ORIGINAL

Establishment of EGF receptor-bearing cell lines by fusion between hamster embryonic palatal cells and mouse Ltk⁻ cells

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Abstract: Hamster embryonic palatal cells, which have functional EGF receptors but a limited life-span, were fused with mouse Ltk-cells, which bear no surface EGF receptors but have an infinite life-span. After HAT selection and cloning, the following three types of cell lines were obtained: i) clones bearing EGF receptors and having a proliferative response to EGF, ii) clones having EGF receptors, but unresponsive to EGF, and iii) clones bearing no EGF receptors and having no proliferative response to EGF. These cells provide appropriate materials for investigations into the role of EGF-receptors in the induction of cell proliferation by EGF. Cells of the first type will be a good source of immunogen for the production of antibodies against embryonic EGF receptors, which will be useful for the examination of the role of EGF in palatogenesis.

Introduction

Epidermal growth factor (EGF) is thought to have some important roles in palate formation in mammalian embryos¹⁻⁵. During palate formation in mouse embryos, the amount of EGF receptors in the palate increases from day 12 to 14 of gestation⁶, suggesting the importance of this factor in normal palatogenesis.

It is of interest to study when and where EGF receptors appear in tissues of developing palates; however, there are very few histological studies of their distribution. Although Neø et al. (1980)⁷ reported the distribution of EGF receptors in organ cultures of dissected palates by using ¹²⁵I-labelled EGF, the detailed distribution was not described. Immunohistochemical examination would provide the answer for determining the precise

distribution of EGF receptors in the palate. Therefore, it is necessary to obtain specific antibodies against embryonic EGF receptors. Usually, A431 cells have been the source of EGF receptors for obtaining antibodies against EGF receptors⁷⁻¹¹, as these human epidermoid carcinoma cells bear an extremely high number of EGF receptors (ca. 2×10^6 per cell), much higher than other cells. It may be possible therefore to study the distribution of EGF receptors in palates of rodent embryos by using polyclonal or monoclonal antibodies against EGF receptors prepared from A431 cells, but only if the antibodies are able to cross-react with EGF receptors of rodent tissues. However, we think that the best way is to prepare antibodies against the embryonic EGF receptors themselves, using receptors in palatal processes of rodents, because EGF might fu-

nction differently throughout development probably by modification of EGF receptor molecules, as suggested by Adamson and Meek (1984)¹²⁾.

As a source of embryonic EGF receptors, we first planned to use diploid palatal cells derived from hamster embryos; however, only a limited amount of EGF receptors can be isolated from these cells because of their limited life-span. Therefore, we decided to establish hybrid cell lines bearing embryonic EGF receptors, by hybridizing diploid hamster palatal cells, which bear embryonic EGF receptors, with mouse Ltk⁻ cells, which are "immortal" and have no surface EGF receptors¹³⁾.

Materials and Methods

Cell culture

Palatal cells were prepared from hamster embryos of 12 days of gestation. Dissected palatal processes were treated with 0.25% trypsin for about 30 minutes at 37°C and then pipetted vigorously, followed by filtration of these dissociated cells through a platinum mesh. After centrifugation to remove trypsin, the cells were cultured in Dulbecco's modified Eagle (DME) medium containing 10% fetal calf serum (FCS). Passages of these cells were performed with 0.05% trypsin containing 0.02% EDTA. Ltk⁻ cells were cultured using the same medium as described above except that 0.25% trypsin was used for passages.

Cell fusion and HAT selection.

The cell fusion technique was performed by using polyethylene glycol, mainly as described by Pontecorvo (1975)¹⁴⁾. Confluent monolayer cultures of both cells in 60mm dishes were trypsinized and collected separately, followed by washing with serum-free DME medium. Then both cells in equal numbers were resuspended in serum-free DME medium, mixed, and centrifuged. After complete removal of the medium, 1.0ml of 42.5% (w/v) polyethylene glycol 1000 was added dropwise with occasional shaking of the tube. The tube was then kept motionless for 5 to 6 minutes, after which the fusion reaction was stopped by the addition of serum-free DME medium. The cells were then centri-

fuged, seeded into a 100mm dish, and cultured overnight, followed by the selection of hybrid cells. The medium was changed to DME containing 10% FCS, 0.1mM hypoxanthine, 0.4 μ M aminopterin, and 0.016mM thymidine (HAT medium) on the day after the fusion event. After two weeks, with feeding every three days, the medium was changed to the normal medium (DME, 10% FCS) and the cultures were continued. After several passages, HAT selection was repeated at least four more times. All passages are performed with 0.25% trypsin.

