to regulate [^{3}H]inositol phosphate production in H357 (Fig. 1B), despite evidence that this pathway was intact following stimulation with bradykinin (a potent agonist of PI-PLC).

Table 1
Characteristics of the normal and tumour-derived human oral keratinocyte cell lines

Cell line	% Inhibition to TGF-β1 [1]	TGF-β Receptor Type [2]			Involucrin expression [3]
		I	II	III	
Normal	76	32.1	6.9	61.0	+++
H103	33	20.3	17.9	61.8	++
H157	18	26.0	9.9	64.1	++
H314	R	13.0	5.8	81.2	0
H357	86	25.6	13.9	60.5	+
H376	90	54.0	2.0	44.0	0/+
H400	88	73.7	16.7	9.6	0

[1] The effect of TGF-β1 (0.1 ng/ml for 24 h) on the normal and tumour-derived human oral keratinocyte cell lines has been reported previously [17]. H357 was inhibited by TGF-β2 and TGF-β3 by 43% and 18%, respectively, whilst H376 was inhibited by TGF-β2 and TGF-β3 by 21% and 15%, respectively (unpublished observations). Values are expressed as a percentage of control (absence of ligand). R, refractory to ligand.

[2] The expression of types I, II and III TGF-β receptors has been examined previously using affinity cross-linking techniques. Autoradiographs were analysed with an integrated densitometer and the receptor type was expressed as a percentage of the total area of the scanning trace on an interactive image analyser [17].

[3] The expression of involucrin has been examined previously by Western blot analysis [19].

Marked increased (+++), increased (++), minimal (+) and absence (0) of involucrin expression.

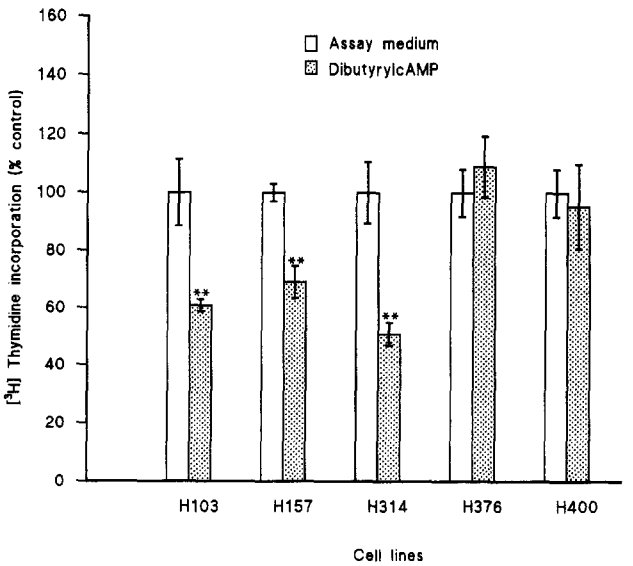

Fig. 3. [^{3}H]Thymidine incorporation following treatment with dibutyryl cyclic AMP (1 mM). Results are expressed as a percentage of untreated controls and are the means and standard deviations of quadruplicate determinations. Statistical correlations were made against the assay medium control. * $P < 0.05$, ** $P < 0.01$.

3.2. cAMP production

Fig. 2 shows the amount of cAMP produced by two tumour-derived human oral keratinocyte cell lines, H357 and H376, following treatment with TGF-β1, TGF-β2 and TGF-β3 or forskolin (a potent activator of adenylate cyclase). The TGF-β isoforms did not modulate intracellular cAMP in either cell line despite the control (forskolin) indicating that this pathway was intact.

Fig. 3 shows the effect of dibutyryl cAMP on thymidine incorporation in tumour-derived keratinocyte cell lines in the absence of TGF-β. The cAMP analogue inhibited (H103, H157, H314) or had no effect (H376, H400) on thymidine incorporation in these cell lines.

