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Original Article

The distribution of epidermal growth factor binding sites in the developing mouse palate

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ABSTRACT The distribution of epidermal growth factor (EGF) receptors in the developing mouse palate was mapped using ^{125}I-EGF labeling of paired palate organ cultures. ^{125}I-EGF binding sites were localized throughout the palate mesenchyme except in a region immediately adjacent to the midline seam. The EGF receptor was detected in all palatal epithelia at the beginning of culture, but as seam formation and subsequent degeneration took place it was down-regulated in the medial edge epithelia. Using submerged culture the mechanism of this down-regulation was investigated by treating with various growth factors such as EGF, basic fibroblast growth factor (bFGF), insulin-like growth factor-II (IGF-II) and transforming growth factors alpha and beta (TGF-α, TGF-ß). Conventional Trowell organ culture was not used because it was observed that the Millipore filter blocked growth factor uptake. All three TGF-ß isoforms accelerated palate fusion and TGF-$ß_1$ reduced ^{125}I-EGF binding throughout the palate, suggesting a potential level of regulation during palatogenesis. Contrary to previous reports, EGF treatment in the absence of serum prevented palatal shelf fusion, and also down-regulated subsequent ^{125}I-EGF binding.

KEY WORDS: *EGF, palate development*

Introduction

The mammalian secondary palate initially develops as bilateral intraoral vertical shelves, growing out from the maxillary processes. Subsequent palate development involves shelf elevation, contact and adhesion of the medial edge epithelia to form a midline seam. Breakdown of this seam to create an intact palate then follows (Ferguson, 1988; Carette and Ferguson, 1992). The formation and later degeneration of the midline epithelial seam is important in normal palatogenesis and has been the subject of much research. Differentiation at this stage of palate formation is controlled by complex epithelial-mesenchymal interactions (Sharpe and Ferguson, 1988), as in many other developing systems. Growth factors, extracellular matrix molecules and their receptors form part of the signalling cascade responsible for mediating such interactions. In particular, the role of epidermal growth factor (EGF) during mammalian secondary palate development has been studied extensively.

Programmed cell death (apoptosis) was originally thought to be the mechanism responsible for degeneration of the palatal midline epithelial seam. EGF added to palatal shelves cultured *in vitro* apparently prevented medial edge epithelial cell death and instead enhanced epithelial cell division and keratinization (Hassell, 1975; Hassell and Pratt, 1977; Tyler and Pratt, 1980; Abbott and Pratt, 1987). The putative embryonic homologue of EGF, transforming growth factor-α (TGF-α) binds to the EGF receptor and mimics the effects of EGF (Lee and Han, 1990). If EGF or TGF-α were physiologically responsible for prevention of seam degeneration by inhibiting cell death, then levels of these growth factors would be expected to decline during normal palatogenesis. However, it is now clear that seam degeneration is more complex than programmed epithelial cell death. The medial edge epithelia have been shown to undergo transformation to mesenchymal cells, with only a small proportion of them dying (Fitchett and Hay, 1989). Epithelial cell migration from the midline seam, either into the mesenchyme (Shuler *et al.*, 1991, 1992; Griffith and Hay, 1992) or into the oral and nasal surfaces of the palate (Carette and Ferguson, 1992), has been shown to be a further mechanism for seam degeneration. It may be that breakdown of the midline epithelial seam involves a combination of all three mechanisms. In the light of these new findings the previously ascribed role of EGF or TGF-α in palatogenesis must be questioned.

Previous work has focussed on elucidating the distribution of EGF or TGF-α peptides, and their receptor, using immunocytochemistry (Abbott and Birnbaum, 1990; Shiota *et al.*, 1990; Dixon *et al.*, 1991). Autoradiography has also been used to localize the sites of ^{125}I-EGF binding in the palate (Abbott *et al.*, 1988).

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IGF-II, insulin-like growth factor-II; TGF-α, transforming growth factor-α; TGF-ß, transforming growth factor-ß.

