

# Modulation of the epidermal growth factor receptor of mouse embryonic palatal mesenchyme cells *in vitro* by growth factors

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**ABSTRACT** A single class of high-affinity receptors for EGF were detected on mouse embryonic palatal mesenchyme (MEPM) cells cultured *in vitro*. The degree of confluence of the cultured cells did not affect the number or affinity of the binding sites. Culture of MEPM cells in the presence of bFGF, IGF-II or TGF- $\beta_1$  induced changes in  $^{125}\text{I}$ -EGF binding. TGF- $\beta_1$  caused a marked reduction in binding to 40% of control levels. This reduction was achieved after 2 h and persisted for 24 h after addition of the growth factor. IGF-II induced a similar reduction but this effect was transitory; after a 12 h pre-treatment with IGF-II, binding was restored to control levels. The effects of bFGF were biphasic. Initially, a short pre-treatment period (3-5 h) with bFGF caused a small reduction in  $^{125}\text{I}$ -EGF binding; longer periods of pre-incubation (24 h) resulted in a large increase in receptor number. Pre-incubation in medium containing both bFGF and TGF- $\beta_1$  resulted in a decrease in EGF binding. Thus, TGF- $\beta_1$  negated the large increase in receptor number induced by bFGF alone. Changes in receptor number were usually, but not always, directly related to changes in the biological activity of EGF, as assessed by a thymidine incorporation assay. This study highlights the possible interactive role of growth factors known to be present in the developing palate.

**KEY WORDS:** TGF- $\beta$ , IGF-II, bFGF, EGF receptor, palate development, growth factors

## Introduction

Mammalian palate development is a complex process involving morphogenetic movement, epithelial-mesenchymal interactions and regionally specific epithelial differentiation (Ferguson, 1988). Of critical importance is the process of formation and disruption of the mid-line epithelial seam resulting in mesenchymal continuity across the palate. Originally, seam degeneration was thought to be entirely the result of programmed epithelial cell death (Hudson and Shapiro, 1973). More recently, evidence has accumulated to suggest that a significant proportion of basal epithelial cells migrate from the epithelial seam (Sharpe and Ferguson, 1988), possibly transforming into a mesenchymal phenotype (Fitchett and Hay, 1989).

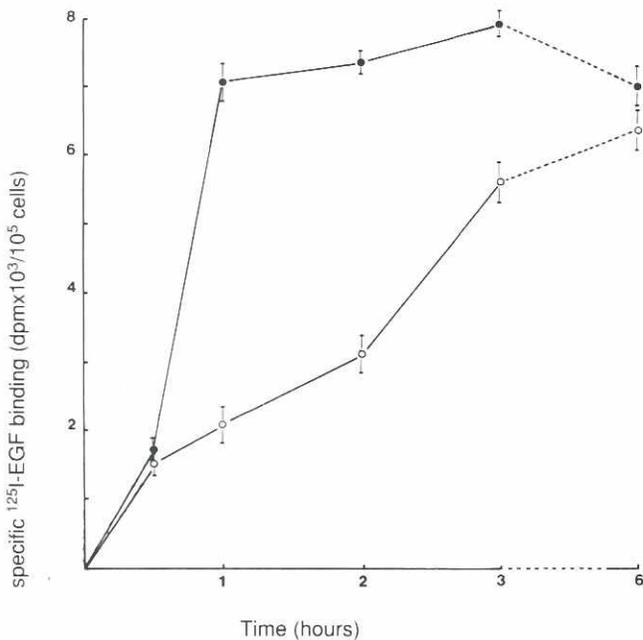
Regionally specific differentiation of the palatal epithelia may be the result of a complex interaction between extracellular matrix molecules and soluble growth factors (Sharpe and Ferguson, 1988). Epidermal growth factor (EGF) prevents medial edge epithelial cell death and normal seam formation, when the epithelium is cultured in contact with an underlying mesenchyme (Hassell, 1975; Tyler and Pratt, 1980). That EGF-like molecules play a physiological role in palate development is suggested by numerous studies which

have shown the presence of EGF, its putative embryonic homologue transforming growth factor- $\alpha$  (TGF- $\alpha$ ) and the EGF receptor in the embryonic murine palate (Abbott *et al.*, 1988; Abbott and Birnbaum, 1990; Shiota *et al.*, 1990; Dixon *et al.*, 1991). In addition, there is an apparent genetic association between variation in the TGF- $\alpha$  locus and orofacial clefting in some human families (Artinger *et al.*, 1989).

If exogenous EGF is capable of modifying medial edge epithelial differentiation to prevent palatal fusion, it follows that normal palatal development may involve down regulation of EGF activity in the medial edge region. Since there is no evidence that EGF and TGF- $\alpha$  transcripts are present in the developing palate, it is unlikely that modulation of transcription or translation of these factors is responsible for regulation of EGF-like activity. A further possibility is the modulation of the EGF receptor. Significantly, teratogens known

*Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IGF-II, insulin-like growth factor-II; MEPM, mouse embryonic palatal mesenchyme; TGF- $\alpha$ , transforming growth factor- $\alpha$ ; TGF- $\beta$ , transforming growth factor- $\beta$ .