4. Discussion

Recent data indicate that murine keratinocyte differentiation is associated with an increase in intracellular calcium and stimulation of inositol phospholipid metabolism [23]. The results of the present study support these findings because baseline levels of inositol phosphates broadly correlated with the amount of involucrin, a marker of terminal keratinocyte differentiation, previously demonstrated in extracts of the cell lines (Table 1) [19]. Normal keratinocytes, H103 and H157 contained the highest level of involucrin, whilst H314, H357, H376 and H400 expressed considerably lower levels, results that reflect the degree of tumour cell differentiation and which parallel the distribution of basal inositol phosphates in the present study. This generalization, however, should be treated with caution because it might have been expected that normal keratinocytes, which express the most involucrin, would also have the highest unstimulated levels of total inositol phosphates. In fact, H157 produced the greatest levels of unstimulated inositol phosphates in this study.

Signal transducing systems associated with TGF-β have largely remained an enigma. In the present study, there was no relationship between the basal levels of total inositol phosphates and the previously reported growth inhibitory response to TGF-β1 in these cell lines [17]. In addition, TGF-β isoforms did not change the levels of inositol phosphates in both normal and tumour-derived human oral keratinocytes. Our results reflected an acute response to TGF-β. Others [12,13] have observed changes in inositol phosphates in response to TGF-β over longer periods of time which may result from non-specific changes. The results of the present study support previous observations in TGF-β inhibited Chinese hamster lung fibroblasts [24], but contrast to findings in TGF-β stimulated rat-1 cells [12]. Recent data indicates that TGF-β1 growth inhibition is mediated by cyclin-dependent kinases and their inhibitors [25]. Furthermore, there is an increasing volume of evidence to show that the control of cellular proliferation and extracellular matrix elaboration by TGF-β1 occur by distinct signal transduction pathways [26]. The results of the present study suggest that neither the inhibition of cell growth or the elaboration of extracellular matrices by TGF-β1 are mediated by changes in inositol phosphate metabolism in human keratinocytes. This suggests that the correlation between basal levels of inositol phosphates and involucrin expression, observed in this study, is independent of TGF-β1. Further, the results of the present study demonstrated no obvious differences between normal and tumour-derived cells, suggesting that the inositol phosphate signal transduction pathways are not compromised by tumour-development.

There is increasing evidence that TGF-β signal transduction is mediated through G-proteins [6–10] and one component of the prototypic G-protein signal transduction pathway is cAMP. The results of the present study, however, indicated that TGF-β1, -β2 and -β3 did not modulate cAMP levels in two tumour-derived human oral keratinocyte cell lines. Similar observations have been reported previously in rat fibroblasts and human thyroid epithelial cells [14,15]. Further confirmation that cAMP is not involved in TGF-β signal transduction in keratinocytes is demonstrated by the cAMP analogue studies. Dibutyryl cAMP, for example, inhibited (H103, H157) or had no effect (H376, H400) on thymidine incorporation in TGF-β-responsive cell lines. In addition, thymidine incorporation was down-regulated by dibutyryl cAMP in a TGF-β unresponsive cell line (H314). Vivien et al. [27], however, demonstrated that a TGF-β-induced G2/M delay in rabbit articular chondrocytes was associated with a decrease in cAMP, whilst Anderson et al. [11] showed that TGF-β1 stimulated adenylate cyclase activity and the cAMP response element binding protein (CREB) in renal epithelial cells. When the inositol phosphate and cAMP data are taken together, the results emphasize the importance of defining cell origin, together with the nature of the mitogenic response to TGF-β, before interpreting ligand induced second messenger systems.

Adenylate cyclase activity is tightly regulated by inhibitory (Gi) and stimulatory (Gs) guanine nucleotide binding proteins. Lack of cAMP elevation in response to TGF-β suggests Gs is unlikely to be activated but a role for Gi cannot be excluded. Indeed, receptors which couple to Gi are known to mediate *ras*-activation and subsequent signalling via MAP kinase [28]. It is currently perceived that both these factors are involved in TGF-β signal transduction [4,5]. Studies are currently underway to examine the role of Gs and Gi in TGF-β signal transduction in our keratinocyte cell lines.

In conclusion, the results of this study indicate that TGF-β signal transduction is not mediated by either inositol phosphates or cAMP in normal and tumour-derived human oral keratinocytes. The diverse biological effects of TGF-β, therefore, are likely to be mediated by multiple signal transduction pathways.

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