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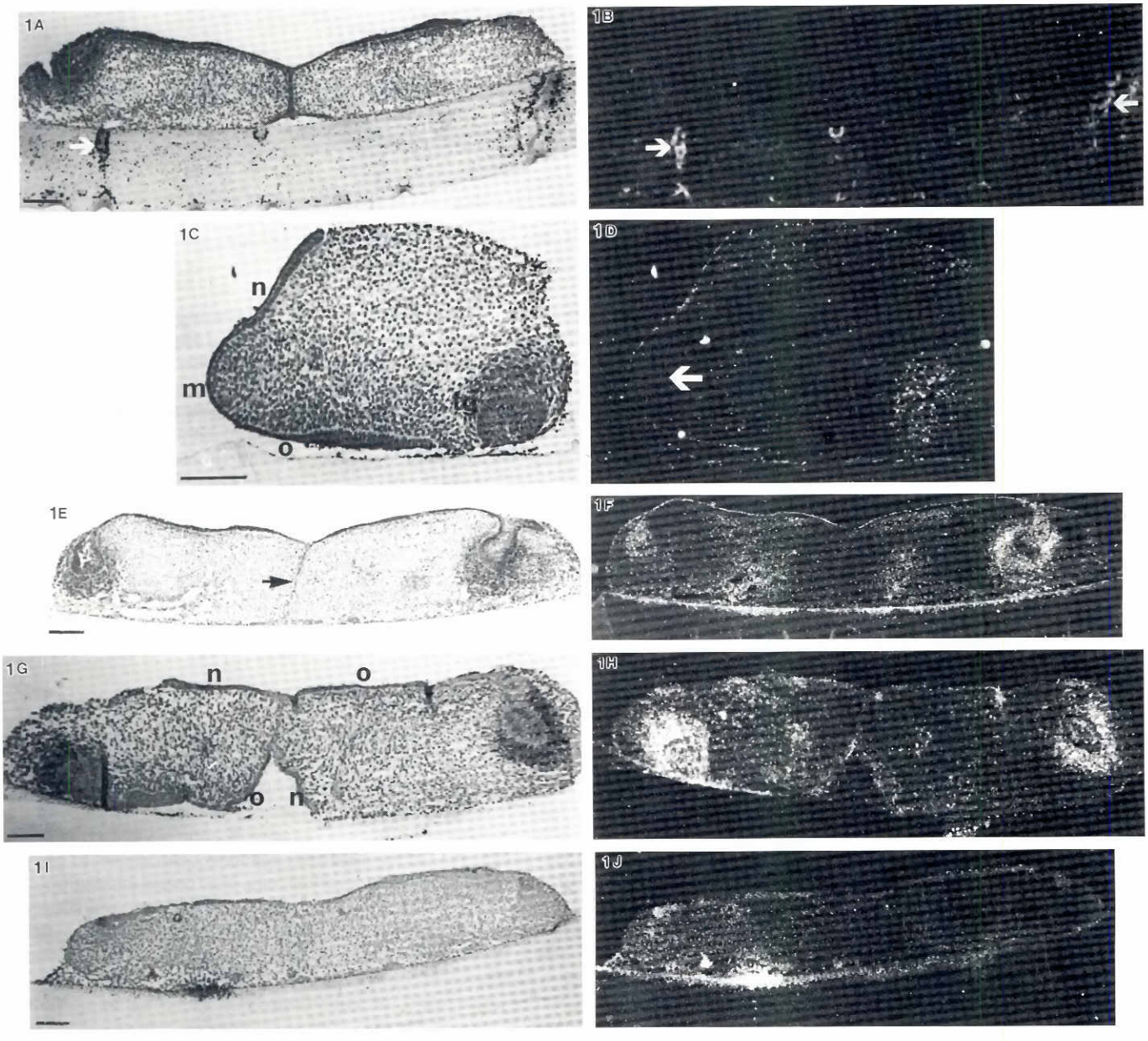

Fig. 1. The distribution of ^{125}I-EGF binding sites in the embryonic murine palate. (A, B). *Light and dark field photographs of a control submerged culture taken at 24 h and incubated in binding medium containing ^{125}I-EGF and a 500-fold excess of unlabeled EGF. No palate tissue was labeled. Note non-specific labeling of Millipore filter (arrowed). Scale bar, 50 μm.* **(C, D)** *Light and dark field photographs of a time 0 submerged culture labeled with ^{125}I-EGF. Note labeling of oral (o), nasal (n) and medial edge (m) palatal epithelia and toothgerm (tg). There was a region of palate mesenchyme immediately adjacent to the medial edge epithelia which was unlabeled (arrowed). Scale bar, 50 μm.* **(E, F)** *Light and dark field photographs of a 48 h submerged culture labeled with ^{125}I-EGF. The medial edge epithelia have now approximated and formed a midline seam. The absence of silver grains adjacent to this seam was now more obvious. The oral epithelia and toothgerm were still labeled. Labeling of the medial edge epithelia was lighter than at time 0. Note the trapping of ^{125}I-EGF at the tissue-filter interface. Scale bar, 50 μm.* **(G, H)** *Light and dark field photographs of a 48 h submerged culture with one palatal shelf oral surface uppermost as usual (o) and the other with its nasal surface uppermost (n). This confirmed that the nasal epithelium does label with ^{125}I-EGF. Scale bar, 50 μm.* **(I, J)** *Light and dark field photographs of a 72 h submerged culture labeled with ^{125}I-EGF. The midline seam has now degenerated. Labeling of the nasal and oral epithelia persisted. There was now labeling throughout the palate mesenchyme including the region of the former seam. Scale bar, 50 μm*

However, there are certain discrepancies in the reports of the precise temporo-spatial distribution of this receptor molecule throughout palatogenesis. Whereas Abbott *et al.* (1988) and Shiota *et al.* (1990) observe a region of mesenchyme adjacent to the midline seam where EGF receptors are not expressed, Dixon *et al.* (1991) do not.

If the EGF receptor and its ligands play an important role in palatogenesis, their expression is likely to be tightly regulated. Little is known about how such regulation is mediated. Previous research has shown that palate mesenchymal cells express the EGF receptor (Nexo *et al.*, 1980; Yoneda and Pratt, 1981; Kukita *et al.*, 1987) and that various other polypeptide growth factors, for example bFGF, IGF-II, and TGF-ß, alter the levels of EGF receptor expression in cell culture (Sharpe *et al.*, 1992).