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**Fig. 1.** The effect of temperature on <sup>125</sup>I-EGF binding to cultured MEPM cells. Incubations were carried out as described in Materials and Methods at 4°C (○) and 20°C (●). At the indicated times specific <sup>125</sup>I-EGF binding was determined. Results are expressed as means (±SEM) of triplicate determinations.

to cause cleft palate *in vivo*, such as retinoic acid and the glucocorticoids, induce an increase in EGF receptor number in both whole palate organ cultures (Abbott *et al.*, 1988) and palatal mesenchyme cells in monolayer culture (Yoneda and Pratt, 1981; Kukita *et al.*, 1987).

Several other growth factors have been mapped to the developing palate. The transcripts for isoforms of transforming growth factor β (TGF-β<sub>1,2,3</sub>) are present in the embryonic murine palate and the expression of each is developmentally and spatially regulated (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). The distribution of the TGF-β<sub>1</sub> and TGF-β<sub>2</sub> peptide in the developing palate have been described (Abbott and Birnbaum, 1990; Gehris *et al.*, 1991; Williams *et al.*, 1991). In addition, acidic and basic fibroblast growth factor (aFGF, bFGF) and IGF-II mRNA, protein and binding protein are present in the medial edge region of the developing murine palate (unpublished observations).

We have investigated the possibility that these factors, endogenous to the developing palate, are capable of modulating EGF receptor expression by mouse embryonic palatal mesenchyme (MEPM) cells.

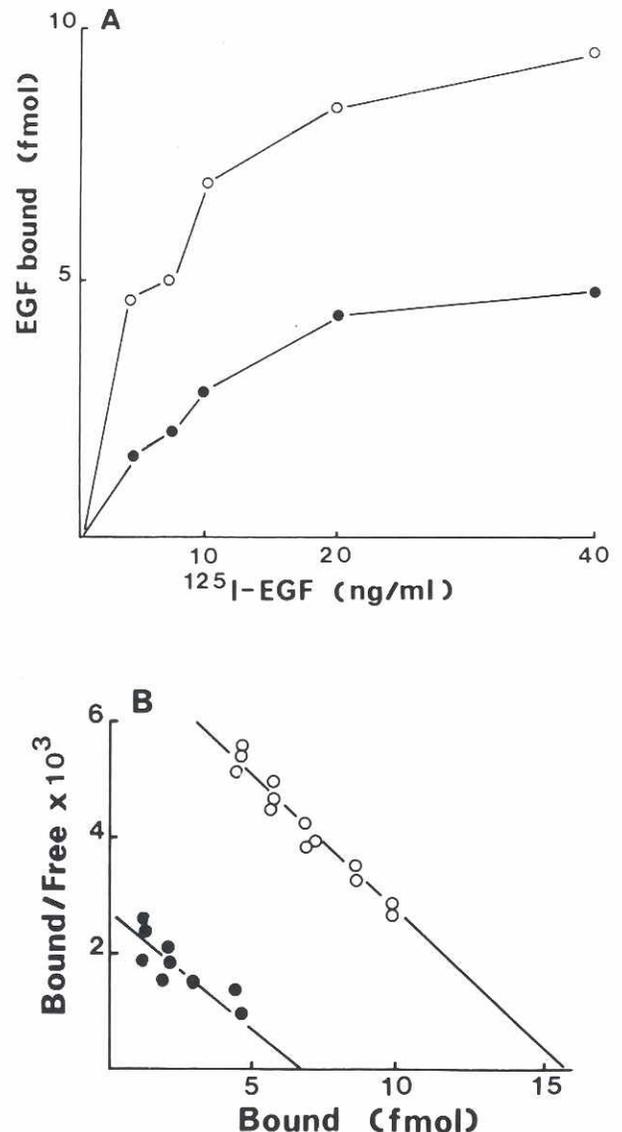
**Results**

**Time course of binding**

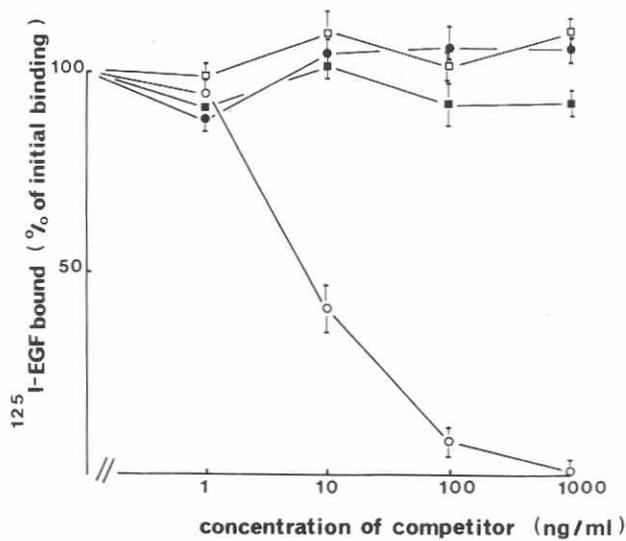
Fig. 1 illustrates the time course of binding of <sup>125</sup>I-EGF to cell surface receptors at 4°C and 20°C. At 4°C, levels of binding remained relatively low until 2 h of incubation, and maximal binding

was achieved at some point between 2 and 6 h. At 20°C binding rapidly reached a maximal level by 1 h of incubation, and remained constant for the rest of the experiment. Nonspecific binding was determined at each time point. Nonspecific binding increased between 10 and 30 min of incubation and then remained constant throughout the experiment.

