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Effects of epidermal growth factor on preimplantation mouse embryos

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Summary. When epidermal growth factor (EGF) was added to the medium for culture of preimplantation embryos, morphological development as determined by microscopic observation was unaffected, but 333 nM-EGF stimulated total uptake of [^{3}H]leucine by late morulae/blastocysts which had been cultured for 24 h from morulae. Incorporation of [^{3}H]leucine into protein by these embryos was increased by 0·33, 3·3 and 33 nM-EGF, following a quadratic relationship producing less stimulation at 333 nM, which may indicate down regulation of receptors. The estimated EC_{50} was ~0·25 nM. Manipulation of the culture period indicated that the embryos responded to EGF at the morula/blastocyst transition period and immunosurgery was used to show that the increased protein synthesis was restricted to the trophectoderm cells. No mitogenic effect was observed. The effective concentration of EGF is close to that of serum and to values which stimulate other tissues. It is suggested that EGF receptors appear at compaction and that EGF may have a role in differentiation of the trophectoderm cells.

Keywords: EGF; preimplantation embryos; protein synthesis; mouse

Introduction

It has been generally accepted that development of fertilized mouse eggs to at least the blastocyst stage requires only a regulated gaseous and osmotic environment and energy substrates. Evidence for this hypothesis is that simple culture media based on Krebs' solution supplemented with pyruvate, lactate and glucose can support growth of mouse and human embryos over this period.

However, there is growing evidence that growth factors and/or hormones are involved in these early stages of development. Newly fertilized embryos produce ovum factor which initiates maternal production of early pregnancy factor (Cavanagh *et al.*, 1982). This ovum factor may be platelet-activating factor (PAF; Orozco *et al.*, 1986), which is also released by the recently fertilized egg (O'Neill, 1985). Insulin alters blastocyst glucose metabolism *in vitro* (Wales *et al.*, 1985), an observation which was confirmed by the demonstration of insulin receptors at about the morula stage (Rosenblum *et al.*, 1986). Insulin specifically increases mouse embryonic protein synthesis during and after compaction (Harvey & Kaye, 1988), the rate of morphological development of mouse embryos during the preimplantation period *in vitro* (Gardner & Kaye, 1984), blastocyst glucose transport rates (Gardner & Kaye, 1984) and fetal growth rate of embryos transferred after culture in its presence during the preimplantation period (Gardner & Kaye, 1986).

Despite these implications for the operation of humoral systems, no effects of recognized growth factors on preimplantation development have been demonstrated, although epidermal growth factor (EGF) receptors have been observed on trophoblast outgrowths (Adamson & Meek,

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1984). EGF is mitogenic on ectodermal and mesodermal cells (Gospodarowicz, 1981) and has been shown to stimulate protein synthesis (Cohen & Stastny, 1968). This study presents evidence for stimulation of protein synthesis by the trophectoderm cells of mouse late morulae/blastocysts. A preliminary report has been presented (Wood & Kaye, 1985).

Materials and Methods

Embryos were collected from superovulated randomly bred Quackenbush mice into M2 medium containing no protein by standard techniques 48 h (2-cell embryos), 72 h (morulae) or 96 h (late morulae/blastocysts) after injection of hCG (Hobbs & Kaye, 1985). They were cultured in 20 μl droplets of BMOC2 medium (Brinster, 1965) containing either EGF or no protein under oil for 24 h (37°C, 5% O_2, 5% CO_2, 90% N_2), before measuring total uptake and incorporation of [4,5-^{3}H]leucine (27 μM, 1 Ci/l: Amersham, North Ryde, NSW, Australia) over 2 h in the same medium. After incubation, embryos were washed through 4 changes of 2 ml Medium M2 containing 1 g bovine serum albumin (BSA)/l. To determine total uptake, individual washed embryos were added to 0·2 ml H_2O, and radioactivity measured. Incorporation was determined after adding 0·1 ml Medium M2 to individual embryos, freezing, thawing, precipitation overnight at 4°C after addition of 0·1 ml 613 mM-CCl_3CO_2H, and collection of the acid-insoluble fraction on glass-fibre discs after washing thoroughly with 20 ml 306 mM-CCl_3CO_2H. Results have been converted to fmol/embryo assuming that the specific radioactivity of the pool used for incorporation is the same as that of the medium. The efficiency of counting was 25·3 (±0·4)% for total uptake and 12·0 (±0·3)% for counting on glass-fibre discs; background was 7 c.p.m.