In this study we have extended these cell culture experiments to elucidate whether the same regulatory mechanisms occur in palate organ culture. We have further investigated the pattern of EGF receptor expression during palate development by utilizing autoradiography to reveal sites of ^{125}I-EGF binding in paired cultures. Autoradiography was chosen in preference to immunocytochemistry. The reported inconsistencies in receptor distribution mentioned earlier may have been caused by non-recognition of the specific antibody-epitope due to masking. By contrast, autoradiography reveals all the EGF receptors capable of binding to their ligand. Many previous studies have used single palatal shelves cultured in isolation (e.g. Gehris and Greene, 1992). In the light of the new findings about medial edge epithelial cell migration, single palatal cultures may not represent normal palate development, as no palatal shelf fusion can take place. Therefore we have used a paired palate culture system in which seam formation and subsequent degeneration can be observed.

Results

Distribution of ^{125}I-EGF binding at different developmental stages

Control cultures, those incubated in binding medium containing ^{125}I-EGF and a 500-fold excess of unlabeled EGF, revealed no silver labeling at any time point above background levels (Fig. 1A,B). This demonstrated that the presence of an excess of unlabeled EGF blocked the specific binding of the radiolabeled ligand.

At time 0 no seam formation had taken place (Fig. 1C,D). Autoradiography revealed silver grains throughout the palate mesenchyme with the notable exception of the mesenchyme immediately adjacent to the medial edge epithelia. Silver grains were particularly concentrated in the bud-stage toothgerm (Fig. 1D). Heavy labeling was present in the stellate reticulum of the enamel organ, but not in the external or internal enamel epithelium. The dental papilla mesenchyme labeled heavily. Labeling was also present in the entire palatal epithelia (nasal, medial and oral).

After 48 h of culture, the medial edge epithelia of the palatal shelves had fused to form a seam (Fig. 1E,F). The pattern of labeling in the palatal mesenchyme was similar to that seen at time 0, but the absence of silver grains in the mesenchyme adjacent to the medial edge epithelia was more obvious. The medial edge epithelia also labeled, but to a lesser extent than at time 0. The oral epithelia were labeled intensely on their surface. The nasal epithelia also labeled, but such localization was difficult to determine due to non-specific concentration of the ligand at the filter/tissue interface (Fig 1F). Combining palatal shelves in culture with one shelf oral surface uppermost as usual and the other nasal surface uppermost confirmed that there was specific labeling of the nasal epithelia (Fig. 1G,H). The density of EGF binding sites on oral and nasal epithelia were similar. Dental tissues labeled as at time 0, except that the dental follicle, which had now differentiated, also labeled heavily. After 72 h in culture, the epithelial seam had broken down and there was mesenchymal continuity across the palate (Fig. 1I). Labeling throughout the mesenchyme was uniform. EGF binding sites were seen in the region of the degenerated seam. Labeling of the oral and nasal epithelia persisted (Fig. 1J).

Growth factor treatment in Trowell organ culture system: suitability of use?

Addition of TGF-α (10 ng/ml) or EGF (20 ng/ml) to the Trowell culture system did not alter EGF binding sites, as revealed by autoradiography (Fig. 2C,D). Such treatment also had no effect on palatal shelf fusion (Fig. 3C,D). This was an unexpected result as previous studies report that treatment with EGF prevented normal seam formation. This phenomenon was therefore investigated further. It was hypothesized that the absence of any response to these two growth factors was due to their inability to penetrate the filter.

Evidence that the Millipore filter blocked the passage of growth factors from the culture medium into the tissue included;

1) Addition of ^{125}I-EGF to the Trowell organ culture medium. ^{125}I-EGF was added to the culture medium in the Trowell system and culture continued for up to 24 h. Palatal cultures were removed at various time points (90 min, 2, 3, 6, 12, 24 h). The tissue was washed, fixed and sectioned, as described for autoradiographic studies, and the distribution of ^{125}I-EGF determined. Palatal tissues were unlabeled at all time points up to and including 24 h, there was some silver labeling of the filter (Fig. 2G,H), implying that the ^{125}I-EGF was unable to penetrate it. Addition of ^{125}I-EGF to the submerged culture medium resulted in an identical distribution of silver labeling to that described earlier including the labeling deficit adjacent to the midline epithelial seam (Fig. 2E,F). In contrast to previous reports which state that serum is required at fairly high concentration in order for EGF to block palatal fusion (Dixon and Ferguson, 1992), treatment of submerged cultures with EGF alone prevented normal seam formation (Fig. 3A,B).

2) Treatment of palatal shelves in Trowell organ culture with EGF (20 ng/ml) and serum did prevent fusion (Fig. 3G,H). This could be due to components of the serum saturating binding sites in the filter, allowing access of EGF through to the tissue, or it may be that EGF is more active in the presence of other factors. However, the use of serum in experimental procedures prevents investigation of the effects of growth factors in isolation or in specific combinations.

3) Addition of EGF or TGF-α to day 13 palates in Trowell organ culture did not result in an increase in glycosaminoglycan synthesis (data not shown). An increase was seen in palates at this stage when a submerged organ culture system was used (Foreman *et al.*, 1991).

These data suggested that the Trowell organ culture system was unsuitable for the investigation of the effects of added growth factors due to poor penetration of the Millipore filter. In consequence, all further experiments utilized the submerged organ culture technique.