It was noticed that at 4°C, after approximately 3-4 h of culture, MEPM cells often became detached from the culture dish. This



**Fig. 2.** The effect of <sup>125</sup>I-EGF concentration on binding to MEPM cells at 20°C. Cells were cultured at a confluent (5 x 10<sup>4</sup> cells/well, ○) or sub-confluent (2.2 x 10<sup>4</sup> cells/well, ●) density. Cells were incubated with the indicated concentration of <sup>125</sup>I-EGF for 90 min at 20°C. (A) Shows saturation curves of specific binding, determined as described in Materials and Methods, to MEPM cells. Each point represents the mean (±SEM) of triplicate determinations. Scatchard plots of the data (B) indicated that maximal binding of EGF in confluent culture was 15.7 fmol EGF, equivalent to 31.4 fmol/10<sup>5</sup> cells. In the sub-confluent cells maximal binding was 6.8 fmol, equivalent to 30.9 fmol/10<sup>5</sup> cells.



**Fig. 3.** Effect of bFGF (□), EGF (○), IGF-II (■) and TGF-β<sub>1</sub> (●) on the specific binding of <sup>125</sup>I-EGF by MEPM cells. MEPM cells were cultured in media containing <sup>125</sup>I-EGF in the presence or absence of competing growth factor at 20°C for 90 min. Each point represents the mean (±SEM) of 4 separate determinations

probably accounts for the greater variability observed at later time points. For this reason subsequent experiments were carried out at 20°C with an incubation time of 90 min.

#### Preliminary binding data

A preliminary experiment was performed to examine the effect of confluence on expression of cell surface receptors for EGF (Fig. 2). MEPM cells were plated onto 24-well culture plates at either  $5 \times 10^4$  or  $2 \times 10^4$  as described in Materials and Methods. Binding studies were carried out in buffer containing increasing concentrations of <sup>125</sup>I-EGF (5-40 ng/ml). It was apparent that saturation of the binding sites occurred at a concentration of approximately 20 ng/ml (Fig. 2A). Scatchard analysis resulted in a rectilinear plot suggesting a single class of high-affinity binding sites (Fig. 2B). In sub-confluent cell culture, maximal binding was 6.8 fmol EGF, in confluent cell culture 15.7 fmol. After correction for cell number (determined from duplicate wells using a Coulter counter) maximal binding of EGF was equivalent to 30.9 fmol/ $10^5$  cells and 31.4 fmol/ $10^5$  cells in sub-confluent and confluent cultures respectively. i.e., the number of binding sites per cell was not significantly affected by the degree of cell confluence.

#### Receptor specificity

Specific binding of <sup>125</sup>I-EGF to MEPM cells was not affected by the presence of unlabeled bFGF, IGF-II and TGF-β<sub>1</sub>, even at very high concentrations (Fig. 3). By contrast, increasing concentrations of unlabeled EGF competed for specific cell surface binding. Percentage binding is expressed as the percentage of specific binding relative to binding in control cells, i.e., cells incubated without the addition of excess EGF. These data confirm the presence of specific

binding sites for EGF and also show that any effect of other growth factors on EGF binding was not due to active competition for binding sites.

#### Receptor modulation

Binding of <sup>125</sup>I-EGF to MEPM cells was modulated by pre-incubation with each of the growth factors studied (Fig. 4A-C). Addition of TGF-β<sub>1</sub>, even at very low concentrations, resulted in a rapid down-regulation of specific EGF binding to approximately 40% of the level in control wells. Percentage binding is expressed as binding relative to those cells cultured in the absence of added growth factors. The effects of TGF-β<sub>1</sub> were maximal 2 h after addition and persisted for at least 24 h (Fig. 4A).

IGF-II caused a similar decrease in cell surface binding (Fig. 4B). The induced down-regulation in binding followed a similar time course to that caused by TGF-β<sub>1</sub>, being maximal at 2 h of pre-incubation. In contrast to the effect of TGF-β<sub>1</sub>, binding of EGF gradually began to rise 4 h after addition of IGF-II until reaching control levels by 12 h of pre-treatment.

bFGF initially had a rather modest effect on the binding of EGF (Fig. 4C). At the highest concentration a maximal reduction to 60% of control values was achieved after 3 h. With increasing pre-incubation time (12 h), binding eventually was restored to control levels. Surprisingly, a longer period of pre-incubation with bFGF, up to 24 h, resulted in a large increase in specific cell surface binding of <sup>125</sup>I-EGF.

