Studies of Mammalian Implantation *In Vitro* (II): Effects of Cytokines from Mixed Lymphocyte Reaction on Proliferation of Murine Trophoblast Cell and Establishment of Its Cell Line

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Abstract. We report here that the supernates of mixed lymphocyte reaction (MLR) enhanced the proliferation of trophoblast cells. The analysis of the factors in MLR supernates revealed that a series of CSF family cytokines such as IL-3/GM-CSF and M-CSF, which seemed to be responsible for stimulation of the proliferation of trophoblast cells, were significantly raised in MLR supernates. At the same time, the effects of recombinant human (rh) IL-3/GM-CSF and M-CSF on the proliferation of the trophoblast cells were investigated. The rhIL-3/GM-CSF gave slight proliferation-promoting effects while the rhM-CSF showed stronger stimulating effect which was comparable to the MLR supernates. Continuous addition of the MLR supernates into the primary culture of trophoblast cells resulted in the establishment of trophoblast cell line. The cells showed the polygonal epithelium like appearances and reacted positively with anti-cytokeratin antibodies but not with anti-vimentin antibodies by means of indirect immunofluorescence (IIF) test.

Key words: Trophoblast cell line, CSF family cytokines, Mixed lymphocyte reaction, Allogeneic lymphocyte therapy.

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Mammalian pregnancy has frequently been termed as the successful nature's semi-allograft. Therefore, the allogeneic stimulation has been known to compromise the fetal survival supported by evidences [1, 2, 3] in animals and man that the prior immunization using donor-type cells can prevent abortion just as organ transplantation rejection is diminished by the prior

immunization with blood transfusion [4]. Despite the seemingly clinical benefit of the allogeneic lymphocyte therapy for the recurrent abortion, the scientific basis of the therapy remained unknown. These set of observations have shed light on the immunological approach to the mechanisms of placenta formation regulated by cytokine network at the feto-maternal interface [5].

Following our previous studies [6] on the effect of the supernates of the decidual cell line (TTK-1) and MLR on murine embryonal development, the

factors from MLR supernates essential for the proliferation of trophoblast cells was precisely analysed and the enhancing effects of rhIL-3/GM-CSF or M-CSF on the proliferation of trophoblast cells was investigated because the effects of cytokines such as IL-3/GM-CSF or M-CSF on the proliferation of placental cells have been suggested [7, 8]. Furthermore, the continuous addition of MLR supernates into the primary culture of trophoblast cells resulted in the establishment of the trophoblast cell line.

Materials and Methods

Collection of MLR supernates

The lymphocytes were isolated from heparinized peripheral blood obtained from several pairs of allogeneic healthy volunteers who differed in their HLA haplotypes, by the centrifugation on Ficoll-Paque gradients. They were washed 3 times with RPMI 1640 (Nissui, Tokyo, Japan) and suspended in the same medium. The two-way MLR with each allogeneic combination at a total density of 2×10^6 cells from 2 lymphocyte donors/well were carried out on 24 well culture plate (Nunc, Denmark) containing with 2 ml of serum free medium HM 606 (Kohjin Biol. Tokyo, Japan). Supernates were collected on 3, 5 and 7 day after the initiation of MLR culture by centrifugation and passed through 0.22 μ m millipore filter to exclude cellular components. Furthermore, the MLR-stimulated cells on each day of culture as described above were washed and recultured for additional 24 h. Each MLR supernate collected was examined for their effects on the proliferation of trophoblast cells in the primary culture. Then, they were pooled for the examination of cytokines, such as IL-3/GM-CSF and M-CSF and used as the sources of growth factors for the long term culture of embryonal trophoblast cells. The one-way MLR with the lymphocytes obtained from 5 different pairs with the recurrent abortion, who had the loss of 3 or

more confirmed pregnancies to the same partner, was also performed prior to immunization with the paternal lymphocytes, and the effects of their supernates on the proliferation of trophoblast cells in the primary culture were examined.

