Gene Gun-Mediated Human Erythropoietin Gene Expression in Primary Cultured Oviduct Cells from Laying Hens

H. Ochiai, H. M. Park, R. Sasaki[1], J. Okumura and T. Muramatsu[2]
Department of Applied Genetics and Physiology, Graduate School of Bioagricultural Sciences, Nagoya University
Nagoya 464-8601, Japan

ABSTRACT : Factors affecting gene gun-mediated expression of the human erythropoietin gene were investigated in primary cultured oviduct cells from laying hens. The human erythropoietin gene was transfected by a gene gun method at 1.25 μg per dish, and cultured in a synthetic serum-free medium for 72 hrs. The concentration of human erythropoietin mRNA was determined by RNA:RNA solution hybridization. In experiment 1, the effect of changing the shooting pressure of DNA-coated microparticles with nitrogen gas was tested at 20 and 60 kgf/cm^2. The results showed that the erythropoietin mRNA concentration was significantly higher at 60 than 20 kgf/cm^2. In experiment 2, the effects of supplementing the medium with fetal calf serum at 10%, and raising the shooting pressure from 60 to 80 kgf/cm^2 on the cell number and erythropoietin gene expression were examined. Although supplementation with fetal calf serum significantly increased the cell numbers compared with no supplemented controls ($p < 0.05$), erythropoietin mRNA concentration per 10^3 cells was not affected. Raising the shooting pressure from 60 to 80 kgf/cm^2 did not affect either of the parameters. In experiment 3, the effect of supplementing ascorbate 2-phosphate at 0.5 mM was tested. The results indicated that the ascorbate supplementation significantly increased the cell number ($p < 0.05$), and tended to increase erythropoietin mRNA concentration ($p < 0.1$). Thus, for human erythropoietin gene expression by using the gene gun method, shooting pressure with nitrogen gas should be sufficient at 60 kgf/cm^2, and supplementation with ascorbate phosphate would be useful to enhance not only the cell proliferation but also erythropoietin gene expression.
(**Key Words**: Human Erythropoietin; Gene Gun; Primary Cultures; Oviduct Cells; Laying Hens)

INTRODUCTION

Erythropoietin (EPO) is known as a principal glycoprotein hormone that stimulates erythropoiesis (Gesundheit et al., 1994). The synthesis of EPO may occur mainly in the kidney (Jacobson et al., 1986). When anemia occurs due to blood loss, EPO mRNA level increases remarkably by a factor of 200 (Bondurant and Koury, 1986). In patients with chronic renal failure, administration of EPO could boost hematocrit values and prevent both anemia and the need for transfusion (Eschbach et al., 1987).

Recent progress in recombinant DNA technology has made it possible to synthesize EPO in cultured cell lines such as Chinese hamster ovary cells (Lin et al., 1985) and baby hamster kidney cells (Tsuda et al., 1990). However, subtle differences in sugar chains of recombinant EPO from naturally purified human EPO might cause antigenicity after repeated administration of recombinant EPO to patients, resulting in poor biological activity. Accordingly, optimal host cell sources for EPO production should be searched.

Because oviduct tubular gland cells of laying hens synthesize and secrete a large amount of egg white proteins, at a rate of approximately 3 to 4 g per entire oviduct a day (Gilbert, 1971), the hen's oviduct cells might serve as a good host cell for producing EPO. However, laying hen's oviduct cells cannot be easily utilized for therapeutic protein production. This is primarily because common DNA transfection methods such as calcium phosphate precipitation and lipofection are not suitable for these fully differentiated cells. Thus, if hen's oviduct cells are to be used, a new DNA transfection technology should be employed.

Of nonviral gene transfection methods, the gene gun provides a simple and convenient means of gene transfer, in principle, to any types of dividing and nondividing

[1] Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan.
[2] Address reprint request to T. Muramatsu.
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cells *in vivo* and *in vitro* (Yang et al., 1990; Muramatsu et al., 1996, 1997a). The gene gun method was thought, therefore, to offer a good opportunity of gene transfer to primary cultures of laying hen's oviduct cells, which appears to reject common transfection procedures such as calcium phosphate transfection and lipofection.