Scatchard analysis, measurement of EGF binding using increasing concentrations of <sup>125</sup>I-EGF (2.5-20 ng/ml), was carried out on cells pre-incubated for either 4 h (Fig. 5A), or 24 h (Fig. 5B) in binding buffer containing the various growth factors. The parallel nature of the resultant Scatchard plots confirmed that modulation of binding was due to changes in receptor number rather than affinity.

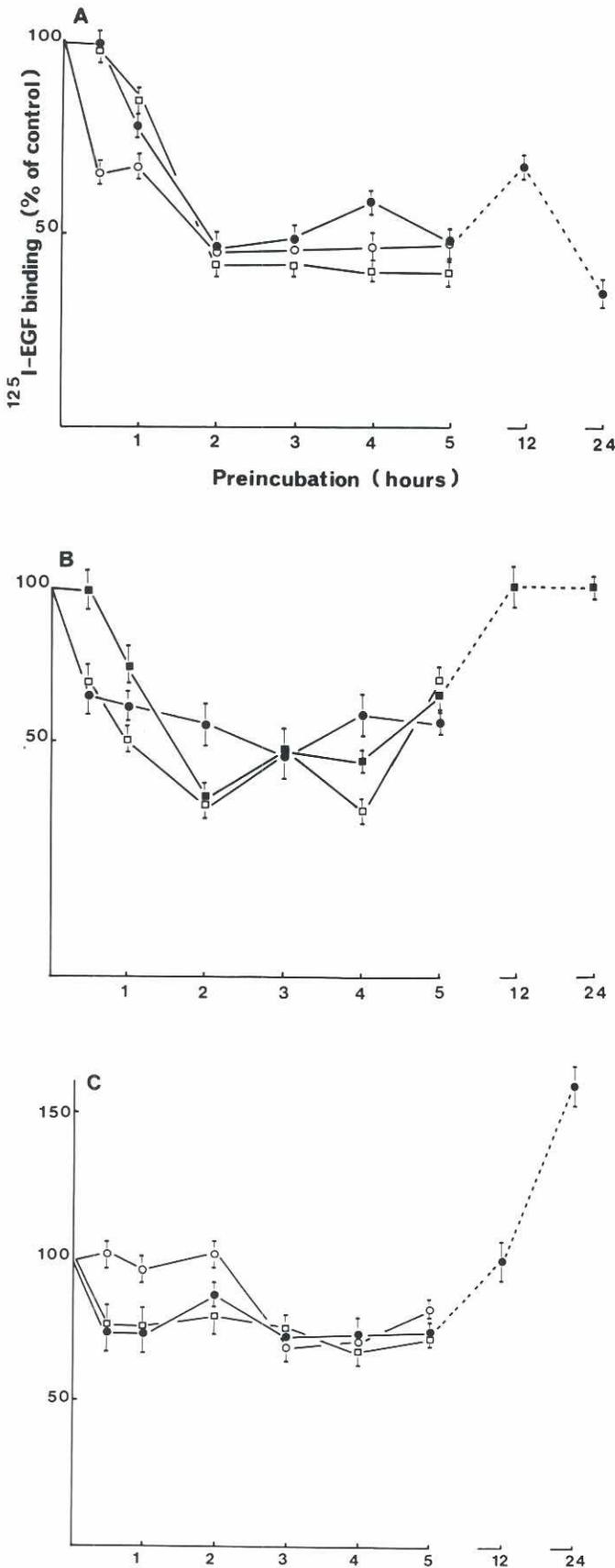
Pre-treatment of MEPM cells with TGF-β<sub>1</sub> and bFGF in combination resulted in a gradual decrease in cell surface receptors for EGF (Table 1). The maximal reduction was not as great as that seen for TGF-β<sub>1</sub> alone (Fig. 4A). This reduction persisted for 24 h after addition of the growth factors, thus TGF-β<sub>1</sub> effectively neutralized the large increase in cell surface binding induced by bFGF alone (Fig. 4C).

TABLE 1

#### THE EFFECT OF PRE-TREATMENT WITH TGF-β<sub>1</sub> AND bFGF ON <sup>125</sup>I-EGF BINDING BY MEPM CELLS

| Period of pre-treatment (hours) | Binding of <sup>125</sup> I-EGF (% of control) |
|---------------------------------|--|
| 0 (control)                     | 100  |
| 0.5                             | 93.2 ± 0.8                                     |
| 1                               | 86.4 ± 1.5                                     |
| 2                               | 90.5 ± 1.7                                     |
| 5                               | 77.9 ± 2.6                                     |
| 24                              | 64.9 ± 0.4                                     |

MEPM cells were cultured in DMEM/F12 containing TGF-β<sub>1</sub> (1 ng/ml) and bFGF (1 ng/ml) for various periods of time (0.5-24 h) as described in Materials and Methods. The cells were washed 3x in binding medium and total and nonspecific binding of <sup>125</sup>I-EGF determined as described in Materials and Methods. All values are the means (±SEM) of triplicate cultures.