Cloning

HAT-selected cells were trypsinized followed by successive dilution of the cell suspension. A small number of these cells (five to twenty cells) were seeded into each of several 10mm dishes and cultured in normal medium for about 2 weeks. Clones were observed by the naked eye and colony positions were marked with a felt pen on the bottom of each dish. Very small pieces of sterile filter paper (1mm $\times$ 1mm) that had been immersed in 0.25% trypsin were placed on each colony, followed by incubation for 10 minutes at 37°C. These filter papers, to which the trypsinized cells adhered, were picked up and placed into 35mm dishes containing normal medium. Then the clones were cultured.

¹²⁵I-EGF binding studies

Studies on the binding of EGF to various cell types were performed as described before¹⁵⁾.

Materials

Mouse Ltk⁻ cells were a kind gift from Dr. S. Shiota of the Department of Oral Microbiology, Kyushu University. Mouse L cells were given by Mrs. A. Kukita of Saga Medical School. Polyethylene glycol 1000 was purchased from Wako Co. Ltd. (Osaka, Japan). Hypoxanthine, aminopterin, and thymidine were obtained from Sigma Chemical Co. (St Louis, Missouri).

Results

After the successive selection with HAT medium, a cell population able to proliferate for a long period of time was obtained. These cells could grow to confluence even if

they were seeded sparsely at a cell number of less than 10^4 cells per 35mm dish. Under our culture conditions, normal diploid fibroblast cells do not proliferate if seeded so sparsely. Therefore, we thought that the cells we had obtained by HAT selection might have the ability to form colonies even when very few were seeded into dishes. After seeding 5 to 20 cells per 100mm dish, cultures were continued for about two weeks without changing the medium. Colonies first became visible after about one week of culture. Cell morphology was not the same among colonies, that is, epithelial cell-like, fibroblast cell-like, and neuronal cell-like colonies were observed. Each colony was picked up as described in MATERIALS AND METHODS and recloned once more. A total of fifty-one clones was thus obtained. As these cells obtained by HAT selection have the ability to form colonies under culture

conditions in which normal diploid fibroblast never form such colonies, it appears they have at least some properties of transformed cells, probably derived from the Ltk⁻ cells.

Cell growth in HAT medium and normal medium.

We have examined the growth properties of the two parental cells and of the obtained clones. Fig. 1 shows the growth curves of the parent cells. Palatal cells derived from hamster embryos can proliferate in HAT medium as in normal medium (Fig. 1a) because normal diploid cells bear thymidine kinase and therefore can utilize the salvage pathway of DNA synthesis even when the *de novo* pathway is blocked by an inhibitor of dihydrofolate reductase, aminopterin, which is included in the HAT medium. The other parent cell, the Ltk⁻, can not proliferate at all in HAT medium (Fig. 1b) because of its deficiency in thymidine kinase.

Fig. 1 Cell growth curves of hamster embryonic palatal cells (a) and Ltk⁻ cells (b). Cells were seeded at a cell number of 10^5 (a) or 5×10^4 (b) per 35mm dish, followed by culture for 1 to 9 days in the normal (○) or HAT (●) medium. The medium was changed on day 1, 3, and 5 of culture. Cells of each dish were trypsinized and counted by haemocytometer. Each point represents the mean value from triplicate dishes (mean value $\pm$ SE).

Fig. 2 Cell growth of typical hybrid clones. The experiment was performed as described in the legend to Fig. 1. (a) B604A and (b) B604B clones. Growth curves in normal medium (O) and in HAT medium (●).

Cell growth of two representative clones, B604A and B604B, of the fifty-one obtained is shown in Fig. 2a, 2b. As is evident, these clones can proliferate in HAT medium as well as in normal medium, indicating that these clones have thymidine kinase.

From the facts that these clones were obtained by cloning twice and that they have thymidine kinase probably derived from palatal cells from hamster embryos, we regard them as hybrids between the two parent cells.

Cell morphology of various clones

As shown in Fig. 3, each clone showed one of a variety of cell morphologies; eg, epithelial cell-like (Fig. 3c), fibroblast-like (Fig. 3d), neuronal cell-like (Fig. 3e), and other types of cell morphology (Fig. 3f, 3g and 3h). There were also some clones that formed characteristic cell aggregates, as shown in Fig. 3i and 3j.