The present study was conducted to examine the possibility of transferring and expressing a gene encoding human EPO to primary cultured oviduct cells from laying hens by using a new transfection technology, the gene gun. Factors affecting the gene expression intensity were investigated such as optimal shooting pressures with nitrogen gas, and supplementing culture medium with fetal calf serum and ascorbate phosphate, because these supplements are known to stimulate cell proliferation and gene transcription (Holley and Kiernan, 1968; Hata and Senno, 1989; Sullivan et al., 1994; Muramatsu et al., 1995).

MATERIALS AND METHODS

Experimental animals

Single comb White Leghorn laying hens at 17 months of age, having an average egg production rate of about 70%, were used throughout the experiments. They were cared for under Guideline of Animal Experimentation, laid down by the Committee of Experimental Animal Care, Nagoya University, Nagoya, Japan.

Experimental procedures

For one experiment, usually one or two laying hens were sacrificed in the morning of the day of transfection by injecting sodium pentobarbital intraperitoneally. The magnum portion of the oviduct was then quickly excised, cut longitudinally, and placed on a glass plate. A portion of oviduct folds which mainly consists of tubular gland cells, was scraped off with a microscope slide glass, and transferred to Petri dish. Subsequently, the mucosal scrapings mainly consisting of oviduct folds were cut into small pieces with fine scissors to give small lumps of tubular gland cells, weighed, and approximately 1 g of tubular cell lumps was mixed with 1 ml of serum-free culture medium. The composition of the culture medium has been described elsewhere (Sanders and McKnight, 1988).

A 200 μl aliquot of the suspension of fine tubular cell lumps thus prepared was spotted onto three-fold gauze placed above a sponge packed 5 cm deep in a 10 ml syringe. Culture medium was then aspirated as much as possible by air pump from the tip of the syringe to prevent the possible splash of the sample when bombarded. While aspirating the air, 5 mg tungsten of a median particle size of 0.36 μm (Bio-Rad, USA) coated with 1.5 μg expression plasmid DNA was shot at the center of the tubular gland cell spots with the Machimpacter (Kansai Paint, Osaka, Japan) by the pressure of nitrogen gas at 20-80 kgf/cm^2 at a distance of 3 cm from the nozzle. The human EPO expression plasmid DNA, pSVhEPX, is shown in figure 1. Subsequently, the DNA transfected oviduct cell lumps were collected by suspending into 5 ml of the culture medium. The cell lumps were pelleted by centrifugation at 80 × g for 5 min, and the supernatant was discarded. This washing procedure was repeated twice. To the resultant pellet, a 800 μl aliquot of the fresh culture medium was added and the cell lumps were genetly mixed. A 200 μl aliquot of the cell suspension which contained about 5 mg of the cell lumps was transferred to a Petri dish having 10 ml of the culture medium. The primary culture of the oviduct cell lumps lasted for 72 hrs. This culture period was chosen since EPO mRNA concentration in primary cultures of oviduct cells from estrogen-stimulated immature chicks continued to increase linearly over the 72-hr period (Muramatsu et al., 1997c).

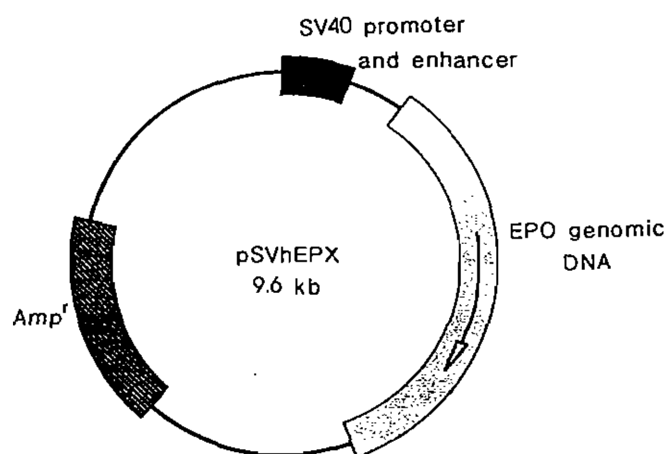

Figure 1. Circle map of pSVhEPX. The SV40 promoter drives the transcription of human erythropoietin (EPO) DNA. Amp^r : ampicillin resistance gene.

