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Epidermal Growth Factor Receptors Increase in Rabbit Embryonal Implantation

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배아착상에 대한 Epidermal Growth Factor 수용체의 동태

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=국문초록=

Epidermal growth facter(EGF)는 내열성이 강하고 분자량이 6045 dalton인 단쇄상의 polypeptide로써, Cohen에 의해 생쥐의 악하선에서 처음 발견된 이래, 여러학자들에 의해 많은 연구가 되어왔다. 인체의 EGF는 urogastrone이라고도 불리우며, 인체의 소변에서 처음 검출되었고 분자구조 및 생리작용이 생쥐의 EGF와 매우 유사한 것으로 판명되었다. EGF의 자세한 작용기전은 확실히 규명되어있지는 않지만 세포의 증식과 분화를 촉진시키며 위산의 분비를 억제시킨다고 알려져 있다. 또한 EGF receptor는 분자량이 170,000~180,000dalton인 세포표면의 polypeptide로써 인체, 쥐, 닭, 소 등의 세포막조직에 특이하게 결합되어 있다. 최근 수년동안 몇몇 학자들에 의해 EGF가 배아와 태아 및 태반의 성장을 촉진시키고 chorionic gonadotrophin과 placental lactogen의 분비를 증진하는데 기여할 것이라고 가정되어 왔다. 그러나 아직까지 배아착상에 대한 EGF의 작용여부에 관해서는 발표된 문헌이 없어 저자는 *radioreceptor assay*를 이용하여 EGF receptor binding과 토끼의 배아착상과의 관계를 규명하고자 임신경과에 따른 착상부위와 비착상부위의 자궁 및 태아측 태반과 모체측 태반을 분리취득하고 receptor binding assay를 시행하여 다음과 같은 결론을 얻었다.

1. 전임신군과 비임신군의 자궁조직의 membrane fraction으로부터 specific한 EGF receptor binding이 관찰되었다.
2. 착상전 임신 3일에 자궁조직의 EGF receptor수는 4.72+0.16(10 mol/μg)로 비임신시보다 의의있게 증가되어 있었고($p<0.01$), 착상시기인 임신 7일에는 착상된 부위에서 20.33+6.58로 훨씬 더 높은 측정치를 나타내었다($p<0.05$).
3. 착상이후 가장 먼저 취득된 임신 14일의 태아측 태반은 모체측 태반의 1.39+0.49에 비해 훨씬 높은 11.94+1.97의 EGF receptor 측정치를 보였다($p<0.01$).
4. 이상의 소견들로 보아 EGF가 토끼의 배아착상에 밀접한 관련이 있을 것으로 추측되며, 이러한 착상전후의 EGF의 작용은 태아측으로부터 일 것으로 예상된다.

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INTRODUCTION

Epidermal growth factor(EGF), a heat-stable, single chain polypeptide of 6045 daltons, was first discovered by Cohen(1962) in the submaxillary gland of mice. Human EGF, also known as urogastrone, was discovered in human urine and is very similar to mouse EGF in molecular structure(Gregory, 1975; Cohen and Carpenter, 1975). Both human and mouse EGF elicit the same biological responses and compete for the same cell membrance receptors(Hollengerg and Gregory, 1977).

The recognized biological actions of EGF are stimulation of cellular proliferation and inhibition of gastric secretion, but its physiologic function has not been well defined. The EGF receptor, a cell surface polypeptide of 170,000~180,000 daltons, binds specifically to cell membranes derived from a variety of human(Rao et al., 1984), rat(O'keefe et al., 1974), mouse(Adamson and Meek, 1984), chicken(Savage and Cohen, 1973) and bovine (Gospodarowicz et al., 1977) tissue.

Some investigators have postulated that EGF has a role in stimulating embryonic and feto-placental growth(Nexo et al., 1980; Adamson et al., 1981; Thorburn et al., 1981) and in stimulation of secretion of chorionic gonadotropin and placental lactogen(Wilson et al., 1984; Morrish and Siy, 1985). However, there are no published data on EGF influence for implantation. Thus, we carried out study and report the results of our investigation to determine the relationship between binding EGF receptors and implantation of rabbit blastocyst.

MATERIALS AND METHODS

Materials

Adult female New Zealand white rabbits weighing between 2.7 and 3.2kg were mated with fertile bucks. The day of mating was designated as day 0. Eight groups of rabbits were studied. In the control group, non-pregnant unmated female rabbits were used and pregnant groups were divided according to the days after coitus(day 3, 5, 7, 14, 18, 24 and 27). Uterine horns were recovered by laparotomy(Khan-Dawood and Dawood, 1984) and the implantation sites of uterine horns were separated from the interimplantion sites as described by Wide and Wide(1979) and placentae were dissected free of fetal membranes and decidua basalis and divided to fetal and maternal placenta. All these specimens were frozen at -70℃.