**Thymidine Incorporation**

Treatment of MEPM cells with EGF or bFGF caused significant increases in incorporation of [<sup>3</sup>H]-thymidine. In contrast TGF-β<sub>1</sub> induced a significant decrease while IGF-II had no significant effect (Table 2).

Typically, addition of a sub-maximal concentration of EGF (1 ng/ml) induced an increase in incorporation of 59% above control values. When EGF (1 ng/ml) was added to MEPM cells which had been pre-treated with various growth factors, the magnitude of response was affected (Table 3). The increase in [<sup>3</sup>H]-thymidine incorporation induced by EGF treatment in cells pre-treated with TGF-β<sub>1</sub> alone (31%), or in combination with bFGF (33%), was less than that in control cells (59%). In MEPM cells pre-treated with bFGF alone, EGF was more effective (76%) than in the control. Interestingly, the largest effect was seen with IGF-II pre-treated cells in which EGF caused a 196% increase in [<sup>3</sup>H]-thymidine incorporation.

**Discussion**

It has been suggested that the soluble growth factors play a major role in normal mammalian secondary palate development (Ferguson, 1988; Sharpe and Ferguson, 1988), possibly via a complex interaction with the extracellular matrix. To date, the bulk of research has focused on the role of EGF, the putative embryonic homologue of EGF, TGF-α and the EGF receptor. EGF is able to modulate normal medial edge epithelial cell differentiation, and prevent shelf fusion *in vitro* (Hassell, 1975). EGF and TGF-α have been localized in the developing embryonic murine palate (Abbott *et al.*, 1988; Abbott and Birnbaum, 1990; Dixon *et al.*, 1991) and it is not clear whether one or both of these factors are physiologically relevant. Both of these factors act via the EGF receptor, which has also been localized in the developing palate (Abbott and Birnbaum, 1990; Shiota *et al.*, 1990; Dixon *et al.*, 1991), and detected in embryonic palate mesenchyme cells *in vitro* (Yoneda and Pratt, 1981; Kukita and Kurisu, 1986; Kukita *et al.*, 1987).

As exogenous EGF/TGF-α can prevent normal medial edge epithelial cell differentiation and mid-line seam formation *in vitro* (Hassell, 1975; Tyler and Pratt, 1980; Abbott *et al.*, 1988), it has been supposed that normal palate development involves a down-regulation of EGF-like activity in the medial edge region at the time of epithelial seam development. Immunocytochemical evidence from developing mouse embryos *in vivo* indicate that the levels of EGF, TGF-α and the EGF receptor are reduced in the mesenchyme of the medial palatal region around the time of seam formation (Abbott and Birnbaum, 1990; Shiota *et al.*, 1990). Evidence that this also occurs in the medial edge epithelia is contradictory (Abbott *et al.*, 1988; Abbott and Birnbaum, 1990; Dixon *et al.*, 1991). Regulation is unlikely to occur at a local transcriptional/translational level. Although the EGF/TGF-α genes are expressed in the pre-

**Fig. 4.** Effect of TGF-β<sub>1</sub> (A), IGF-II (B) and bFGF (C) pre-treatment on specific binding of <sup>125</sup>I-EGF by MEPM cells. Cells were pre-treated for the indicated times in media containing various concentrations (0.1 (○), 1 (●), 10 (□) or 100 (■) ng/ml) of growth factor at 37°C. After the pre-incubation period specific binding of <sup>125</sup>I-EGF to MEPM cells was determined as described in Materials and Methods. Each point is the mean (±SEM) of 4 separate determinations.

TABLE 2

**THE EFFECT OF EGF, TGF- $\beta$ 1, bFGF AND IGF-II ON [ $^3$ H]-THYMIDINE INCORPORATION BY MEPM CELLS**

| Medium                    | Stimulation of [ $^3$ H]-thymidine incorporation (%) |
|---------------------------|--|
| Control                   | 100  |
| EGF (0.1 ng/ml)           | 126.4 $\pm$ 6.2                                      |
| (1 ng/ml)                 | 160.6 $\pm$ 4.2                                      |
| (10 ng/ml)                | 201.2 $\pm$ 14.3                                     |
| bFGF                      | 178.8 $\pm$ 10.4                                     |
| TGF- $\beta$              | 40.0 $\pm$ 4.6                                       |
| IGF-II                    | 102.0 $\pm$ 5.3                                      |
| EGF + bFGF                | 111.8 $\pm$ 5.4                                      |
| EGF + TGF- $\beta$        | 54.4 $\pm$ 2.4                                       |
| EGF + IGF-II              | 209.8 $\pm$ 5.2                                      |
| EGF + bFGF + TGF- $\beta$ | 52.6 $\pm$ 2.3                                       |

MEPM cells were plated onto Falcon 24-well plates in DMEM/F12 + 2.5% DCS as described in Materials and Methods. Experimental media consisted of DMEM/F12 + 1% DCS  $\pm$  EGF (0.1, 1 or 10 ng/ml), bFGF (1 ng/ml), TGF- $\beta$ 1 (1 ng/ml) or IGF-II (100 ng/ml) and EGF (10 ng/ml) in combination with the other growth factors. The cultures were pulsed with [ $^3$ H]-thymidine (1  $\mu$ Ci/ml) for the last 2 h (from 22-24 h) of culture after addition of growth factors. Incorporation was determined as described in Materials and Methods. All values are means ( $\pm$ SEM) for 5 cultures.

implantation and early post-implantation murine embryo there is no evidence that EGF/TGF- $\alpha$  transcripts are present at embryonic days 12-15 (reviewed by Lee and Han, 1990), the time of palate development. However, there are no specific expression data for the palate. Moreover, specific tissue effects of EGF activity are likely to be modulated by localized mechanisms. One likely possibility is modulation of EGF activity at the receptor level.