Growth factor regulation of EGF binding

In submerged culture treatment of palates with TGF-α or EGF blocked the fusion process. Silver particle localization following ^{125}I-EGF labeling of these treated cultures was reduced to background levels (Fig. 2A,B). Treatment of the submerged cultures with various other growth factors was investigated by both routine histology to analyze their effects on palatal seam fusion and by ^{125}I-EGF labeling and autoradiography to determine any changes to the pattern of EGF binding sites. Addition of IGF-II (100 ng/ml) or bFGF (1 ng/ml) had

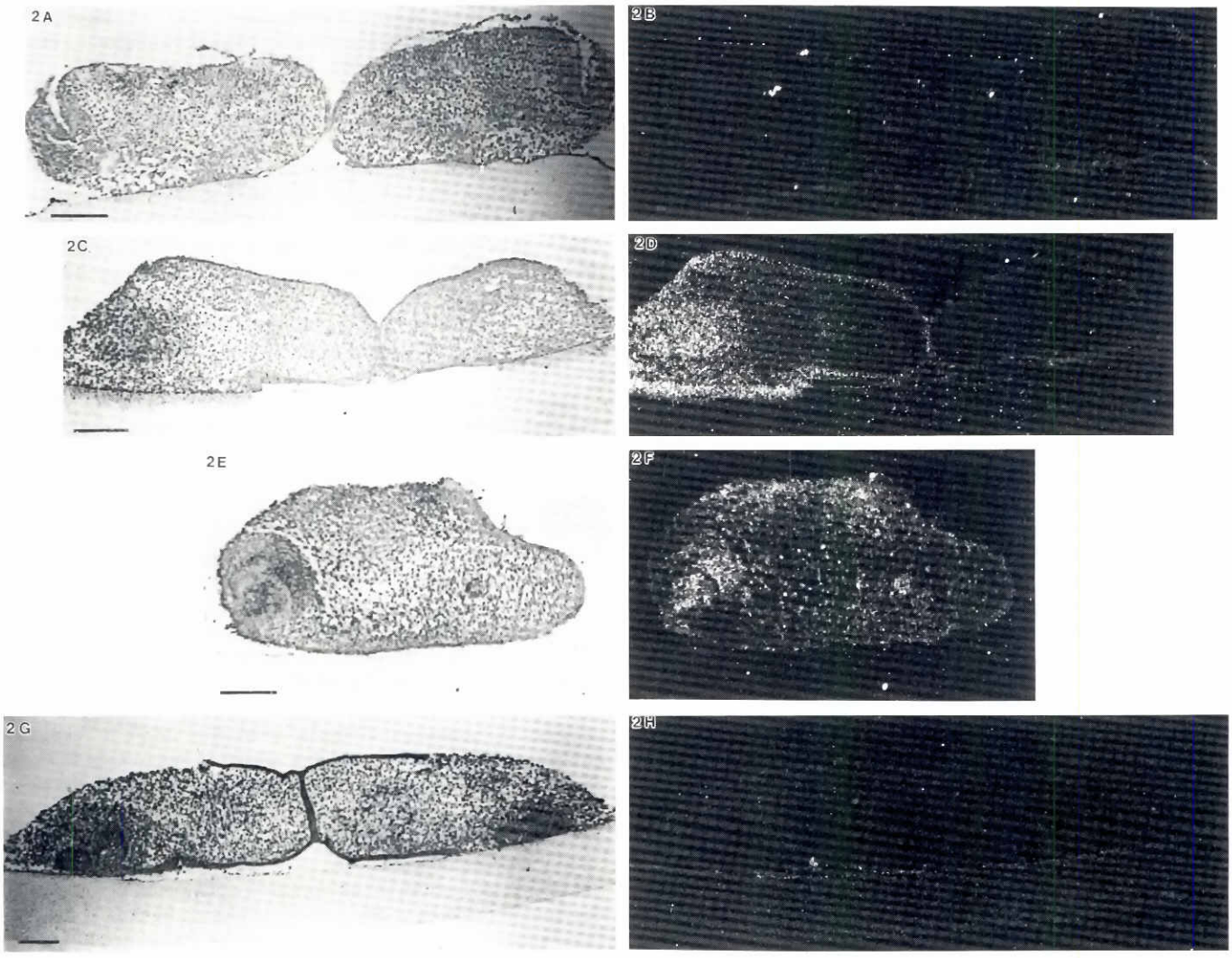

Fig. 2. The binding of ^{125}I-EGF to embryonic palatal shelves under Trowell and submerged organ culture conditions. (A, B) *Light and dark field photographs of a submerged culture treated with 10ng/ml TGF-α for 24 h prior to labeling with ^{125}I-EGF. Silver labeling was reduced to background levels. Scale bar, 50 μm.* **(B, C)** *Light and dark field photographs of a Trowell culture treated with 10ng/ml TGF-α for 24 h prior to labeling with ^{125}I-EGF. The labeling was the same as that seen in untreated cultures; there was labeling of all palatal epithelia and the toothgerm showed the usual characteristic pattern. TGF-α treatment did not down regulate EGF binding, unlike in submerged culture (2A, 2B), suggesting that the added TGF-α did not penetrate the filter into the tissue. Scale bar, 50 μm.* **(E, F)** *Light and dark field photographs of a submerged culture labeled for 90 min in the presence of ^{125}I-EGF. The palatal epithelia were labeled, as were the toothgerms. Scale bar, 50 μm.* **(G, H)** *Light and dark field photographs of a Trowell culture incubated for 24 h in culture media containing ^{125}I-EGF. There was no labeling of the palate tissue, but there was labeling of the Millipore filter. Scale bar, 50 μm*

no effect on the pattern of EGF binding sites as shown by autoradiography and both additions resulted in normal palatal fusion.

