Synergistic Effects of Insulin-Like Growth Factor II (IGF-II) with Leukemia Inhibiting Factor (LIF) on Establishment of Rat Pluripotential Cell Lines

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ABSTRACT. To examine effects of IGF-II on establishment of pluripotential diploid cells from rat embryos, we cultured blastocysts in medium containing mouse LIF with or without IGF-II. Combination of mouse LIF (5,000 units/ml) and rat IGF-II (100 ng/ml) promoted growth of inner cell mass (ICM) and was effective for establishment of pluripotential cell lines derived from the ICM. The cell lines indicated colony forms different from the rat ES cell lines. However, they showed morphological alteration to adult-like tissue cells, formed embryoid body in suspension culture, and thus, seemed to retain a pluripotent characteristics. The rat IGF-II is useful for establishing of pluripotential cells efficiently.—**KEY WORDS:** ES cell, IGF-II, rat.

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Leukemia inhibiting factor (LIF) was used as growth-stimulating and differentiation-inhibiting agent to establish embryonic stem (ES) cells [14]. Likewise, basic fibroblast growth factor (bFGF) was used to establish embryonic germ-line (EG) cells [8]. These studies indicate that other biological factors can act to establish pluripotential cell lines. In our previous study [15], we demonstrated that a fraction of buffalo rat liver cell conditioned medium (BRL-CM) was effective on establishment of ES-like cells. Free form of rat insulin-like growth factor II (rIGF-II) [7, 10] was considered to be contained in this fraction. IGF-II is a peptide hormone belonging to the insulin family. Several studies reported that the IGF-II had a function relating to embryogenesis in rodents [1, 2, 9, 11, 16]. In this report, we examined effects of rIGF-II on blastocysts cultured to know whether the rat pluripotential cells could be established by the factor.

Method for rat blastocyst culture was the same as that in our previous study [15]. Hatched blastocysts were recovered from two strains of rats; Wistar and ACI. To culture hatched blastocysts, we prepared three kinds of embryo culture medium, i.e., mouse LIF (mLIF) alone, mixture of mLIF and human IGF-II (mLIF/hIGF-II) and mixture of mLIF and rIGF-II (mLIF/rIGF-II). For purpose of determining a minimum effective concentration of rIGF-II, hatched blastocysts were cultured in three kinds of mixture, containing 5,000 units/ml of mLIF and either 1,000 ng, 100 ng or 10 ng/ml of rIGF-II. On the basis of results obtained from the preliminary experiment (data not shown), the concentration of 100 ng/ml of either rIGF-II or hIGF-II to 5,000 units/ml of mLIF was employed. On day 1 in culture, there was no significant difference in attachment rate of blastocysts to feeder cells. Morphology revealed various types of ICM growth on days 3 and 4 in attached blastocysts obviously categorized into three groups such as ICM⁻, ICM[±] and ICM⁺ (Fig. 1). As shown in Table 1, only mLIF/rIGF-II yielded the high rate growth of the ICM⁺, whereas mLIF alone and mLIF/hIGF-II did not. To establish cell lines from these

proliferated ICMs, whole ICMs categorized as ICM⁺ were directly isolated and subjected to further culture at the same concentration of mLIF/rIGF-II. Two to 3 days later, the expanded ICMs were dissociated into small cell clusters by gentle pipetting. The cell clusters attached to a fresh feeder cell layer and showed colony-like proliferation (Fig. 2). By this culture, 6 ICM-derived cell lines were established from 7 isolated ICMs of Wistar strain and 5 from 8 ICMs of ACI strain, respectively. The colony form of the ICM-derived cells was different from that of rat ES cells [5]. However, some interesting findings were obtained. First, the cells were differentiated into endoderm-like cells in the medium containing only mLIF without rIGF-II (Fig. 3a). Second, they altered their morphology to adult-like tissue cells, such as fibroblast-, neuron- and cardiovascular-like cells, when the cells were cultured without these factors (Table 2). The cardiovascular-like cells were identified by spontaneous strokes of the reconstructed cell masses. Third, formation of embryoid body-like structure was observed in suspension culture (Fig. 3b). These findings indicate that the ICM-derived cells maintain the pluripotency for *in vitro* differentiation as ES cells.