The number of cells in embryos was determined by counting Feulgen-stained nuclei (Humason, 1972) in air-dried spreads of embryos (Tarkowski, 1966). Inner cell masses (ICMs) were isolated from blastocysts by the immunosurgical method of Harlow & Quinn (1979) using rabbit antiserum raised against mouse spleen cells. Statistical analysis was by Student's *t* test or analysis of variance unless indicated otherwise. Values are means (±s.e.m.). Electrophoretically homogeneous EGF prepared from mouse salivary glands (Waters *et al.*, 1987) was a gift from Dr M. Waters.

Results

Response to EGF

Initial studies surveyed 2-cell embryos, morulae and late morulae/blastocysts for effects of adding 33 nM-EGF to the culture medium for 24 h. There was no effect on the morphological development of embryos of any of the three stages examined over the next 24 h. At least 90% of all embryos progressed to the next developmental stage or beyond. Similarly, there was no significant effect on total uptake of leucine by any stage (Table 1).

However, adding 33 nM-EGF to the medium used for culture of morulae for 24 h increased leucine incorporation in the resulting late morulae/early blastocysts by 38% compared to controls (Table 1, $P = 0·003$). There was no effect in 4/8-cell embryos previously cultured for 24 h with EGF from the 2-cell stage. The 8% increase for expanded blastocysts was not statistically significant (Table 1).

Effective concentration of EGF

Concentrations of 0, 0·33, 3·3, 33·0 and 333·0 nM-EGF were examined for the effects of their presence during 24 h culture of morulae on uptake and incorporation of leucine by the resulting late morulae/blastocysts. The presence of 333 nM-EGF in the medium increased uptake by 50% ($P < 0·05$, Table 1).

The results for incorporation of leucine (Table 1) were examined by analysis of variance (Table 2). There was a significant effect of varying the concentration of EGF in the culture medium on protein synthesis in the resulting late morulae/early blastocysts ($P = 0·0001$), but this effect was confounded by significant variation between experiments ($P = 0·0003$) and a significant interaction between these main effects ($P < 0·0001$). Examination of the results indicated a distinct relationship between concentration and stimulation of protein synthesis, and so the variability due to the concentration effect was partitioned between linear, quadratic, cubic and quartic relations using

Table 1. The effect of 24-h treatment with EGF on total uptake and incorporation of [^{3}H]leucine by mouse embryos (fmol/embryo/2 h)

		Initial stage†		
		2-cell	Morula	Blastocyst
Control				
Uptake		252 ± 187	677 ± 10	2500 ± 231
Incorporation		79 ± 8	285 ± 20	276 ± 35
EGF				
0·33 nM	Uptake	ND	653 ± 141	ND
	Incorporation	ND	352 ± 16	ND
3·3 nM	Uptake	221 ± 16	883 ± 107	ND
	Incorporation	83 ± 8	397 ± 18	ND
33 nM	Uptake	209 ± 18	725 ± 107	2817 ± 197
	Incorporation	77 ± 9	379 ± 24	296 ± 33
333 nM	Uptake	ND	1015 ± 146*	ND
	Incorporation	ND	301 ± 10	ND

Values are mean ± s.e.m.; results are from 4–9 experiments with measurements on 3–12 individual embryos in each.

†Embryos were collected at the indicated stages and cultured for 24 h before uptake or incorporation of [^{3}H]leucine was measured. ND, not determined. *$P < 0·05$. ANOVA for incorporation of [^{3}H]leucine by late morulae/blastocysts is presented in Table 2.

orthogonal polynomials to determine the nature of this relationship. This analysis showed that 94·5% of the variability in the concentration–response curve could be ascribed to a quadratic relation indicating maximum response at 3·3 nM-EGF (Fig. 1, $P < 0·001$). Since there was a significant interaction between main effects, the significance of this concentration–response was tested against the interaction mean square and was significant at $P < 0·025$. Thus, despite the interaction between main effects, there was still a significant quadratic relationship between the concentration of EGF and the protein synthetic rate. This relation estimates the EC_{50} at ~0·25 nM-EGF (Fig. 1). When acid-soluble uptake was calculated by difference, 3·3 and 333 nM-EGF stimulated uptake into this fraction by late morulae/blastocysts derived from morulae which had been cultured 24 h. However, these increases were not significant at $P < 0·05$.