However, administration of TGFß$_1$ during culture resulted in a dramatic decrease in binding sites for EGF. After 24 h of culture in the presence of TGFß$_1$, there were no detectable EGF binding sites in the toothgerm and very low binding levels throughout the palate mesenchyme (Fig. 4C,D). After 48 h, there remained no binding sites throughout the palate mesenchyme and epithelia, but palatal fusion had taken place. Indeed, addition of TGF-ß$_1$, ß$_2$, or ß$_3$ caused an increase in the rate of palatal fusion which was complete by 48 h of culture (Fig. 4B).

Discussion

This study has demonstrated the localization of binding sites for EGF within the developing embryonic murine palate and the regulation of EGF binding by other growth factors. Our original experimental plan was to carry out a series of incubations using conventional Trowell organ culture at the liquid/gas interface. However, although this system proved adequate for control cultures we became aware that penetration of the filter was a problem when exogenous growth factors were added to the culture medium.

The inability of growth factors to penetrate a Millipore filter has not been reported previously. However, the vast majority of previous

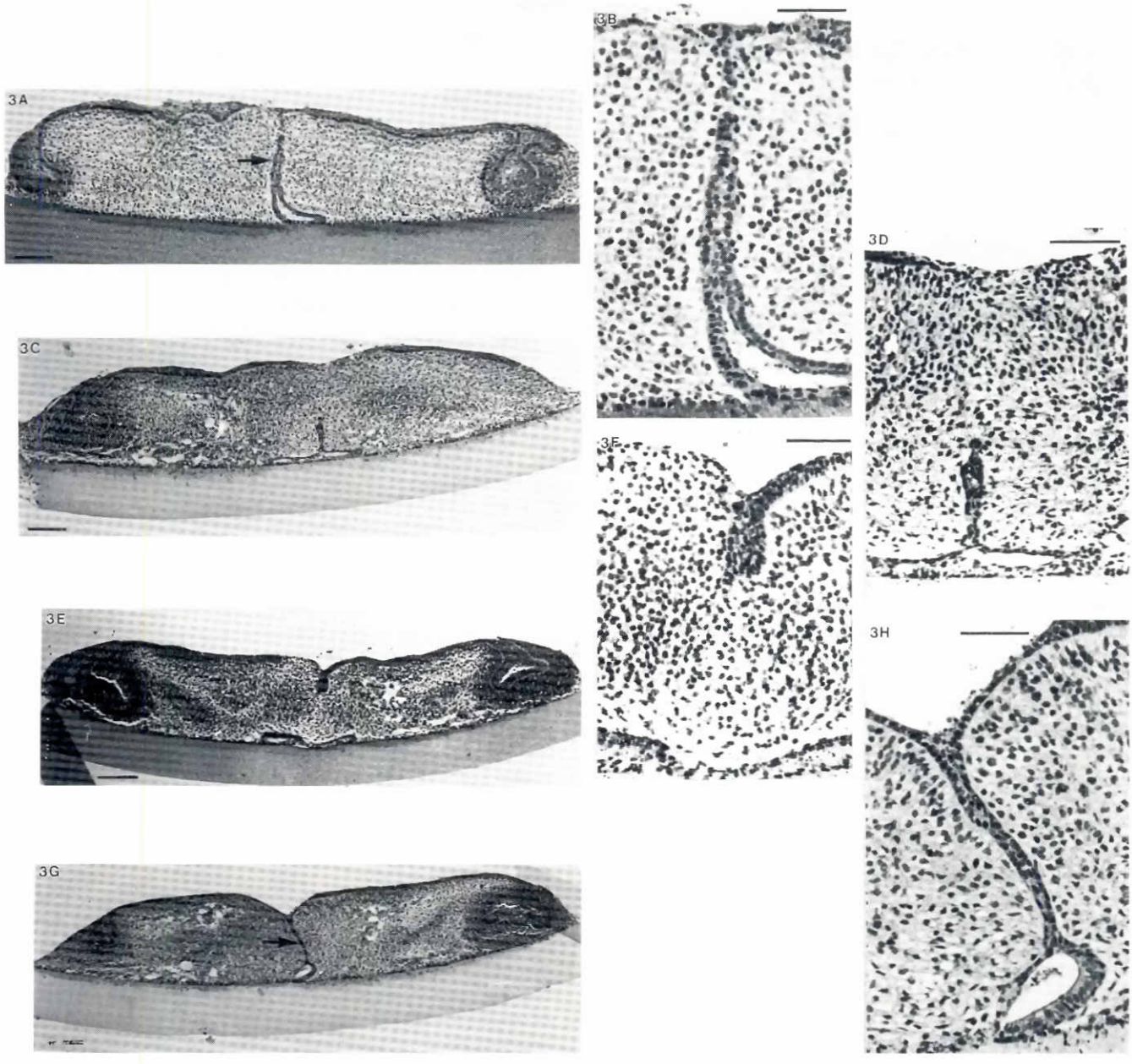

Fig. 3. The effects of EGF on palatal fusion under Trowell and submerged culture conditions. (A) *Light field photograph of a submerged culture treated with 20 ng/ml EGF for 72 h. The medial edge epithelia persisted (arrowed); no fusion occurred. Scale bar, 50 μm.* **(B)** *Higher power magnification of the medial edge region. Scale bar, 100 μm.* **(C)** *Light field photograph of a Trowell culture treated with 20 ng/ml EGF for 72 h. The medial edge epithelia have degenerated and fusion was normal. Scale bar, 50 μm.* **(D)** *High power magnification of medial edge region demonstrating mesenchymal continuity. These cultures were identical to control Trowell cultures, for example 3E. Scale bar, 100 μm.* **(E)** *Light field photograph of a 72 h control Trowell culture. Scale bar, 50 μm.* **(F)** *High power magnification of the medial edge region. Scale bar, 100 μm.* **(G)** *Light field photograph of a Trowell culture treated with 20 ng/ml EGF in the presence of 1% DCS for 72 h. Fusion did not occur and the medial edge epithelia persisted (arrowed). Scale bar, 50 μm.* **(H)** *Higher power magnification of the medial edge region. Scale bar, 100 μm*