In experiment 1, the effect of changing the shooting pressure of DNA-coated microparticles with nitrogen gas on EPO gene expression was tested at 20 and 60 kgf/cm^2. In experiment 2, the effects of supplementing the medium with fetal calf serum at the final concentration of 10% on the cell number and EPO gene expression were examined. In experiment 3, the effect on the cell number and EPO gene expression of supplementing the culture medium with magnesium ascorbate 2-phosphate (Wako Pure Chemical Industries Ltd., Osaka, Japan) was tested at 0.5

mM. After the 72-hr culture period, the oviduct cell lumps were harvested, and cell extracts were obtained as described previously (Muramatsu et al., 1995, 1997b).

Chemical and statistical analyses

The concentration of EPO mRNA was determined quantitatively by RNA : RNA solution hybridization, a variant of RNase protection assay (Durnam and Palmiter, 1983) as modified by Sanders and McKnight (1985). For the RNA : RNA solution hybridization, EPO riboprobe was prepared from the bottom strand of the 840-bp DNA sequence encoding the full length of human erythropoietin cDNA derived from pZipNeoSV(X)EPO. The full length EPO cDNA was isolated by *Sau* 3A1 digestion, and subcloned into the multiple cloning site of pGEM-11Zf (−) cloning vector (Promega, Madison, WI, USA) to give pGEPO2, from which the EPO antisense RNA was transcribed *in vitro*. Cell numbers were calculated from the DNA concentration as 2.5 pg/cell according to McKnight and Palmiter (1979).

The data were treated by ANOVA, and singificance of difference between means was tested by a protected LSD method by using General Linear Model Procedures of SAS (SAS Institute, 1985).

RESULTS

The effect of changing the shooting pressure on the intensity of EPO gene expression is shown in figure 2 (experiment 1). EPO mRNA concentration was significantly increased by raising the shooting pressure of nitrogen gas for the gene gun from 20 to 60 kgf/cm^2 ($p < 0.05$).

In experiment 2, the effects of both increasing shooting pressure from 60 to 80 kgf/cm^2, and supplementing the serum-free medium with fetal calf serum to give a final concentration of 10% were tested. The results are given in figure 3. The cell numbers were significantly increased by supplementing with 10% fetal calf serum, while there was no effect by raising the shooting pressure from 60 to 80 kgf/cm^2. When the concentration of EPO mRNA was expressed as femto gram level per 10^3 cells, neither increasing the shooting pressure from 60 to 80 kgf/cm^2 nor supplementing with fetal calf serum gave sigificant impacts. Therefore, the shooting pressure would be sufficient at 60 kgf/cm^2, and fetal calf serum was effective only in cell proliferation but not the EPO gene expression intensity per 10^3 cells.

Figure 4 represents the effect of supplementing with ascorbic acid on the cell number and EPO gene expression. The ascorbate supplementation significantly increased the cell number ($p < 0.05$), and tended to increase the concentration of EPO mRNA ($p < 0.1$) in the primary cultures of oviductal cells from laying hens.

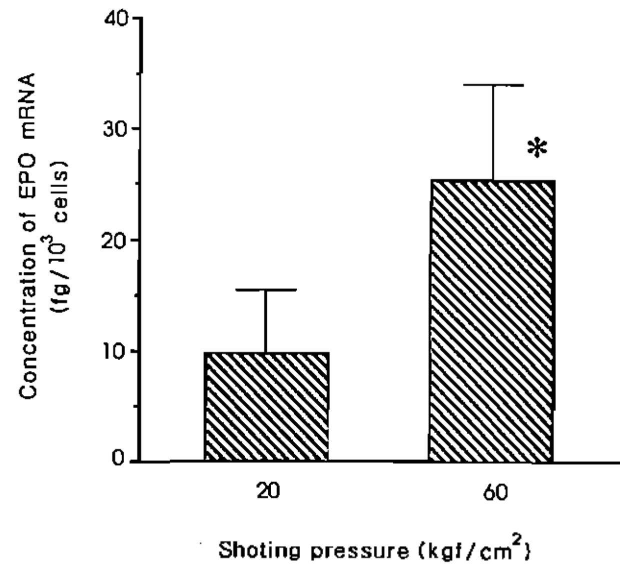

Figure 2. Effect of shooting pressure on the concentration of human erythropoietin (EPO) mRNA expressed in the primary cultured oviduct cells of laying hens. Cells were transfected by a gene gun method with pSVhEPX containing the human EPO DNA at 1.25 μg per dish, and cultured in a synthetic serum-free medium with insulin (50 ng/ml), 17β-estradiol (2×10^{-7}M), corticosterone (1×10^{-6}M) and progesterone (1×10^{-8}M). The concentration of human EPO mRNA was determined by RNA : RNA solution hybridization. Values are means ± SEM of 4 replicates. * Significantly different from the 20 kgf/cm^2 value at $p < 0.05$.