Examination of the effects of MLR supernates and rhIL-3/GM-CSF or M-CSF on the proliferation of the embryonal trophoblast cells

The primary culture of trophoblast cells were prepared by washing out the embryonal inner cell mass at 120 h after they had been cultured from 2-cell stage embryos (7/well), in each well of a 96-well culture plate (Nunc, Denmark) as described in the session of Materials and Methods of the preceeding paper (I) [9]. Then, the culture medium was replaced with RPMI1640 supplemented with 30% FCS (RPMI1640/30 in the following). The properly diluted test solutions such as MLR supernates, serum free medium HM606, rhIL-3/GM-CSF and M-CSF were added to the medium. Then, 48 h later, one microcurie (μ Ci) of 3 H-TdR (NEN Res Prod, Boston, U.S.A.) was added to each well of the trophoblast cell culture. The radioactivity of 24 h pulse was counted in a liquid scintillation counter (Beckman, CA, U.S.A.) following the harvest of the trophoblast cells on glass filter by cell harvester (Laboscience, Tokyo, Japan).

Preparation of polyclonal antibodies against rhIL-3/GM-CSF

The rhIL-3/GM-CSF was prepared by Kirin Brewery Co., Ltd. using E coli expression systems and rhM-CSF was also prepared by Otsuka Pharmaceut. Co., Ltd., using CHO cell expression systems. Polyclonal antibodies against each cytokine were prepared as follows. New Zealand White rabbits were immunized by subcutaneous injections of each cytokine biweekly for 8 weeks using 200 μ g of the cytokine per injection. The first immunization was performed by the cytokine emulsified in complete Freund's adjuvant and the subsequent boosts were done by administering incomplete Freund's adjuvant i.v.. The sera were collected from blood drawn 3 days after the final boost. The titer of each sera was determined by measuring their reactivity with the respective antigen by enzyme linked immunoassay (EIA) method.

Abbreviation

Mixed lymphocyte reaction: MLR, Indirect Immunofluorescence: IIF, 3 H-Thymidine: 3 H-TdR, Fetal calf serum: FCS, Granulocyte macrophage-colony stimulating factor: GM-CSF, Macrophage-colony stimulating factor: M-CSF, Interleukin-3: IL-3, Fluorescein isothiocyanate: FITC, Enzyme linked immunoassay: EIA, i.v.: intravenous, The cell line established from a patient with erythroleukemia: TF-1.

Assessment of cytokines in MLR supernates

IL-3 and GM-CSF activities in MLR supernates were assayed by examining the effects upon the proliferation of human erythroleukemia TF-1 cell line [10]. The cell suspension was seeded into 96 well culture plate at a density of 10^5 cells/ml and 50 μ l of MLR supernates were added. Cells were cultured for 72 h in the presence or absence of anti rhGM-CSF and anti rhIL-3 antibody diluted at a ratio of 1:500. After 72 h, ^3H -TdR (0.5 μCi /well) was added and the culture was continued for the following 6 h. The culture was harvested with the cell harvester and the amount of radioactivity was measured in a scintillation counter (Beckman, CA, U.S.A.). The M-CSF activity in MLR supernates was also examined by the radio-immunoassay (RIA); ^{125}I -labelled standard M-CSF was prepared by Iodogen method.

Rabbit anti M-CSF antibody (Genzyme, U.S.A.) was used as the primary antibody and goat anti-rabbit IgG (EYLab, CA, U.S.A.) as the second antibody in the RIA. Instead of MLR supernates, serum free medium (HM 606) was used for the control sample.

Long term culture of the embryonal trophoblast cells

Trophoblastic outgrowth cells obtained by the procedures previously described [9] was cultured further on the 4-well culture plate (Nunc, Denmark) in the special medium which was consisted of the mixture of RPMI 1640/30 and pooled MLR supernates as described previously. Two, 4, 6, 8, 10, and 12 months after the initiation of culture, the trophoblast cells grown were subcultured for the examination of its functional and morphological marker by transferring them into a larger scale culture bottle.