palate culture studies have included serum in the culture medium (reviewed by Dixon and Ferguson, 1992) and we found that EGF with the addition of serum in the Trowell system led to a prevention of palatal fusion. Perhaps proteins in serum block non-specific binding sites in the filter and so allow EGF access to the tissue. However, serum is undefined and variable and therefore does not permit the investigation of growth factors in isolation or in specific combinations. While untreated cultures from both Trowell and submerged systems exhibited identical ^{125}I-EGF binding patterns, studies involving pretreatment with soluble growth factors were carried out in submerged culture, because of the apparent filter penetration problems.

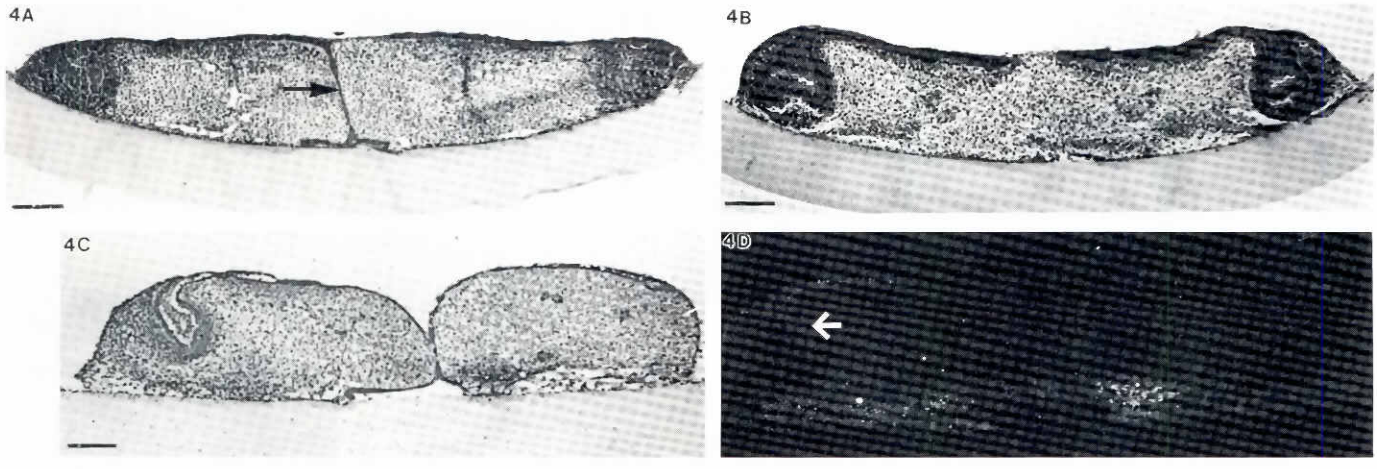

Fig. 4. The effects of TGFß on palate fusion and EGF binding sites. (A) *Light field photograph of a 48 h control submerged culture. Note formation of the midline seam (arrowed). Scale bar, 50 μm.* **(B)** *Light field photograph of a submerged culture treated with 10ng/ml TGF-ß$_3$ for 48 h. Note that accelerated degeneration of the midline seam has occurred resulting in mesenchymal continuity. Similar results were observed using TGF-ß$_1$ and -ß$_2$ (data not shown). Scale bar, 50 μm.* **(C, D)** *Light and dark field photographs of a submerged culture treated with 10ng/ml TGF-ß$_1$ for 24 h. Silver labeling was generally reduced. Note the reduction in labeling of the toothgerm (arrowed) when compared to control cultures (see Fig. 1F,H). Scale bar, 50 μm*

Most previous *in vitro* studies have made use primarily of single palatal shelves cultured in isolation. The differentiation of the medial edge epithelia in these cultures may not reflect what occurs *in vivo*. The use of single palatal shelves stems from the fact that cell death can be seen in the medial edge epithelia (Ferguson *et al.*, 1984). This was thought to be the mechanism by which the palate fused *in vivo*. It was therefore suggested that the medial edge epithelia could differentiate normally *in vitro* without coming into contact with another palatal shelf (Ferguson *et al.*, 1984). However, in the light of new findings, it is thought that the palate fuses *in vivo* by a mechanism of epithelial cell transformation and migration rather than cell death (Fitchett and Hay, 1989; Shuler *et al.*, 1991, 1992; Carette and Ferguson, 1992; Griffith and Hay, 1992). It may be that this does not occur unless there is adhesion between two palatal shelves (Griffith and Hay, 1992). The cell death seen in single palate organ cultures may be the result of a failure to make this contact. Adhesion molecules, such as those present in desmosomes, which may be responsible for the initial adhesion between two palatal shelves, may also be part of the subsequent signalling mechanism for medial edge epithelial cell transformation/migration. It would seem, therefore, that as a paired organ culture system is available, its use is preferable to the technique of culturing single palatal shelves in isolation.