DISCUSSION

It has been recognized that DNA transfection to tubular gland cells from laying hens is difficult and virtually impossible, although the cells are abundant and available in the fully developed magnum segment of the laying hen's oviduct. There may be at least two reasons. Firstly, unlike the oviduct cells from estrogen-stimulated immature chicks, the primary cultures of laying hen's oviduct cells do not appear to be responsive to steroid treatment, since ovalbumin mRNA levels in the oviduct cells from laying hens continuously decreased during the culture period, reaching to a minimal value within a few days (Muramatsu et al., 1995). Even by supplementing the medium with estrogen and corticosterone, this gradual decrease was not counteracted, while using the same treatment, substantial induction of ovalbumin mRNA has been found in the primary cultured oviduct cells from estrogen-stimulated immature chicks (Sanders and

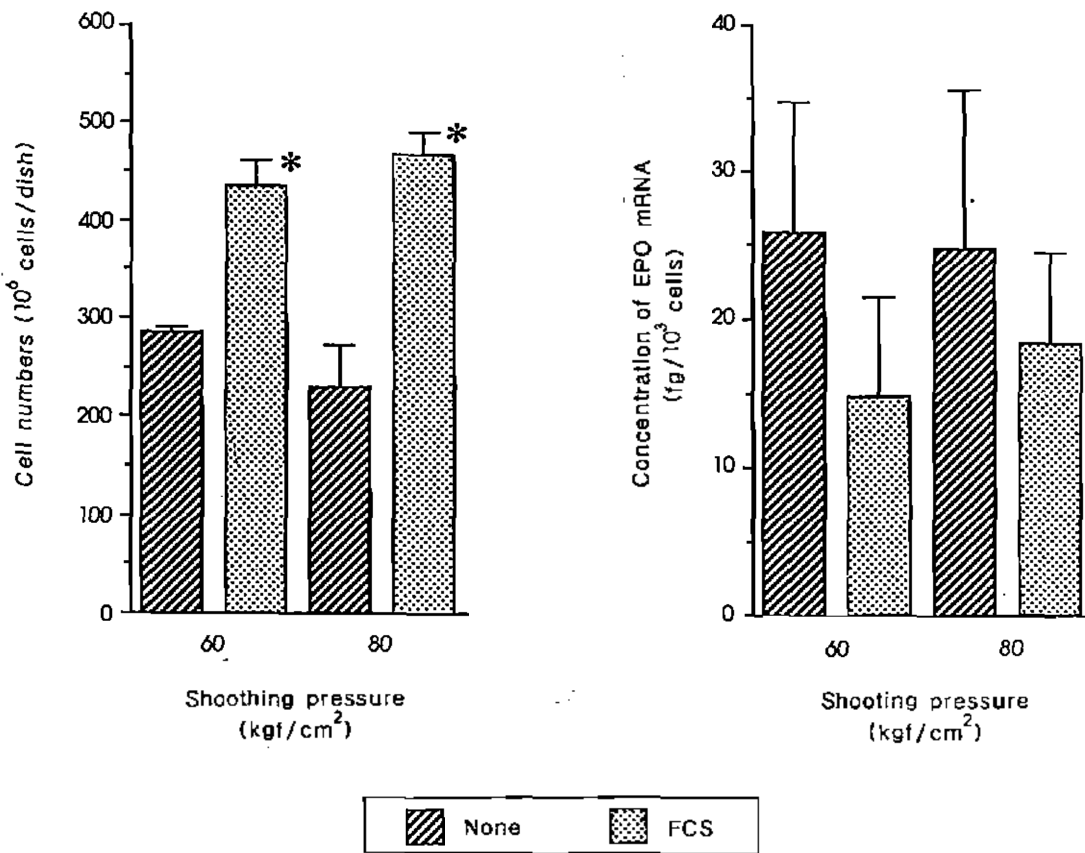

Figure 3. Effects of supplementing with fetal calf serum (FCS) and shooting pressure on the concentration of human erythropoietin (EPO) mRNA expressed in the primary cultured oviduct cells of laying hens. Cells were transfected by a gene gun method with pSVhEPX containing the human EPO DNA at 1.25 μg per dish, and cultured in a synthetic serum-free medium with insulin (50 ng/ml), 17β-estradiol (2×10^{-7}M), corticosterone (1×10^{-6}M) and progesterone (1×10^{-8}M). The concentration of human EPO mRNA was determined by RNA : RNA solution hybridization. The cell number prior to the culture was $28.3 \pm 4.7 \times 10^6$ cells per dish which was not significantly different from the FCS-free values. Values are means$\pm$SEM of 4 replicates. * Significantly different from the corresponding FCS-free values at $p < 0.05$.