Some support for this theory is given by the observation that the teratogen retinoic acid, which prevents palatal fusion in organ culture (Newall and Edwards, 1981), acts on EGF receptors causing their expression to persist in the medial edge region (Abbott *et al.*, 1988). In addition, retinoic acid has been shown to increase the specific binding of [ $^{125}$ I]-EGF to MEPM cells *in vitro* (Kukita *et al.*, 1987). A similar increase in EGF binding was induced by the glucocorticoid dexamethasone, a cleft palate inducing compound, in a human embryonic palate mesenchyme cell line (Yoneda and Pratt, 1981). However, the relevance of this data must be questioned since this cell line has since been shown to be dissimilar to mammalian cells derived from primary palatal explants (Linask *et al.*, 1991). Such MEPM cells do not appear to respond to glucocorticoids in a similar way (Kukita and Kurisu, 1986).

In this study we have examined the effects of various growth factors, known from immunocytochemical studies to be endogenous to the developing mouse palate, on the expression of EGF receptors by MEPM cells in culture. We found that MEPM cells, derived from explants of embryonic day 13 palatal shelves possessed a single class of high affinity receptors, specific for EGF, the binding characteristics of which were in close agreement with previously published observations (Kukita and Kurisu, 1986; Kukita *et al.*, 1987). Each of the growth factors examined had an effect on EGF

binding characteristics. TGF- $\beta$ 1 induced a rapid down-regulation in receptor binding to 40% of control levels and the effect persisted for up to 24 h after treatment with TGF- $\beta$ 1. IGF-II caused a similar reduction but its effects were transitory; binding levels had returned to control values 12 h after addition of the growth factor. The effects of bFGF were biphasic. Initially, bFGF pre-treatment (3-5 h) induced a small decrease in EGF binding. After 12 h, binding was restored to the level of the control, but longer periods of pre-incubation with bFGF (24 h) resulted in a large increase in EGF receptor number. Co-incubation of TGF- $\beta$  with bFGF negated the positive effect of bFGF on EGF binding.

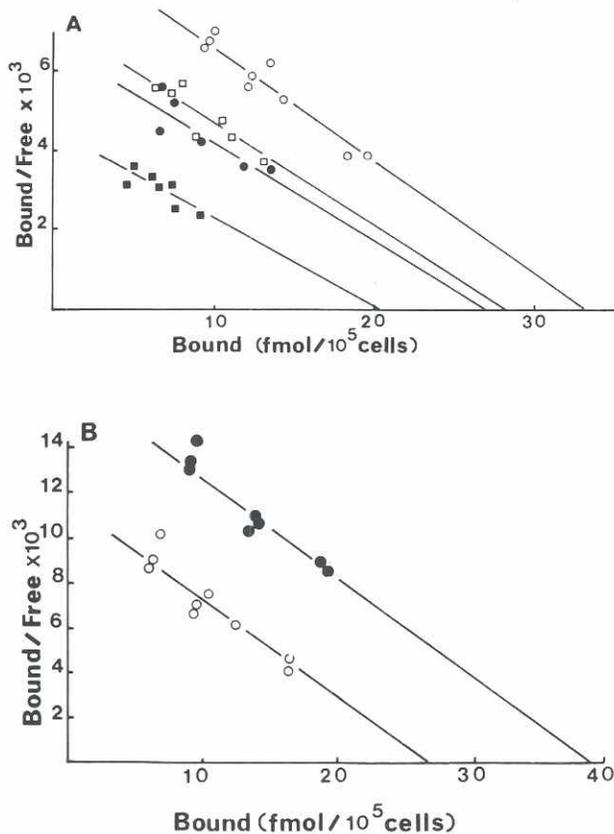
Of these growth factors the interaction of TGF- $\beta$  with other growth factors is the best documented (Sporn *et al.*, 1987). Indeed, the synergistic interaction of TGF- $\beta$  with EGF to stimulate aspects of transformed behavior in NRK fibroblasts were among the earliest experiments performed to characterize TGF- $\beta$  as a novel growth factor (Anzano *et al.*, 1983). The effects of TGF- $\beta$  on the EGF receptor appear to be dependent on cell type. In the NRK fibroblast cell line it has been reported that TGF- $\beta$  reduces EGF binding (Massague, 1985). However, this is a short-term response (up to 6 h). The effects of TGF- $\beta$  on this cell line are biphasic; longer-term exposure to TGF- $\beta$  (16-24 h) resulted in a 3-fold increase in EGF binding (Assoian, 1985). The increase in EGF receptor number is at least partly a result of *de novo* synthesis of receptor proteins (Thompson *et al.*, 1988). Long-term pre-treatment of rat hepatocytes (12-24 h) with TGF- $\beta$  caused a similar increase in EGF binding (Shiota *et al.*, 1986). By contrast TGF- $\beta$  had no effect on the EGF binding characteristics of Mv1Lu epithelial cells (Like and Massague,