Table 2. ANOVA table of the effect of 24-h treatment with EGF on incorporation of [^{3}H]leucine by mouse morulae†

Source	SS	d.f.	MS	MS/MS_e	*P*	$MS/MS_{A \times B}$	*P*
Exps (A)	11 250 381	8	1 406 298	3·9063	0·0003	1·0406	ns
[EGF] (B)	8 628 733	4	2 157 183	5·9920	0·0001	1·5962	ns
linear	319 263	1	319 263	1·1276	ns	4·2331	ns
quadratic	8 154 153	1	8 154 153	22·6497	<0·001	6·0336	<0·025
cubic	138 060	1	138 060	2·6076	ns	9·7889	ns
quartic	17 257	1	17 257	20·8768	ns	78·3138	ns
(A × B)	33 786 560	25	1 351 462	3·7539	<0·001		
Error (C)	74 522 250	207	360 011				
Total	135 409 870	245	552 693				

†Untransformed data from 9 experiments each containing 5 concentrations of EGF with 7 missing plots were analysed by ANOVA.

SS(B) was partitioned using orthogonal polynomials. ns, Not significant.

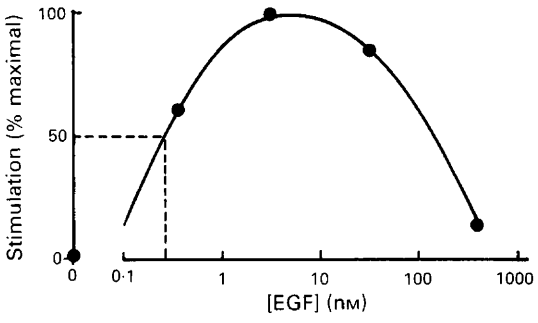

Fig. 1. Stimulation of protein synthesis in late morulae/blastocysts obtained by culture with EGF. Morulae were collected and cultured with 0, 0·33, 3·3, 33·0 or 333 nM-EGF for 24 h before assay of [^{3}H]leucine incorporation. Results (from Table 1) are expressed as (%) maximal stimulation above that level obtained in embryos from culture without EGF.

To avoid possible deleterious effects of BSA on preimplantation development (Caro & Trounson, 1984) and the possibility that contaminants of the relatively impure preparations of BSA may confound the results, most experiments were performed in protein-free media. However, it was also possible that the embryos utilized the EGF not specifically as a growth factor but simply as a source of protein. The effects of adding BSA at an equimolar concentration were therefore examined. Addition of 3·3 nM-BSA to the medium in which morulae were cultured for 24 h did not alter incorporation by the resultant embryos 232 ($\pm$84) compared with 239 ($\pm$84) fmol/embryo/2 h). Similarly, in 3 experiments in which blastocysts were cultured for 3 h in the absence or presence of 3·3 nM-BSA there was no effect on incorporation.

Ontogeny of the response in morulae

Culture of 2-cell embryos for 48 h in the presence of 3·3 nM-EGF caused a 20% stimulation of protein synthesis ($P < 0{\cdot}02$, Table 3), but there was no effect on total uptake in the resulting late morulae/blastocysts.

To see whether the stimulation by EGF could be produced in a shorter time, freshly collected late morulae/blastocysts were cultured in the absence or presence of 3·3 nM-EGF for 3 h before measuring leucine uptake and incorporation. Culture in control medium had no effect on total uptake, but incorporation was reduced by 13%, although this was not significant when compared to freshly collected embryos. However, incorporation was increased 38% by addition of EGF to the medium ($P < 0{\cdot}05$, Table 3). This activity was 20% greater than, but not statistically different from, that of embryos which were assayed immediately after collection.

Total uptake and incorporation by late morulae/blastocysts which had been cultured for 24 h from morulae in protein-free medium were 42% ($P < 0{\cdot}001$) and 64% ($P < 0{\cdot}01$), respectively, of those of freshly collected embryos (Table 3). The addition of 3·3 nM-EGF to the medium for culture of morulae to blastocysts had no effect on total uptake, which was only 55% ($P < 0{\cdot}01$) of that by freshly collected embryos; but increased the rate of incorporation to 89% of the value for freshly collected embryos and there was no significant difference between these two groups.