Receptor Preparation

All tissues were thawed quickly and washed with chilled 0.9 percent saline just before homogenization. The tissue was then homogenized for 30 sec. with a Tissumizer homogenizer(Tekmar Company, Cincinati, Ohio) at 4 ℃ in 10mM Trishydrochloride buffer(pH7.0), containing 250mM sucrose and 1mM $CaCl_2$. The homogenate was centrifuged at 800×g at 4℃ for 15 minutes. The resulting supernatant was again centrifuged at 100,000×g at 4℃ for 60 minutes to get the plasma membrane fraction. The 100,000×g pellet was then resuspended in 5mM Tris-HCl buffer (pH7.0), containing 125mM sucrose, 0.5mM $CaCl_2$, 75mM NaCl and 0.5% bovine serum albumin. The protein content in homogenate aliquots was determined by the method of Lowry et al(1951) using BSA as the standard and stored at -70℃ until used.

Reagents

Mouse EGF and rabbit EGF antisera were obtained from Collaborative Research Inc. (Bedford, Massachusetts). Sodium iodide I-125 solution was obtained from Amersham Corp.(Arlington Height, Illinois).

Receptor Binding Studies

^{125}I-mouse EGF was prepared by iodinating mouse EGF with ^{125}I (specific activity 16.9mCi/μg iodine; Amersham Corp.) according to the chloramine-T method(Hunter et al., 1963). Free unincorporated iodine was removed by absorption with Hycel resin beads (Hycel Inc., Houston, Texas). The radioiodinated mixture was purified by gel filtration on a Sephadex G-25 column(0.9 by 17Cm). The specific activity of final purified ^{125}I-mouse EGF varied between 160 and 210μCi/μg.

Fifty microliter aliquots of plasma membrane preparation containing 300-600μg homogenate protein were incubated for 2 hours at 22℃ with 50μl of increasing concentration of ^{125}I-EGF(about 3,000-50,000cpm), 5mM Tris-HCl buffer(pH7.0; containing 125mM sucrose, 0.5mM $CaCl_2$, 75mM NaCl and 0.5% bovine serum albumin) with or without 50μl of 100-fold excess unlabelled EGF. The total incubation volume was 200μl. The reaction was terminated by the addition of 200μl chilled 10mM Tris-HCl buffer(pH7.0). The mixture was then centrifuged at 10,000×g for 30 minutes at 4℃. The supernatant was aspirated and the pellets were counted for 1 minute in a γ-counter(Beckman Instruments Inc., Palo Alto, California) with a counting efficiency of approximately 93% for ^{125}I. The EGF receptor specific binding to ^{125}I-mouse EGF was analyzed by five point Scatchard plots(Scatchard, 1949) for each individual tissue specimen and the respecitive binding parameters, including the number of binding sites, the association constant(Ka) and the dissociation constant(Kd) were determined.

Statistical analysis

Differences in EGF receptor concentrations before and after mating, from one day to another, between implantation and interimplantation sites of the uterus and between fetal and maternal placentae were analyzed for statistical significance by means of nonpaired Student's t test and a two-tailed p value. P values>0.05 were considered to be not significant.

RESULTS

Epidermal growth factor receptors in uterine tissues

The levels of EGF binding capacity in the implantation and interimplantation sites of rabbit uterus are shown in Fig. 1. The membrane fraction of uterine tissue had detectable EGF receptors as measured by the specific binding of ^{125}I-labeled EGF. The EGF receptor level was $1.77\pm0.39\times10^{-19}$mol/$\mu$g prorein before mating and increased significantly to $4.72\pm0.16\times10^{-19}$mol/$\mu$g of protein 3 days after mating($p<0.01$) and decreased to $1.88\pm0.21\times10^{-19}$mol/$\mu$g of protein 5 days after mating and increased again markedly to $20.33\pm6.58\times10^{-19}$mol/$\mu$g of protein 7 days after mating(implantation time) in the implantation sites($p<0.05$), but remainde low(1.85 ± 0.51) 7 days after mating in the interimplantation sites. At day 7 postcoitum, embryonic implantation sites were