IIF test with anti cytokeratin and anti vimentin serum

Cells were fixed with 75% ethanol and treated with rabbit anti-vimentin (Transformation Res., MA, U.S.A.) and anti-cytokeratin antibodies (Dako Corp., CA, U.S.A.) followed by the incubation with the secondary FITC labelled F(ab')₂ of goat anti-rabbit IgG (EYLab, CA, USA).

Analysis of karyotypes in the established trophoblast cell line

Colcemid (0.05 $\mu\text{g}/\text{ml}$) was added to the culture of trophoblast cell line and incubated for 3 h. Then, cells were removed from the substratum by the treatment with trypsin (Mochida, Tokyo, Japan) solution (0.02% W/V). The cell suspension was centrifuged at 1000 rpm for 5 min and the pellets were treated with the hypotonic solution containing 1% sodium citrate for 15 min at room temperature.

The fixative solution (1:3 of glacial acetic acid and methyl alcohol) was slowly added to the cell suspension and gently shaken for 15 min. Then, the suspension of fixed cells was centrifuged at 1000 rpm for 5 min. Two to 3 drops of cell suspension was pipetted onto a wet glass slide. The specimens were immediately dried by gently warming the slide over a flame, and a cover slip was mounted on the slide for observation and storage with Tissue-TekII (Miles Lab., U.S.A.).

Results

Effect of MLR supernates on the proliferation of trophoblast cells

As shown in Fig. 1, the supernates of one-way MLR between a couple of husband and wife with recurrent abortion, on 3 day or 7 day after the initiation of culture, showed enhancing effect on the growth of the primary culture of murine embryonal trophoblast cells in a dose dependent manner. Its growth rate was 10 times higher than that of the control.

MLR supernates collected on 3 day of culture exhibited stronger stimulatory activity to the proliferation of trophoblast cells than MLR supernates on 7 day of culture.

Those facts suggested that the proliferation-stimulating factors in the trophoblast cells were produced on 3 day of culture more abundantly than on 7 day of culture. The other MLR supernates from the recurrent abortive pairs and/or healthy pairs gave almost the same results as those described above (data not shown).

Assay of cytokines in MLR supernates

The content of IL-3/GM-CSF in MLR super-

nates was assessed by means of the proliferation assay using human erythroleukemia TF-1 cell line, growth of which shows the complete dependence

on GM-CSF or IL-3. The highest activity was detected in the 3-day MLR supernates as shown in Fig. 2.

The 3-day MLR-stimulated lymphocytes were recultured for additional 24 h after washing. The supernates from them also gave the maximal effects on the proliferation of TF-1 cells compared with the 5- or 7-day supernates. Furthermore, as shown in Fig. 3, the addition of both anti IL-3 and anti GM-CSF antibody to the 3-day MLR supernates significantly inhibited the proliferation of TF-1 cells, although the anti IL-3 or anti GM-CSF antibodies added separately inhibited only moderately the proliferation of the cells, implying that those cytokines were indeed present in the MLR supernates.

The actual content of M-CSF in the MLR supernates was assayed by RIA, which revealed that the concentration of M-CSF was also significantly elevated in the 3-day MLR supernates compared with that of control as shown in Fig. 4.

* The 25% addition of supernates from singleculture at 3 day was used as the control (100).

Fig. 1. Effect of one-way MLR supernates on proliferation of trophoblast cells. MLR supernates were added to the primary culture of trophoblast cell at various concentrations (% V/V). Each column and vertical bar represents the mean $\pm$ SD of triplicate experiments as the percentage of control.

Fig. 2. Effect of MLR supernates of various intervals on proliferation of TF-1 cells. Each column shows the additional effect of the 3-day (MLR3), 5-day (MLR5), 7-day MLR supernates (MLR7). MLR3', MLR5', MLR7' represents MLR supernates from the recultured lymphocytes for additional 24 h. For details of the experiments, see the text.