TABLE 3

**THE EFFECT OF EGF ON [ $^3$ H]-THYMIDINE INCORPORATION BY MEPM CELLS AFTER PRE-TREATMENT WITH GROWTH FACTORS**

| Pre-treatment     | [ $^3$ H]-thymidine incorporation (dpm/well) | % control |
|-------------------|--|-----------|
| NONE              | 2246 $\pm$ 231                               |           |
| + EGF             | 3574 $\pm$ 143                               | 159       |
| bFGF              | 2778 $\pm$ 258                               |           |
| + EGF             | 4878 $\pm$ 275                               | 176       |
| TGF- $\beta$      | 1545 $\pm$ 131                               |           |
| + EGF             | 2018 $\pm$ 91                                | 131       |
| bFGF/TGF- $\beta$ | 1660 $\pm$ 50                                |           |
| + EGF             | 2206 $\pm$ 90                                | 133       |
| IGF-II            | 2217 $\pm$ 159                               |           |
| + EGF             | 6565 $\pm$ 474                               | 296       |

MEPM cells were plated onto Falcon 24-well plates in DMEM/F12+2.5% DCS as described in the Materials and Methods. The cells were cultured for 24 h in DMEM/F12 containing bFGF (1 ng/ml), TGF- $\beta$ 1 (1 ng/ml), bFGF+TGF- $\beta$ 1, or IGF-II (100 ng/ml). The cells were washed 3x in DMEM/F12 and thymidine incorporation, in the presence or absence of EGF (1 ng/ml), determined as described in Materials and Methods after 24 h. All values are the means ( $\pm$ SEM) of 5 separate determinations.



**Fig. 5.** Effect of bFGF, IGF-II and TGF- $\beta$  on  $^{125}\text{I}$ -EGF binding capacity of cultured MEPM cells. Cells were pre-treated for 4 h (A) or 24 h (B) in the presence or absence (○) of bFGF (1 ng/ml, ●), IGF-II (100 ng/ml, ◻) or TGF- $\beta_1$  (1 ng/ml, ■). After pre-treatment the specific binding of increasing concentrations of  $^{125}\text{I}$ -EGF (2.5–20 ng/ml) was determined as described in Materials and Methods, and expressed as a Scatchard plot.

1986), while TGF- $\beta$  treatment resulted in a decrease in high-affinity binding sites for EGF in several endothelial cell lines (Takehara *et al.*, 1987). Changes in EGF receptor number in the NRK cells, whether reduction (6 h after TGF- $\beta$  treatment) or increase (24 h), were mirrored by changes in the biological effectiveness of EGF, as assayed by thymidine incorporation (Assoian, 1985). This is not always the case. TGF- $\beta$  blocks the cellular response of the Mv1Lu line to EGF although receptor number and affinity and the signal transduction ability of the receptor are unaffected (Like and Massague, 1986). In rat hepatocytes, the increase in EGF receptor number induced by TGF- $\beta$  is accompanied by a reduction in the biological effectiveness of EGF (Shiota *et al.*, 1986). We therefore investigated the effects of the observed changes in EGF receptor number induced by growth factor pre-treatment on the biological response of MEPM cells to EGF. Biological activity was assayed by measuring the ability of EGF to stimulate the incorporation of [ $^3\text{H}$ ]-thymidine into MEPM cells pre-treated for 24 h with the various growth factors. It is of course possible that there may be a bifurcation in the biological response to EGF after growth factor pre-

treatment. In endothelial cells, although TGF- $\beta$  inhibits the ability of EGF to induce the expression of several specific competence genes (c-myc, JE, KC) its effects on others (c-fos) is unaffected (Takehara *et al.*, 1987). It is therefore possible that other biological responses to EGF, such as a stimulation of extracellular matrix production (Silver *et al.*, 1984; Turley *et al.*, 1985; Pisano and Greene, 1987; Foreman *et al.*, 1991), may be affected differently, or not at all, by growth factor pre-treatment. With this caveat, it was apparent that in general the changes in receptor number induced by growth factor pre-treatment were mirrored by changes in the biological activity of EGF. Pre-treatment with TGF- $\beta_1$  alone, or in combination with bFGF, resulted in a reduced response to EGF. Pre-treatment with bFGF alone was accompanied by an increase in the activity of EGF. Interestingly, pre-treatment with IGF-II, which has no long-term effect on EGF number or affinity, was accompanied by a large increase in the effect of EGF. This enhancement of EGF activity was not the result of a direct synergistic interaction between the two factors since concurrent incubation of MEPM cells with EGF and IGF-II did not have a significantly greater effect on [ $^3\text{H}$ ]-thymidine incorporation than EGF alone. Since changes in the biological effectiveness of EGF do not always involve coincident alteration in binding parameters, caution must be exercised in interpreting studies based on assumptions about the activity of EGF/TGF- $\alpha$  from the degree of localization of these factors, and their receptors, in specific regions of the palate (Abbott *et al.*, 1988; Abbott and Birnbaum, 1990; Shiota *et al.*, 1990; Dixon *et al.*, 1991).