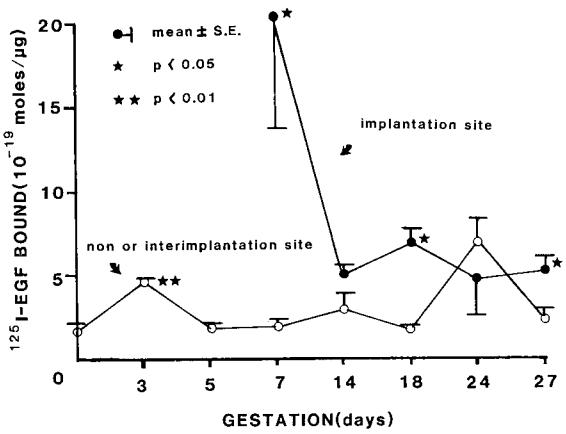

Fig. 1. ^{125}I-EGF binding to implantation and interimpantation site of rabbit uterus.

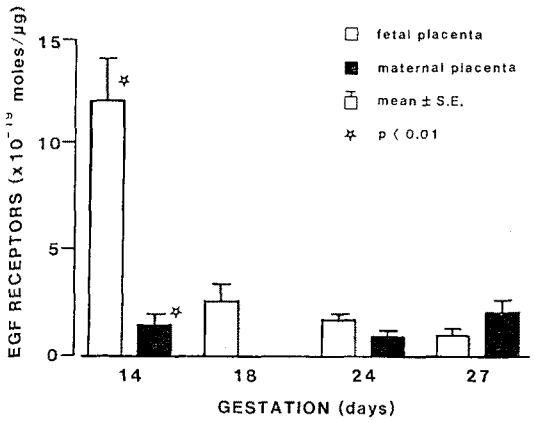

Fig. 2. EGF receptor binding to fetal and maternal placenta of rabbit.

observed from all pregnant uterine tissues. At day 14 postcoitum, the EGF receptor concentration of implantation sites was $4.73 \pm 0.73 \times 10^{-19}$mol/$\mu$g of protein, showing marked decrease compared to day 7 postcoitum. And thereafter EGF receptors remained significantly higher in implantation sites than in nonpregnant control group and interimplantation sites on days 18, 24 and 27($p<0.01$, $p<0.05$).

Epidermal growth factor receptors in placental tissues

Fig. 2. shows the number of EGF receptors in the fetal and maternal placental tissues of rabbits. At days 14 after mating, fetal and maternal placenta were separated from uterine implantation site. EGF receptor number was measured from all placental tissues excluding maternal placental tissue on days 18 after mating. On day 14, the number of EGF receptors measured from the fetal placenta was $11.94 \pm 1.97 \times 10^{-19}$mol/$\mu$g of protein, which was significantly higher than the control group and the maternal placental tissue($p<0.01$), but a decreasing value was observed on day 18, 24 and 27. However, specific binding of iodinated EGF was not present on day 18 of maternal placenta and binding capacity of maternal placenta showed decreased levels compared to fetal placenta from days 14 through 24 after mating. But, on day 27, immediately before delivery, maternal placenta showed the higher EGF receptors compared to fetal placenta.

DISCUSSION

Recently there are new methods introduced in dealing with infertility such as in vitro fertilization and embryo transfer that are well accepted. However, further development has been limited due to difficulty in embryonal implantation as well as rather high failure rate of pregnancy even after successful fertilization and in vitro development(Edwards and Fishel, 1987). The exact mechanism of implantation is not fully understood but it is postulated that embryo, uterine tissue and intrauterine secretion are interrelated by well organized hormonal control that may regulate the embryo implantation(Biggers, 1981). Especially, around the time of implantation, maternal blood flow has no direct effect on fetus and there is no any embryonic blood flow itself. Thus, it may be autocrine or paracrine hormonal effect rather than endocrine.

It has been postulated that rabbit embryonal implantation occurs on days 7 after mating and that implantation site is controlled by chorionic gonadotropin, chorionic gonadotropin like structure(Haour and Saxena, 1974; Fujimoto et al., 1975; Asch et al., 1979; Ellinwood et al., 1979), or estrogen secreted by embryo itself(Dickman et al., 1976; Psychoys, 1976; Dickman, 1979). However, the recent studies done by Khan-Dawood and others with the rabbit confirmed that β-HCG and β-HCG receptors are present in embryo but not β-HCG receptors in uterine tissue(Khan-Dawood and Dawood, 1984). The same authors additionally suggested that there were the signifi-

cantly increased level of estrogen receptors in the implantation site and the estrogen concentration in cellular fluid at the same location was not increased(Khan-Dawood and Dawood, 1984). Furthermore, Martel and Psychoyos(1981) reported that mouse embryo did not produce estrogen and there were a few estrogen receptors in embryonal implantation sites.