Mitogenic response to EGF and EGF receptor amounts of hybrid cells

As shown in Table 1, hamster palatal cells

have receptors for EGF and their growth was stimulated by 10ng/ml EGF. In contrast, Ltk⁻ cells have no receptors for EGF and their growth was not stimulated by EGF at all. Some hybrid cell clones have receptors for EGF and their growth is clearly stimulated by EGF (clones A524fib, B604A, B604B, C528A, HA527A, HA527B, I531B, NI531D, NI531E). Other clones have receptors for EGF; however, their growth was not stimulated by EGF (clones A527B, A605F, C528-B, F528B, F528C, I531E). There were also some clones that have no EGF receptors, nor was their growth stimulated at all by EGF (clones E528A, E531C, RO605A). In order to visualize the relationship between the amounts of EGF receptors and the mitogenic response to EGF in these clones, we have plotted the data of Table 1 in graphic form in Fig. 4. Only clones bearing more than about 10 fmol of ¹²⁵I-EGF binding sites per 10⁵ cells (about 6 × 10⁴ binding sites per cell) could be stimulated in their growth by EGF. Thus some threshold may exist in

Fig. 3 Phase-contrast micrographs of various clones. (a) Hamster palatal cells, (b) Ltk-cells, (c) A605F, (d) A524fib, (e) I531E, (f) NI531D, (g) B604B, (h) C528B, (i), and (j) E531C. Original magnification was $\times 2000$ (a-h, j) or $\times 100$ (i).

the number of EGF receptor needed for the stimulation of cell growth.

Discussion

We have obtained hybrid cell clones between hamster embryonic palatal cells and mouse Ltk⁻ cells by cell fusion using polyethylene glycol 1000 followed by HAT selection and cloning. Since they have the ability to form clones and proliferate in HAT medium, these clones are thought to have inherited some of the properties of both parent cells. Although it might be thought that these clones are Ltk⁺ revertants of Ltk⁻ cells, this is probably not the case since the hybrids bear EGF receptors. It is strongly suggested that these receptors have come from the palatal cell parent, because mouse L cells (tk⁺), the origin of Ltk⁻ mutant cells, have no detectable surface receptors for EGF according to our ¹²⁵I-EGF binding experiment. Of the hybrid cells we have obtained, those bearing many functional receptors for EGF and having high saturation densities (e.g., B604A, B604B, HA527A, and HA527B) could be utilized as sources of hamster embryonic EGF receptors for the preparation of antisera a-

gainst the receptors.

Adamson and Meek (1984)¹²⁾ reported that the number of EGF receptors increased during mouse development in all tissues they had, examined, but that the affinity of the receptors declined in carcass and placenta and remain relatively unchanged in brain and liver. Therefore they suggested EGF that EGF might function differently throughout development, probably by modification of EGF receptor molecules, and they proposed that such changes in the nature of EGF receptors during development could occur only by alteration of their carbohydrate moiety. Therefore, we think it important to get antibodies specific for embryonic EGF receptors and to study the changes in distribution of embryonic EGF receptors and their biochemical properties for the purpose of understanding palatogenesis from the stand-point of endocrinology.

According to our growth data on these hybrid cells, only those which have more than about 6×10^4 binding sites per cell expressed positive responses, i.e., cell growth, to EGF, suggesting that some threshold may exist in the amount of EGF receptors

Table 1 Mitogenic responses of selected clones to EGF and amounts of EGF receptor. Each clone was seeded at a cell number of 5×10^4 per 35 mm dishes followed by culture for 1 day. Then the medium was changed to DME medium containing 10 % FCS with or without 10 ng/ml EGF and cultured for more 6 days; the cells were then trypsinized and counted by haemacytometer. Medium changes were performed on day 3 and 5 of culture. Experiments were done in triplicate, and each datum of cell growth is the mean value $\pm$ SE. The mean differences in the cell number of cultures treated with EGF and those without EGF were assessed by Student's t-test. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$)

Confluent monolayer of each clone were incubated at room temperature for 4 h in 0.2 ml of the binding medium containing 50 ng/ml ^{125}I -EGF with (non-specific binding) or without (total binding) an excess of unlabelled EGF (5 $\mu\text{g/ml}$). The amount of non-specific binding was subtracted from that of the total binding to give the specific binding. Each datum indicates the amount of specific binding per 10 cells. The specific radioactivities of ^{125}I -EGF used were 235, 198 and 169 cpm per fmol. Experiments were performed in duplicate.