Using ^{125}I-EGF to localize the EGF-receptor during murine palatogenesis revealed that the pattern of expression was regulated both spatially and temporally. ^{125}I-EGF binding sites were found in the palatal shelf mesenchyme except in the region immediately adjacent to the medial edge epithelia and subsequent midline seam. Labeling of palatal epithelia was also temporally regulated. At time 0 h, oral, nasal and medial edge epithelia had EGF binding sites. As the palatal shelves fused (time 24 h), the level of EGF binding sites in the medial edge epithelia declined. This pattern was similar to previous autoradiographic and immunocytochemical studies. The absence of EGF receptors in the mesenchyme adjacent to the medial edge was reported by Shiota *et al.* (1990), but not by Abbott *et al.* (1988) or Dixon *et al.* (1991).

In summary, our major findings are that there is an absence of EGF binding sites in the mesenchyme underlying the medial edge epithelia, the region in which any putative epithelial-mesenchymal interactions are occurring. All palatal epithelia bind EGF at time 0, although binding in the medial edge epithelia declines to low levels with time. This may be because as the seam degenerates by migration there are fewer epithelial cells in this region. In the fused palate EGF binding is uniform across the palatal mesenchyme.

The pattern of expression of EGF binding sites in the developing tooth germ (Fig. 1D,F) matches exactly that reported previously (Partanen and Thesleff, 1987). This supports the validity of our findings in the palate.

Of the growth factor treatments only TGF-ß and EGF/TGF-α elicited any effect. Routine histology at 24, 48 and 72 h revealed that EGF/TGF-α treatment prevents palatal fusion. Our data contradict those of Dixon and Ferguson (1992) who suggested that EGF required the presence of serum (or some growth factor component of serum) in order to prevent palatal fusion. We believe this to be incorrect as in our submerged system EGF and TGF-α were equally effective in serum-containing and serum-free medium. The earlier study used palatal shelves cultured on a collagen matrix (Dixon and Ferguson, 1992): since collagen may bind or trap a wide range of growth factors (Smith *et al.*, 1982) this may have led to the absence of an effect of EGF/TGF-α except in the presence of serum.

As evidenced by routine histology at 24, 48 and 72 h of culture TGF-ß$_1$, ß$_2$ or ß$_3$ treatment appeared to accelerate the fusion process (Fig. 4B), a phenomenon observed by other authors (Dixon and Ferguson, 1992; Gehris and Greene, 1992). An explanation for enhanced palatal fusion may be that, as in other systems such as bronchial epithelia, TGF-ß$_1$ accelerates desmosome formation (Yoshida *et al.*, 1992). Treatment with TGF-ß$_1$ led to a change in the pattern of EGF binding sites. After treatment with TGF-ß$_1$ a marked reduction in EGF binding occurred. This reduction to virtually undetectable levels was apparent in both the epithelia and mesenchyme of the palate (Fig. 4D). Treatments with TGF-ß$_1$ for up to 48 h maintained the down-regulation in EGF binding. These data

support our previous observation that TGF-ß$_1$ treatment of palate mesenchyme cells *in vitro* led to a marked down-regulation of EGF binding sites (Sharpe *et al.*, 1992). This reduction in EGF binding sites corresponded with a decrease in responsiveness to EGF. TGF-ß isoforms are endogenous to the developing murine palate, and their expression appears to be independently regulated (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). At late day 13, early day 14, TGF-ß$_1$ and ß$_3$ mRNA begin to be expressed in the medial edge epithelia, TGF-ß$_2$ mRNA in the mesenchyme immediately underlying the medial edge. These regions correspond to the areas where EGF receptor density is lowest.

Given the available evidence: (i) TGF-ß reduces EGF binding in organ and cell culture; (ii) reduction in EGF binding corresponds to a decrease in EGF activity; (iii) the area of TGF-ß isoform expression correlates with the region of lowest EGF binding, it is tempting to speculate that endogenous TGF-ß is involved in suppressing EGF activity in the medial edge region perhaps facilitating shelf fusion. Convincing evidence for this hypothesis may be obtained by ablating endogenous TGF-ß from the developing palate and observing the effects on EGF binding and shelf fusion. Such experiments, using neutralizing antibodies and anti-sense oligodeoxynucleotides, are ongoing in this laboratory.