Location of response

To determine which cells of the blastocyst were responding, morulae were collected, cultured for 24 h in the absence or presence of 3·3 nM-EGF and labelled for 2 h (as above), before inner cell masses were isolated and leucine incorporation was measured and compared with that of similarly treated controls. Whole blastocysts from the same group which were held at room temperature

Table 3. Uptake and incorporation of [^{3}H]leucine by freshly collected late morulae/blastocysts and those derived from 3-, 24- or 48-h culture with or without 3·3 nM-EGF (fmol/embryo/2 h)

Embryos*	EGF	Uptake	Incorporation
Freshly collected	—	1600 ± 110[b,d]	446 ± 23[c]
Cultured			
3 h	−	1700 ± 350	389 ± 52[a]
	+	2100 ± 310	535 ± 52[a]
24 h	−	677 ± 10[b]	285 ± 20[c,e]
	+	883 ± 107[d]	397 ± 18[e]
48 h	−	550 ± 65	259 ± 21[f]
	+	709 ± 72	354 ± 33[f]

Values are mean ± s.e.m.

*Embryos were collected fresh and assayed immediately or collected as late morulae/blastocysts and cultured for 3 h with (+) or without (−) 3·3 nM-EGF or collected as morulae and cultured for 24 h (these data from Table 1) or collected as 2-cell embryos and cultured for 48 h before assay. Values with the same superscript are significantly different: a, $P < 0.05$; b, $P < 0.001$; c,d, $P < 0.01$; e, $P < 0.05$; f, $P < 0.02$. Three experiments were performed on freshly collected embryos and those cultured for 48 h. There were 6–9 experiments at 3 and 24 h. Each experiment contained 6–9 embryos assayed individually.

under the same conditions whilst the immunosurgery was performed (2 h) were examined at the same time. These had 74% of the incorporation rates of embryos assayed immediately after labelling. This reduced activity probably indicates protein turnover (Brinster *et al.*, 1979). Nevertheless, in these experiments, EGF increased incorporation by whole blastocysts by 22% ($P < 0.02$, Table 4). Incorporation into the inner cell mass was the same in both groups and contributed 54–60% of the total incorporation by blastocysts for both groups. However, when the incorporation by trophectoderm cells was calculated by difference, those isolated from blastocysts treated with EGF were 49% more active ($P < 0.05$). This difference accounted for 89% of the stimulation observed in whole blastocysts.

Table 4. Site of the response to 3·3 nM-EGF

	Whole embryo (fmol/embryo/2 h†)	Inner cell mass (fmol/ICM/2 h)	Trophectoderm (fmol/TE/2 h)
Control	201 ± 13	122 ± 10	79 ± 8
EGF	245 ± 10**	133 ± 10	118 ± 8*

†Morulae were cultured for 24 h and [^{3}H]leucine incorporation was measured. ICMs were isolated from some blastocysts before collection of the acid-insoluble fraction; trophectoderm incorporation was calculated by difference. Values are mean ± s.e.m. from 4 experiments each containing 8–12 individual measurements. **$P < 0.02$; *$P < 0.05$.

Mitotic effect

The mean number of cells in embryos derived by 24 h culture of morulae with 3·3 nM-EGF was slightly higher, 50·7 (±2·9) compared with 47·0 (±2·8) cells/embryo (3–5 experiments each with

3–11 embryos assayed in each), but this difference was not significant at $P < 0.05$. The number of cells in freshly collected blastocysts was 59·2 (±2·9).

Discussion

Most of these experiments were performed in the absence of any protein other than EGF. This procedure was deliberately chosen to exclude possible confounding factors introduced by the BSA. It was possible that the EGF was used by the embryos as a source of protein, with resulting alterations in metabolism, but the addition of equimolar BSA had no effect on embryonic protein synthesis. Therefore, in view of these results, the dose-dependence and stage-specificity (discussed below), we conclude that the observed effects of EGF follow from its binding to specific receptors and not from non-specific nutritive effects on the embryos.