Effects of rhIL-3, GM-CSF or M-CSF on the proliferation of trophoblast cells

As shown in Table 1, rhIL-3/GM-CSF showed the slight enhancement of the proliferation of

Fig. 3. Effect of MLR supernates and its neutralization with anti-cytokine antibody on proliferation of TF-1 cells. Left column shows the effect of the 3-day MLR supernates on the proliferation of TF-1 cells. Right column shows the neutralizing effect of anti rhIL-3 and anti rhGM-CSF antibodies upon the proliferation-stimulating activities of the supernates.

Fig. 4. Radioimmunoassay of M-CSF in MLR supernates. Each column shows the amount of M-CSF in the 3-day (MLR3), 5-day (MLR5) and 7-day MLR supernates (MLR7). Serum free medium (HM 606) was used as the control.

trophoblast cells (1.7 times of control at 4 $\mu\text{g/ml}$ of IL-3 and 1.8 times of control at 9 $\mu\text{g/ml}$ of GM-CSF). The rhM-CSF gave more prominent proliferative effect on the growth of trophoblast cells in the primary culture (2.8 times of control at 7.5 $\mu\text{g/ml}$ of M-CSF), which was comparable to MLR supernates (2.6 times of control at 50% dilution). The effects of rhM-CSF clearly showed the dose dependency.

Establishment and identification of trophoblast cell line

As shown in Fig. 5a,b, the continuous addition of MLR supernates to the primary culture of trophoblast cells promoted the proliferation of trophoblast cells. Gradually, the polygonal epithelium like cells became morphologically detectable in the focus nest of the tissue culture of this cell line as shown in Fig. 5c. The cells continued to proliferate over 12 months after the initiation of the primary culture. By IIF test with anti-cytokeratin and anti-vimentin antibodies, cytokeratin of this cell line was clearly stained as shown in Fig. 6a compared to the negative control as shown in Fig. 6b. Vimentin was not stained in this cell line. Furthermore, analysis of the karyotype of the cell line revealed that the cells contained all acrocentric chromosomes and the modal number of chromosome was 41 as shown in Fig. 7.

Discussion

The concept that uterine local secretions may be involved in growth of the fetus has been recently formulated by indicating that the autocrine and paracrine growth factors which may directly in-

Table 1. Effect of rhCSF family on proliferation of trophoblast cells

GM-CSF	50%	(9 $\mu\text{g/ml}$)	:	5705 cpm*
	25		:	3361
	12.5		:	4510
IL-3	50	(8 $\mu\text{g/ml}$)	:	4244
	25		:	5397
	12.5		:	5198
M-CSF	50	(7.5 $\mu\text{g/ml}$)	:	8782
	25		:	3948
	12.5		:	2775
MLR Sup.**	50		:	8217
Cult. Med.***	50		:	3112

The rhGM-CSF, IL-3 and M-CSF was added into the primary culture of trophoblast cells in the dose-dependent manner as shown in parenthesis and their effects on the proliferation of trophoblast cells was examined by the incorporation of $^3\text{H-TdR}$.

*: $^3\text{H-TdR}$ incorporation of trophoblast cells from 7 blastocystic embryos / well.

**: Supernates from MLR in serum free medium (HM606).

***: Culture medium from fresh serum free medium (HM606).

fluence the growth of fetuses, extra-embryonic membranes and the formation of placentae [11, 12, 13]. Most likely, they could also include various cytokines that regulate the immunological reactions against the semi-allogeneic fetus. Among them, the best candidates for such uterine growth factors are CSF family cytokines because they were originally shown to be present at high concentrations in the pregnant mouse uterus [14, 15].

The majority, if not all, of this CSF activity might be M-CSF which regulates the proliferation and differentiation of trophoblast cells [16]. Pollard *et al.* [17] reported that a pregnancy defect in the osteopetrotic (op/op) mouse is due to the deficiency of macrophages and osteoclasts followed by a complete absence of M-CSF.