In our experiment, the number of EGF receptors was significantly higher than that in non-pregnant control group at day 3 after mating($p<0.01$). These data are in agreement with Khan-Dawood and Dawood (1984) in which estrogen and estrogen receptors measured from rabbit uterine flushing and uterine tissue showed similarly increased levels on days 3 after mating. This characteristics as well as the marked increasing EGF receptors on day 7(implantation time) of implantation sites suggests that EGF may play an important role in the preparation of embryonal implantation on uterine tissue.

The exact mechanism of EGF effect on embryonic implantation is unclear. But Wilson et al.(1984) and Morrish and Siy(1985) reported that EGF stimulates the secretion of HCG and HPL from cultured term human placenta and Bahn et al.(1980) confirmed that EGF in cultured choriocarcinoma cell causes increased production of progesterone and Greene and Lloyd(1985) also emphasized that EGF stimulates mesenchymal cell of embryonic palate to increase the capacity of prostaglandin synthesis. Thus, further studies on the interaction between EGF and other hormones such as HCG, HPL, estrogen, prostaglandin and progesterone may broaden the knowledge on embryonic implantation. Moreover, in the present investigation with placenta, the level of EGF receptors in fetal placenta were significantly higher than the maternal placenta especially on days 14 after mating. Thus, we postulate that the source of EGF may be derived from fetal side rather than maternal one. However, our results contradicts with Rao et al. (1985), who confirmed that EGF receptors are present on the microvillous plasma membrane near the maternal blood flow at term human placenta using autoradiography. This contradiction may be the result of using only term placenta. Because we obtained similar result on term rabbit maternal placenta(day 27) having higher EGF receptors to fetal placenta.

Smith and Talamantes(1986) revealed that the EGF receptors of mouse placenta were lowest in number on days 14 after mating compared to day 10 and day 17. Brown et al.(1987) measured the EGF receptors in 35 human placentas at last trimester and confirmed that EGF receptor binding capacity increased with respect to the duration of pregnancy. Our results also reveals that the EGF reseptors of fetal placenta were maximum in number on days 14 after mating and had a decreasing levels on day 18, 24 and 27. On the other hand, the EGF receptors of maternal placenta was maximum in number on days 27 after mating.

In conclusion, our study in the rabbit indicates : (1) Specific EGF receptor binding was detected from uterine and placental tissues; (2) On day 3 postcoitum(before implantation). EGF receptors of uterine tissue were significantly higher in number than control group's(unmated); (3) On day 7 postcoitum(implantation time), the number of EGF receptor was maximum at the implantation site of uterine tissues; (4) On day 14 after mating(after implantation), the EGF receptors of fetal placenta was markedly higher in number compared to maternal placenta; (5) Thus, our data suggest that EGF plays a major role in the implantation

of rabbit embryo and the source of EGF is derived from fetal side.

인 용 문 헌

Adamson ED, Deller MH, Warshaw JB : Functional EGF receptors are present on mouse embryo tissues. *Nature* 1981, 291, 656-659.

Adamson ED, Meek J : The ontogeny of epidermal growth factor receptors during mouse development *Dev Biol* 1984, 103, 62-70.

Asch RH, Fernandez EO, Siler-Khodr TM, Pauerstein CJ : Evidence for a human chorionic gonadotropin-like material in the rabbit blastocyst. *Fertil Steril* 1979, 32, 697-702.

Bahn RS, Speeg KV, Ascoli M, Rabin D : Epiermal growth factor stimulates production of progesterone in cultured human choriocarcinoma cells. *Endocrinology* 1980, 107, 2121-2123.

Biggers JD : Cell biology of the developing egg. In : Glasser SR, Bullock DW(Eds) : Cellular and Molecular Aspects of Implantation. Plenum Press, New York, 1981, pp.39-42.

Brown MJ, Cook CL, Henry JL, Schultz GS : Level of epidermal growth factor binding in third-trimester and term human placentas. *Am J Obstet Gynecol* 1987, 156, 716-720.

Cohen S : Isolation of a mouse submaxillary protein accelerating incisor eruption and eyelid opening in the newborn animal. *J Biol Chem* 1962, 237, 1555-1562.

Cohen S, Carpenter G : Human epidermal growth factor isolation and chemical and biological properties. *Proc Natl Acad Sci USA* 1975, 72, 1317-1321.

Dickman Z : Blastocyst estrogen : an essential factor for the control of implantation. *J Steroid Biochem* 1979, 11, 771-775.

Dickman Z, Dey SK, Sengupta J : A new concept : control of early pregnancy by steroid hormones originating in the preimplantation embryo. *Vitam Horm* 1976, 25, 17-19.