In summary, this paper provides evidence that the activity of EGF/TGF- $\alpha$  in the developing mammalian palate may be modulated by endogenous growth factors at the EGF receptor and post-receptor level. Such interactions may be important in the co-ordinate response of the palate to the multitude of growth factors known to localize *in vivo*.

## Materials and Methods

### 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. Briefly, 10  $\mu\text{l}$  of 0.5M phosphate buffer, pH 7.5, containing 1  $\mu\text{g}$  EGF and 10  $\mu\text{l}$  chloramine-T (0.5 mg/ml) were added to 1 mCi  $\text{Na}^{125}\text{I}$  (Amersham). After 30 sec, 10  $\mu\text{l}$  sodium metabisulfite (4 mg/ml in  $\text{H}_2\text{O}$ ) was added. The mixture was chromatographed on a Sephadex G-25 column pre-equilibrated with 0.05M phosphate buffer, pH 7.5 containing 2 mg/ml BSA. The specific activity of the [ $^{125}\text{I}$ ]-EGF was 80–100  $\mu\text{Ci}/\mu\text{g}$  protein.

### Cell culture

Mouse embryonic palatal mesenchyme (MEPM) cells were derived from primary explants of embryonic day-13 palatal shelves as described in Sharpe *et al.* (1992). Cell culture was routinely carried out in standard medium: Dulbecco's modified Eagles medium (DMEM) and Hams F12 medium (1:1 vol.) containing 2 mM glutamine and 1% penicillin-streptomycin (Gibco), supplemented with 2.5% donor calf serum (DCS, Imperial Laboratories, Andover) in a 5%  $\text{CO}_2$  in air, 37°C environment.

### Binding Studies

MEPM cells, at low passage (3–5), were plated onto Falcon 24-well culture plates at  $5 \times 10^4$  cells per well in DMEM/F12 containing 2.5% DCS. Cells were allowed to attach overnight and were then rinsed twice in binding buffer. All studies were carried out in 0.5 ml of HEPES binding buffer (128 mM NaCl, 5 mM KCl, 5 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{CaCl}_2$ , 50mM HEPES, pH 7.5, 2 mg/ml BSA).

For time course studies, binding medium contained  $^{125}\text{I}$ -EGF (5 ng/ml) and cells were incubated at 4°C, or 20°C for various periods of time. The experiment was stopped by removal of the experimental medium and subsequent washing (6X) with ice-cold binding buffer. The remaining radioactivity was solubilized using 0.5 ml NaOH (0.5M) at 37°C for 1 h. Counts were detected using a LKB 1282 gamma counter.

In competition studies, various concentrations of EGF (Flow, Herts), bFGF, IGF-II and TGF- $\beta$  (British Biotechnology, Oxford) were included in the binding buffer. Cells were incubated at 20°C for 90 min. in the presence of  $^{125}\text{I}$ -EGF (5 ng/ml). Binding of labeled EGF was detected as above.

In further studies, MEPM cells were pre-incubated at 37°C in DMEM/F12 containing growth factors for various times, ranging from 0.5-24 h. After the requisite pre-incubation period the cells were washed 3X in binding buffer and were subsequently incubated at 20°C for 90 min in binding buffer containing  $^{125}\text{I}$ -EGF (5 ng/ml). Binding of labeled EGF was determined as above.

Nonspecific binding was determined by measuring the amount of binding which occurred in the presence of a 100-fold excess of unlabeled EGF. Specific binding was determined by subtraction of nonspecific binding from total binding. Nonspecific binding was routinely less than 15% of specific binding and was not affected by growth factor treatment.

In each experiment, for each experimental treatment, duplicate wells were included for determination of cell number using a Coulter counter (Coulter Electronics).

#### Thymidine incorporation assay

MEPM cells were plated onto Falcon 24-well culture plates at  $5 \times 10^4$  cells/well in DMEM/F12 containing 2.5% DCS. Cells were allowed to attach overnight and were washed 3X with serum-free DMEM/F12. Experimental medium (DMEM/F12 supplemented with 1% DCS±growth factor) was added for a 24 h incubation period at 37°C. For the last 2 h of incubation [ $^3\text{H}$ ]-thymidine (Amersham, specific activity 29 Ci/mmol) was added to the medium (1  $\mu\text{Ci}/\text{ml}$ ). Cells were fixed in 3 washes with 5% TCA and remaining radioactivity was solubilized with 0.5ml of NaOH (0.5M) at 37°C for 1 h. Counts were detected using a Beckman 9800 scintillation counter.

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