IL-3/GM-CSF also stimulates the proliferation of placental cells as shown by Athanassakis *et al.* [7]. Based on these facts, Wegmann [18] have proposed the immunotrophic hypotheses that GM-CSF and other T-cell lymphokines produced as a consequence of T-cell activation by the allogeneic fetus have a role in promoting placental growth.

Furthermore, in their recent report [8], it was revealed that cytokines of the CSF family, including IL-3/GM-CSF, increased the chances of fetal survival when injected into abortion-prone mice (CBA/J $\times$ DBA/2) reducing resorption rates in their mating combination. In the clinical fields, it

Fig. 5. Phase contrast photomicrography shows the processes of the establishment of trophoblast cell line (a; the primary culture of trophoblast cells, b; three months in culture, c; twelve months in culture: $\times 100$).

Fig. 6. Trophoblast cell line cells were stained strongly with anti-cytokeratin antibodies (a) but not stained at all with anti vimentin antibodies (b), as revealed by IIF method ($\times 400$).

has been suggested that the defective recognition of fetal alloantigens by the maternal immune system is associated with recurrent abortion and that this may be prevented by boosting the maternal immune system with paternal or third-party lymphocytes although the mechanisms of this process is not yet clear. On the other hand, the allogeneic stimulation *in vitro* (MLR) as described in our previous studies [19, 20] has been considered to induce the production of a series of macrophage and T helper cell factors, including a wide range of cytokines. Taking these facts

together, we tried to investigate the mechanisms of the allogeneic lymphocyte therapy in recurrent abortive patients by examining the effect of MLR products on the proliferation of trophoblast cells *in vitro*. MLR response of the recurrent abortive pairs prior to immunization with paternal lymphocytes was not different from that of healthy pairs at all as previously reported by Smith *et al.* [21].

The analysis of the factors in the MLR supernates revealed that a series of CSF family cytokines such as IL-3/GM-CSF and M-CSF, which were considered to be responsible for the trophoblast

Fig. 7. All karyotypes of the trophoblast cell line cells consist of the acrocentric chromosomes and the modal number of chromosomes was 41.

cell proliferation and differentiation, were induced in MLR supernates. The results suggested that the cytokines like IL-3/GM-CSF and M-CSF induced by maternal immunocompetent cells, which infiltrated into decidual tissue and was activated by the paternally derived fetal antigens in normal pregnancy or therapeutically sensitized by the allogeneic paternal lymphocytes in the recurrent abortive patients, act upon the proliferative potential of the trophoblast cells.

Furthermore, we revealed that the proliferation of murine trophoblast cells in the primary culture was enhanced prominently by rhM-CSF and slightly by rhIL-3/GM-CSF. These experimental results *in vitro* corresponded clearly with those of the cytokine therapy for the abortion-prone mice demonstrated by Chaouat *et al.* [8] in which not only rhM-CSF but also rhIL-3/GM-CSF appeared to cross react beyond the species barrier and act on murine trophoblast cells mostlikely through their altered receptors [22] for these cytokines.

The addition of MLR supernates into the primary culture of trophoblast cells at the optimal condition enhanced the proliferation of tropho-

blast cells and its continuous addition resulted at last in the establishment of trophoblast cell line. This cell line produced the placental hormones in its culture supernates such as chorionic gonadotropin (0.13 ng/ml) and progesterone (9.6 ng/ml). This cell line also demonstrated the polygonal epithelium like profiles in its phase contrast microscopic morphology and the cytokeratin positive in its cytoskeleton.

By these characterizing studies, this cell line was definitely revealed to be of trophoblastic nature.

Our present studies, together with others, gave the *in vitro* experimental basis to the clinical trials of allogeneic lymphocyte therapy in recurrent abortive patients and the technical basis to the establishment of trophoblast cell line.

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