As exogenous EGF prevents shelf fusion, it would appear that, *in vivo*, either its expression, or that of TGF-α, is at very low levels or that their activity must be down-regulated in the medial edge region for normal fusion to occur. If this is the case, why the medial edge epithelia should express EGF receptors at all must be explained. Even though the medial edge region may express relatively few receptors, EGF clearly acts on medial edge cells. Indeed, even after TGF-ß treatment and down-regulation of EGF binding to levels undetectable by autoradiography, subsequent treatment with EGF still prevents palatal fusion (data not shown). In addition, reports that genetic variation at a site close to the TGF-α locus is linked to cleft palate (Ardinger *et al.*, 1989; Chevenix-Trench *et al.*, 1991) are further evidence that TGF-α/EGF-like activity is under strict control during normal palate development. Although binding is generally agreed to be reduced in the medial edge region, significant levels of EGF, TGF-α and EGF receptor peptides have been consistently detected in the murine palate during palatal fusion (Abbott and Birnbaum, 1990; Shiota *et al.*, 1990; Dixon *et al.*, 1991). It is difficult to explain the presence of EGF/TGF-α in the palate at these embryonic stages since transfer of maternal EGF across the placenta has not been demonstrated and the murine embryo is not reported to express EGF/TGF-α mRNA from embryonic day 11-16 (reviewed by Lee and Han, 1990). Perhaps the EGF/TGF-α detected represents growth factors synthesized at an earlier time point and stored within the palate, or possibly small pockets of cells exist capable of synthesizing these factors at levels below the limits of available detection methods.

The problem is therefore to explain the presence of a factor known to prevent medial edge fusion, and also the expression of receptors for that factor, in the medial edge. Perhaps the answer is that palatal development is a multi-step integrated process. Thus EGF/TGF-α and the EGF receptor may be important in the early stages of palatal shelf outgrowth. Subsequent palatal fusion may be a two-step process. First, medial edge cells of apposing palates must come into contact and form cell-cell attachments leading to seam formation. Second, those cells transform/migrate (Fitchett and Hay, 1989) either into the mesenchyme (Shuler *et al.*, 1991, 1992; Griffith and Hay, 1992) or onto the oral and nasal surfaces (Carette and Ferguson, 1992). One could hypothesize that EGF/TGF-α is not required for, indeed actively prevents, the first fusion event but is involved in the second migratory event. EGF/TGF-α activity is probably regulated by other growth factors expressed during palatal development resulting in a smoothly integrated and coordinated system.

Materials and Methods

Embryos

Mice (MF 1 strain) were mated overnight and the day of finding a vaginal plug designated day zero of embryonic development. On embryonic day 13 pregnant mice were killed by chloroform overdose and the embryos aseptically removed. Palatal shelves (Theiler stage 21; Theiler, 1972) were then dissected from the embryonic heads.

Organ culture procedures

Trowell culture system

Palatal shelves were placed on Millipore filters with their medial edges in contact. The filters were positioned on grids and placed in Falcon organ culture dishes over 0.75 ml media (Minimum Essential Medium, containing 40 µg/ml ascorbic acid, 2 mM glutamine, 50 µg/ml glycine, 1% penicillin/streptomycin; MEM). The cultures were maintained at 37°C in an air incubator for 6 h to allow the explants to attach to the filters. At this time point, designated time 0, the original media was replaced with experimental media: MEM±EGF (20 ng/ml), or bFGF (1 ng/ml), or IGF-II (100 ng/ml), or TGF-α (10 ng/ml) or TGF-ß$_1$, ß$_2$, or ß$_3$ (10 ng/ml). After 24, 48 or 72 h the cultures were removed. Ten cultures were used for each treatment at each time point.

Submerged culture system

Explanted palatal shelves were positioned on filters as above and allowed to attach for 6 h using the conventional Trowell culture system. At this point, they were removed into 96-well plates and individually submerged in 20 µl Dulbecco's Modified Eagles' Medium/ Hams' F12 (DMEM/F12)±EGF (20 ng/ml), or bFGF (1 ng/ml), or IGF-II (100 ng/ml), or TGF-α (10 ng/ml) or TGF-ß$_1$, ß$_2$, or ß$_3$ (10 ng/ml). Surrounding wells were filled with sterile distilled water to ensure adequate humidity. Cultures were maintained at 37°C in a 5% CO_2 incubator for 24, 48 or 72 h, with media changes every 12 h. Ten cultures were used for each treatment at each time point.

Iodination of EGF

EGF was purified according to the method of Gregory (1975) and was a gift from Dr. S. Crosby (Hope Hospital, Manchester). EGF was iodinated using the chloramine-T method as described in Sharpe *et al.* (1992). The specific activity of the [^{125}I]-EGF was approximately 80-100 µCi/µg protein.

^{125}I-EGF binding and autoradiography

The filters and attached palatal shelves were submerged in binding medium (MEM, 120 mM Hepes, 0.1% BSA) containing 5 ng/ml ^{125}I-EGF for 90 minutes at room temperature as described by Partanen and Thesleff (1987). Control cultures were incubated as above, but a 500-fold excess of cold EGF was added to compete with the labeled ligand.

After this incubation, the cultures were washed extensively; 6x10 minutes in binding medium and 2x30 minutes in PBS at 4°C. The cultures were then fixed in 4% paraformaldehyde overnight at 4°C, before being prepared for histology by dehydrating through a graded series of alcohols, clearing in chloroform and embedding in fibrowax (Raymond A. Lamb, London).

7 µm histology sections of each culture were cut and mounted on 3-aminoptopyltriethoxysilane-coated slides.

Representative slides from each culture were dewaxed in xylene, dipped in Ilford K5 emulsion and incubated at 4°C for 4-8 weeks before development. After development, the sections were lightly stained with hematoxylin before being viewed and photographed under dark and light field using a Leitz Dialux microscope.

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