These rates of total uptake and incorporation of leucine are similar to those previously reported (Brinster, 1971; Epstein & Smith, 1973; Borland & Tasca, 1974). Brinster (1971) and Epstein & Smith (1973) showed that, for leucine, incorporation was independent of external concentration and so may be taken as a measure of protein synthesis. The similarity between the incorporation rates in this study and those reported earlier confirms Brinster's (1971) observation that the absence of protein from the culture medium has no effects on protein synthesis. This was also confirmed in our experiments to test that the deletion of BSA from the media had no effect on protein synthetic rates.

It is unlikely that the stimulation by EGF of protein synthesis results from anything other than a direct effect of EGF on the embryo. The concentrations that were stimulatory (0·33, 3·3 and 33 nM) are close to the levels found in female mouse serum (0·02–0·1 nM: Perheentupa *et al.*, 1985) and human amniotic fluid (0·1–1 nM: Ladda *et al.*, 1979) and have produced effects in other tissues (0–2 nM: Adamson *et al.*, 1981; 3·3 nM: Grove & Pratt, 1984; 0·5–5 nM: Dekel & Sherizly, 1985). The decreased stimulation with 333 nM-EGF may arise from toxic minor contaminants of the preparation, although it was shown to be electrophoretically homogeneous. However, similar quadratic responses have been reported for stimulation of DNA synthesis in post-implantation tissues and attributed to 'receptor down regulation', a criterion which identifies specific EGF receptors (Adamson *et al.*, 1981; Adamson & Warshaw, 1982). Such a conclusion in this case would support the hypothesis that mouse morulae possess EGF receptors.

Embryos first become responsive to EGF during the morula–blastocyst transition since the stimulation in late morulae/blastocysts derived from 24 h culture of 8-cell embryos/morulae was the same as that caused by exposure for 48 h from 2-cell embryos and by exposure of blastocysts for only 3 h, but exposure during the 2–4/8-cell period was ineffective (Tables 1 & 3). It is during this time that the embryo greatly increases its protein synthetic rate and differentiation into trophectoderm and inner cell mass cells begins. Our results show that most of the stimulation occurs in the trophectoderm cells. Although access of EGF to the inner cell mass may be restricted, this seems unlikely, since EGF was present very early in the morula/blastocyst transition and tight junctions do not mature until after the 16-cell stage (Fleming & Pickering, 1985). EGF receptors have been observed during development on trophoblast outgrowths, but not on inner cell mass derived cells, 7 days after hCG (Adamson & Meek, 1984). It therefore appears that the receptors for EGF may be restricted to trophectoderm cells in the blastocyst and expressed early in their differentiation from the morula. If this is so, then this conclusion would support the observation of specific binding of EGF to trophoblast outgrowths (Adamson & Meek, 1984) which are derived from trophectoderm cells.

It has been reported that rabbit blastocyst ion transport is stimulated by EGF (Nielson *et al.*, 1986). Since trophectoderm cells are responsible for most of the ion transport of blastocoele expansion it is likely that this observation reflects the presence of EGF receptors on equivalent cells in the rabbit and suggests this as a function of EGF stimulation. Recently Na^+/K^+-ATPase has

been shown to appear in mouse morulae at the time of blastocoele expansion (Watson & Kidder, 1988), supporting a role for EGF regulation of blastocoele ion transport. Receptors for EGF were shown to be localized to human syncytiotrophoblast cells, paralleling the local production of hCG (Maruo & Mochizuki, 1987). These authors suggest that, since EGF has been shown to stimulate hCG production by placental tissue *in vitro*, EGF may induce differentiation of function rather than proliferation in these cells which are derived from the trophectoderm cells of the blastocyst.

EGF may be important in stimulating growth and functional differentiation of trophectoderm cells which proliferate more rapidly than inner cell mass cells (Handyside & Hunter, 1986). This is consistent with its role as a stimulator of epithelial cells. However there was no statistically significant evidence for specific stimulation of cell division by EGF. It is possible that the design of our experiments, which examined all morphologically normal embryos, prevented the slight increase observed from achieving statistical significance.

Our results show that EGF stimulates protein synthesis *in vitro* to levels not significantly different from those of late morulae/blastocysts developing *in vivo*. EGF may therefore have a function in normal development of the blastocyst, since it is not a normal constituent of the media used for culture of preimplantation mouse embryos.

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