McKnight, 1985; Muramatsu et al., 1995, 1997b). It may mean that in the primary cultures of laying hen's oviduct cells, the medium lacks some essential constituents such as growth factors which are not required for the oviduct cell culture from estrogen-stimulated immature chicks. Secondly and more importantly, the laying hen's oviduct cells are usually covered with a thick layer of egg white proteins that are secreted continuously. The egg white proteins that are not easily washed off, and therefore prevent foreign genes to be transfected efficiently by a common procedure such as calcium phosphate precipitation and lipofection that are good for oviduct cells from estrogen stimulated and withdrawn immature chicks (Sanders and McKnight, 1988; Park et al., 1995; Muramatsu et al., 1997b).

Under the circumstances described above, the gene gun was chosen to transfect foreign DNA. To the best knowledge of the authors, this is the first report in which the human EPO gene was expressed in primary cultures of laying hen's oviduct cells. The culture period was fixed at 72 hrs because human EPO mRNA increased continuously for up to 72 hrs when the EPO gene expression plasmid was transfected to the primary cultures of oviduct cells from estrogen-stimulated immature chicks (Muramatsu et al., 1997c). Because gene transfection efficiency by a gene gun method is critically affected by both the velocity of DNA-coated microparticles and the distance from the target cell, optimal shooting gas pressure to launch the microparticles was investigated by fixing the distance at 3 cm. The results from experiments 2 and 3 indicated that 60 kgf/cm^2 should be sufficient to have reasonably high gene expression intensity (figures 2 and 3). In order to improve the culture conditions, the effects of supplementing the