The present study revealed a unique activity of rIGF-II to improve growth of rat ICM and proliferation of rat ICM-derived cells and at the same time to inhibit differentiation of the cells. In our previous study, low molecular fraction (<M.W. 5000) from BRL-CM treated with acetic acid, in which rIGF-II is supposed to be contained abundantly, was effective to establish rat ICM-derived cell lines [15]. These cells showed the same morphology and pluripotency as those of the ICM-derived cells we reported here. This correspondence indicates that the rIGF-II is one of the factors cooperating with mLIF in establishment of the rat pluripotential cell lines. The evidences for the effects of rIGF-II in this study can be summarized as follows; (1) ICM growth was observed in the presence of rIGF-II, (2) In the presence of rIGF-II, the ICM-derived cell lines were established at a high rate, (3) Rat ICM-derived cells showed rapid and stable proliferation when they were cultured with rIGF-II, (4) The rat ICM-derived cells stopped proliferation and showed differentiation when rIGF-II was eliminated from the culture medium. It is, however, unlikely that rIGF-II

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Fig. 1. Morphological differences in growth of the ICM cells derived from rat hatched blastocysts on day 3 and 4 of culture. The ICM growth is categorized by the following 3 criteria; ICM⁻: absence of ICM of blastocysts attached to feeder layer (a); bar 100 μ m, ICM[±]: obscure growth of ICM cells on trophectoderm cells (b); bar 100 μ m, ICM⁺: apparent growth of ICM cells as doom-like proliferation (c); bar 100 μ m.

independently acts on these cells. We assume that different intracellular signals are independently induced by binding of LIF [6] and IGF-II [12, 13] to each characteristic receptor and then these signals collaboratively work to promote overall functions for the pluripotent cells.

Fig. 2. The colony of the rat ICM-derived cells. The small spherical cells proliferate and form colonies 4 days after dissociation of the ICMs; bar 100 μ m.

Fig. 3. Morphological alterations of the established rat ICM-derived cells. The cells are differentiated into endoderm-like cells in the medium containing only mLIF without rIGF-II and proliferation of the cells is prevented (a); bar 100 μ m. The cells form embryoid bodies in suspension culture with DMEM containing 10% FCS (b); bar 200 μ m.

The present study demonstrated that rIGF-II was much more effective than hIGF-II although only 5 amino acids are different in these two peptides. These 5 unmatched

Table 1. Effects of the factor(s) on ICM growth of rat blastocysts

Strain of rats	Factor(s) contained in medium	No. of blastocysts ^{a)}		Growth of ICM ^{c)}		
		Cultured	Attached ^{b)}	ICM ⁻	ICM [±]	ICM ⁺
Wistar	mLIF	9	6(67) ^{d)}	6	0	0(0) ^{e)}
	mLIF and hIGF-II	13	10(77)	10	0	0(0)
	mLIF and rIGF-II	14	14(100)	0	2	12(86)
ACI	mLIF and rIGF-II	14	14(100)	0	2	12(86)

a) Hatched blastocysts collected on day 3 post-coitum.

b) Blastocysts attached to feeder layer on day 1 in culture.

c) Observed on day 4 in culture; refer to Fig. 1 for classification of growth.

d) Percentage of attached/cultured blastocysts.

e) Percentage of ICM⁺/attached blastocysts.Table 2. Comparison of *in vitro* differentiation of each cell line

Medium	Origin	Cell lines	Differentiated cell types			
			Fibroblast-type	Neuron-type	Myocardium-type	Endoderm-type
DMEM with 10% FCS	Wistar	WES 110	+++	++	+	—
		WES 135	+++	+	+	—
	ACI	AES 8	+++	+	+	—
		AES 10	+++	++	+	—
DMEM with 10% FCS and mLIF	Wistar	WES 110	—	—	—	+++
		WES 135	—	—	—	+++
	ACI	AES 8	—	—	—	+++
		AES 10	—	—	—	+++

The appearance rate is described comparatively as follows;

+++ : Frequently observed.

++ : Usually observed.

+ : Rarely observed.

— : Not observed.

amino acids, especially 4 in C-domain (amino acid No. 32, 33, 35 and 36) [3, 7] are assumed to affect growth of the rat ICM. The difference in this region may affect the efficiency of binding to the receptors or its inhibitory binding protein.

The novel ability of IGF-II described in this study may provide a clue to cytokine-related embryogenesis. The combination of LIF and IGF-II will be useful in establishment of the ES cell lines from other mammalian species. Therefore further studies will be necessary to analyze function of IGF-II in embryogenesis and to characterize the ICM-derived cells in pluripotency to produce chimeras with them.

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