Edwards RG, Fishel SB : The human uterus in the luteal phase and early pregnancy. In : Edwards RG, Purdy JM(Eds) : Human Conception In Vitro. Academic Press, New York, 1987, pp.257-288.

Ellinwood WE, Seidel GE Jr, Niswender GD : Secretion of gonadotropic factors by the preimplantation rabbit blastocyst. *Proc Soc Biol Med* 1979, 161, 136-141.

Fujimoto S, Euker JS, Riegel GD, Dukelow WR : On a substance cross reacting with luteinizing hormones in the preimplantation blastocyst fluid of the rabbit. *Proc Jpn Acad Sci* 1975, 51, 123-128.

Gospodarowicz D, Moran JS, Owashi ND : Effect of fibroblast growth factor and epidermal growth factor on the rate of growth of amniotic fluid derived cells. *J Clin Endoclinol Metab* 1977, 44, 651-659.

Greene RM, Lloyd MR : Effect of epidermal growth factor on synthesis of prostaglandins and cycle AMP by embryonic palate mesenchymal cells. *Biochem Biophys Res Comm* 1985, 130, 1037-1043.

Gregory H : Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature* 1975, 257, 325-327.

Haour F, Saxena BB : Detection of a gonadotropin in rabbit blastocyst before implantation. *Science* 1974, 185, 444-445.

Hollenberg, MD, Gregory H : Human urogastrone and mouse epidermal growth factor share a common receptor sites in cultured human fibroblast. *Life Sci* 1977, 20, 267-274.

Hunter FC, Greenwood WM , Glover JS : The preparation of ^{131}I -labelled human

growth hormone of high specific radioactivity. *Biochem J* 1963, 89, 114-123.

Khan-Dawood FS, Dawood MY : Chorionic gonadopin receptors and immunoreactive chorionic gonadotropin in implantation of the rabbit blastocyst. *Am J Obstet Gynecol* 1984, 148, 359-365.

Khan-Dawood FS, Dawood MY : Implantation of the rabbit blastocyst; sequential changes in estradiol and progesterone and their receptors. *Am J Obstet Gynecol* 1984, 150, 450-445.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ : Protein measurement with the folin phenol reagent. *J Biol Chem* 1951, 193, 265-275.

Martel D, Psychoyos A : Estrogen receptors in the nidatory sites of the rat endometrium. *Science* 1981, 211, 1454-1455.

Morrish DW, Psychoyos A : Modulation of human chorionic gonadotropin and placental lactogen secretion by epidermal growth factor in serum-free culture of normal human term placental cells. *Clin Res* 1985, 33, 28A.

Nexo E, Hollengerg MD, Figuerova A, Pratt RM : Detection of epidermal growth factor-urogastrone and its receptor during fetal mouse development. *Proc Natl Acad Sci USA* 1980, 77, 2782-2785.

O'Keefe E, Hollenberg MD, Cuatrecasas P : Epidermal growth factor characteristics of specific binding in membranes from liver, placenta and other target tissues. *Arch Biochem Biophys* 1974, 164, 518-526.

Psychoyos A : Hormonal control of uterine receptivity for nidation. *J Reprod Fertil (suppl)* 1976, 25, 17-19.

Rao CV, Carman FR, Chegini JN, Schultz GS : Binding sites for epidermal growth factor in human fetal membranes. *J Clin Endoclinol Metab* 1984, 58, 1034-1042.

Rao CV, Carman FR, Mitra SB, Chegini JN, Cook CL, Schultz GS : Topography of human placental receptors for epidermal growth factor. *J Biol Chem* 1985, 260, 1705-1710.

Savage CR, Cohen S : Proliferation of corneal epithelium induced by epidermal growth factor. *Exp Eye Res* 1973, 15, 351-366.

Scatchard G : The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 1949, 51, 660-672.

Smith WC, Talamantes F : Characterization of the mouse placental epidermal growth factor receptor : Changes in receptor number with day of gestation. *Placenta* 1986, 7, 511-522.

Thorburn GD, Waters MJ, Young IR : Epidermal growth factor : a critical factor in fetal maturations? CIBA Foundation Symposium 86 : The Fetus and Independent Life. Pitman, London, 1981, pp172, 198.

Wide L, Wide M : Chorionic gonadotropin in the mouse from implantation to term. *J Reprod Fertil* 1979, 57, 5-9.

Wilson EA, Jawad, MJ, Vernon MW : Effect of epidermal growth factor on hormone secretion by term placenta in organ culture. *Am J Obstet Gynecol* 1984, 149, 579-580.