cell	-EGF ($\times 10^{-5}$ cells/dish)	+EGF	$\frac{+EGF}{-EGF}$	^{125}I -EGF bound (fmol/ 10^5 cells)
Hamster palatal cells (parent)	6.58 ± 0.26	19.56 ± 0.33	2.97***	36.32
Ltk ⁻ cells (parent)	23.38 ± 0.91	23.29 ± 0.56	1.00	0.00
A 524 fib	5.77 ± 0.12	6.64 ± 0.16	1.15**	10.49
B 604 A	7.26 ± 0.20	10.24 ± 0.26	1.41***	20.89
B 604 B	9.22 ± 0.29	12.46 ± 0.29	1.35***	18.92
C 528 A	7.93 ± 0.15	9.32 ± 0.12	1.18***	10.81
HA 527 A	8.85 ± 0.23	13.39 ± 0.26	1.51***	16.01
HA 527 B	11.56 ± 0.30	15.72 ± 0.21	1.36***	15.82
I531 B	2.86 ± 0.09	3.21 ± 0.08	1.12*	8.67
NI531 D	4.64 ± 0.20	5.61 ± 0.15	1.21**	26.86
NI531 E	3.81 ± 0.02	5.64 ± 0.16	1.48***	22.00
A 527 B	6.03 ± 0.14	5.70 ± 0.15	0.95	2.52
A 605 F	3.98 ± 0.06	3.87 ± 0.15	0.97	0.81
C 528 B	7.78 ± 0.17	7.66 ± 0.16	0.98	6.27
F 528 B	6.94 ± 0.11	6.88 ± 0.20	0.99	4.68
F 528 C	6.73 ± 0.10	6.60 ± 0.20	0.98	1.06
I531 E	2.84 ± 0.08	2.98 ± 0.03	1.01	9.64
E 528 A	6.34 ± 0.10	6.19 ± 0.16	0.98	0.00
E 531 C	7.70 ± 0.10	7.21 ± 0.09	0.94	0.00
RO 605 A	9.48 ± 0.17	8.61 ± 0.28	0.91	0.00

required for the stimulation of cell growth in these cells. Adamson and Rees (1981)¹⁶⁾ stated in their review that no relationship was apparent between EGF receptor number and proliferative responses to EGF when a comparison was made of the data on various types of cells studied by many researchers. The reason why some relationship was not seen between these two parameters is not clear, but, we think that we could see some positive correlation between them

because the clones we have obtained had similar properties to each other.

The cell morphology was quite variable between clones; however, the reason for this is not clear. The population of hamster embryonic palatal cells themselves might be heterogeneous or certain genes may have been activated by cell fusion, resulting in the appearance of different cell morphologies. Knapp et al. (1985)¹⁷⁾ reported that in relation to the expression of genes coding for keratin

Fig. 4 Relationship between response to EGF and amounts of EGF receptors in various clones are shown in Table 1. The amounts of EGF receptors on each clone was plotted against the degree of stimulation of cell growth by EGF (+EGF/-EGF) on the ordinate.

抄録：生理活性のある EGF 受容体をもつが細胞分裂能が有限のハムスター胎仔の口蓋突起細胞と、EGF 受容体はないが無限増殖能を持つマウス Ltk⁻ 株細胞を融合させ、HAT 培地で選択したところ、次の 3 群のクローンが得られた。(1) EGF 受容体をもち、EGF によって細胞増殖が促進されるクローン、(2) EGF 受容体をもつが、EGF に反応しないクローン、(3) EGF 受容体をもたず、EGF に反応しないクローン。これらのクローンは、EGF による細胞増殖促進における EGF 受容体の役割を解明する為の研究に有用な材料を提供するものと考えられる。第 1 群のクローンは、胎仔細胞の EGF 受容体に対するモノクローナル抗体を作成するさいの抗原の良好な採取源となると考えられる。この抗体を用いて、口蓋形成における EGF の役割を追求したい。

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filaments there was a range of morphological differences among hybrid clones between He-La cells and fibroblasts derived from a human male fetus with Lesch-Nyhan disease and having a deficiency in hypoxanthine phosphoribosyl transferase.

Many of the hybrid cell clones we have obtained in this work should provide appropriate material to reveal the role of EGF-receptors in the induction of cell proliferation by EGF, and they should also provide a sufficient amount of embryonic EGF receptors for use as immunogen and for antibody screening to identify monoclonal antibodies against hamster embryonic EGF receptors.

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