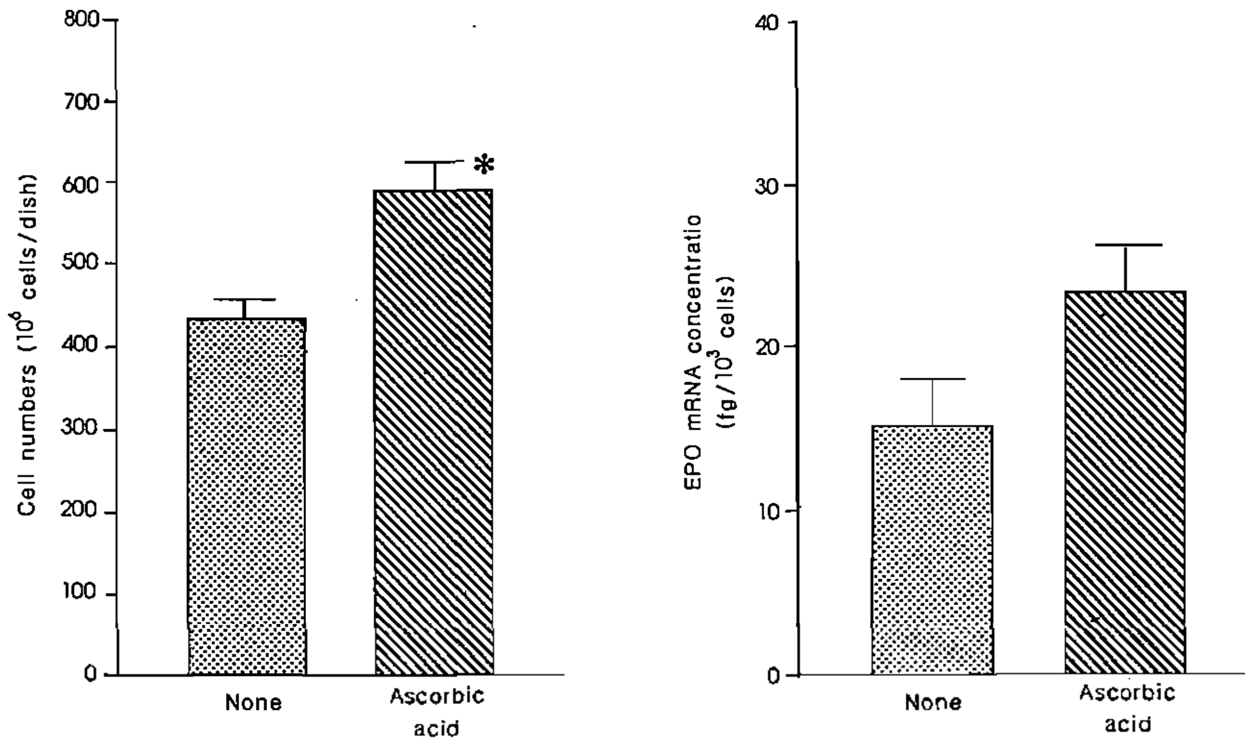

Figure 4. Effect of supplementing with ascorbic acid on the concentration of human erythropoietin (EPO) mRNA and cell numbers in the primary cultured oviduct cells of laying hens. Cells were transfected by a gene gun method with pSVhEPX containing the human EPO DNA at 1.25 μg per dish, and cultured in a synthetic serum-free medium with insulin (50 ng/ml), 17β-estradiol (2×10^{-7}M), corticosterone (1×10^{-6}M) and progesterone (1×10^{-8}M). Magnesium ascorbate phosphate was added to the medium at 0.5 mM. The concentration of human EPO mRNA was determined by RNA : RNA solution hybridization. The cell number prior to the culture was 44.1$\pm$2.5 (10^6 cells per dish), which was not significantly different from the ascorbate-free value. Values are means$\pm$SEM of 4 replicates. * Significantly different from the ascorbic acid-free value at $p < 0.05$.

medium with fetal calf serum and ascorbate phosphate were tested. The reason of testing these substances was that the former was known to enhance the proliferation of 3T3 cells (Holley and Kiernan, 1968), and the latter was found to increase not only the cell proliferation but also gene transcription of skin fibroblasts (Hata and Senno, 1989). Indeed, in our previous studies with primary cultured oviductal cells from estrogen-stimulated immature chicks, the ascorbate phosphate was found to substantially induce ovalbumin mRNA in the presence of estrogen and corticosterone (Muramatsu et al., 1995, 1997b). Ascorbate phosphate rather than ascorbic acid was used as the former is resistant to oxidation, and hence is thought to be a long-lasting vitamin C in culture (Hata, 1997).

As was expected, fetal calf serum increased the cell number when compared with the cell number prior to the culture or after the culture with serum-free medium, but did not affect EPO gene expression per 10^3 cells. In contrast, ascorbate phosphate increased the cell number compared with the cell number prior to the culture or after the culture with ascorbate-free medium. It also elevated, though to a modest extent, EPO gene expression per 10^3 cells. In this sense, ascorbate phosphate may be more potent than fetal calf serum for the total production of EPO gene transcripts. Although the cell number was increased by these supplements, it was not known whether or not the number of differentiated tubular gland cells was actually increased. The nature of change in the cell number should be further characterized in the future.

The present results indicated clearly that human EPO gene was expressed in oviductal cells from laying hens. However, neither the presence of sugar chain nor the biological activity of the human EPO protein could be determined, since the gene expression intensity was not large enough to allow the above anlalysis in our primary culture system. Before making comparison between oviductal cells from laying hens and other cell sources such as Chinese hamster ovary cells (Lin et al., 1985) or baby hamster kidney cells (Tsuda et al., 1990) as a host for human EPO production, the present gene expression system with oviduct cells may have to be further

improved.

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