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# The Effects of Fetal Bovine Serum, Epidermal Growth Factor, and Retinoic Acid on Adult Rat Islets Embedded in Collagen Gels

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**Abstract** The induction of proliferation of adult rat islets was investigated under various culture conditions. The islets were isolated from male Sprague-Dawley rats and subsequently embedded in collagen gels, which mimic the *in vivo* three-dimensional surroundings. During the culture period, the effects of heterologous serum (fetal bovine serum, FBS), epidermal growth factor (EGF), and retinoic acid (RA) on islet growth were examined with respect to the morphological and total DNA content changes. To investigate these changes at the cellular level, whole mount immunocytochemistry using specific antibodies for insulin and glucagon was performed. The results showed that (i) collagen gels as an extracellular matrix can maintain islets in a similar way to that *in vivo*, (ii) heterologous serum (FBS) had stimulatory effects on islet proliferation in a dose-dependent manner, (iii) RA had inhibitory effects on islet proliferation induced by the serum in a dose-dependent manner, (iv) EGF had weak inhibitory effects on islet proliferation induced by the serum except at the concentration of 10 nM where its effect was not significant, and (v) whole mount immunocytochemistry revealed that newly proliferated islet cells were mainly β- and α-cells.

**Key words:** Pancreatic islet, collagen gel, whole mount immunocytochemistry

Diabetes is a major health problem worldwide: in the United States alone, approximately 13 million people are afflicted and 2–3% of the total population in many countries including Korea, Denmark, etc. suffer from the disease [31]. Although insulin has been considered as a miracle by many diabetic patients, it has several drawbacks. First of all, most diabetic patients (80–90%) are afflicted with type II or non-insulin-dependent diabetes mellitus (NIDDM), in which insulin resistance of insulin-responsive cells is prevalent [24, 30]. For patients with type I or insulin-dependent diabetes mellitus (IDDM), current treatments with insulin, self-monitoring, and exercise are difficult and successful only in a relatively small number of highly motivated individuals. In addition, insulin treatment cannot prevent microvascular or macrovascular complications such as retinopathy, nephropathy, neuropathy, and so on [16].

Although islet transplantation as an improved treatment begins to be successful, not enough human tissues will be available to meet the demand. At present, the islet sources for transplantation are exclusively obtained from human cadavers. However, only 4,000 cadaver pancreata could be available yearly in the United States while approximately 35,000 new cases of type I diabetes are diagnosed each year [1]. Therefore, *in vitro* growth of adult human islets is very important for both transplantation and various biological studies.

Through extensive studies, it has been well established that the growth rate of rat pancreatic β-cells is significantly higher during late gestation and the neonatal period than that after weaning, with little change beyond 30–40 days of age [8, 18, 28]. Recent studies, however, demonstrated that human adult islets could be proliferated under appropriate extracellular matrices and growth factors [11, 14, 25].

In this study, we have examined the possibility of whether rat adult islets could be proliferated *in vitro*, and investigated the effects of medium supplements such as fetal bovine serum, epidermal growth factor, and retinoic acid on the growth of rat islets.

## Materials and Methods

### Materials

Reagents of analytical grade and deionized water prepared by a Milli-Q Water Purification System (Millipore, Bedford, U.S.A.) were used. Dulbecco's modified Eagle's medium (DMEM), newborn calf serum (NCS), fetal bovine serum (FBS), and RPMI 1640 tissue culture medium were

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obtained from Gibco BRL (Grand Island, NY). Collagenase type XI, epidermal growth factor (EGF), retinoic acid (RA), calf thymus DNA, and Hoechest 33258 dye were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Guinea pig antiserum to porcine insulin, rabbit antiserum to porcine glucagon, peroxidase-conjugated rabbit anti-Guinea pig immunoglobulins, normal nonimmunized rabbit serum as a negative control, and AEC substrate chromogen system were purchased from DAKO Corp. (Carpinteria, U.S.A.). Peroxidase-conjugated goat anti-rabbit immunoglobulins were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, U.S.A.).

### Animals and Preparation of Pancreatic Islets

The animals used were 10-week old male Sprague-Dawley rats weighing 250 g, which were purchased from Dai-Han Animal Center (Eum Sung, Chungbuk, Korea). Pancreatic islets were isolated by collagenase digestion with minor modifications [17]. In brief, the rat was decapitated, the common bile duct was cannulated and freshly prepared collagenase solution (consisting of 0.7 mg/ml collagenase type XI, 0.1 mg/ml DNase I, and DMEM) was injected into the pancreatic duct. The pancreas was excised from the surrounding organs, e.g. duodenum and spleen, and moved to pre-warmed DMEM quickly. After the pancreas was incubated for 18 min at 37°C, the reaction was stopped by adding cold DMEM supplemented with 10% (v/v) NCS. After brief centrifugation for 2 min (300 ×g, at 4°C), the supernatant was decanted and the pellet was resuspended in DMEM with 10% (v/v) NCS. The suspension was filtered through a 500-μm mesh, and the final filtrate was diluted with appropriately 10% (v/v) FBS-supplemented RPMI 1640, and examined for islets under a dissecting microscope.

### Preparation of Collagen Solution and Collagen Gel

Approximately 0.25 g of collagen fibers dissected from Sprague-Dawley rat tail was placed on a petri dish and irradiated with UV light for 24 h for complete sterilization. Type I collagen was solubilized by stirring it for 48 h at 4°C in 300 ml of 1:1,000 (v/v) sterile acetic acid solution. The resulting solution was filtered through a sterile triple gauze and centrifuged at 16,000 ×g for 1 h at 4°C to remove undissolved fibers. The supernatant was aliquoted and stored at -80°C until use. The gels of reconstituted collagen fibers were prepared by simultaneously raising the pH and ionic strength of the collagen solution according to a modified method originally described by Elsdale and Bard [5]. This was achieved by quickly mixing 7 volumes of cold collagen solution with 1 volume of 10X RPMI 1640 medium and 2 volumes of sodium bicarbonate (11.76 mg/ml) in a sterile conical tube kept on ice to prevent immediate gelation. The cold mixture was then dispensed into 12-well plates (Falcon Plastics, Div. of Bioquest, Oxnard, U.S.A.) and allowed to gel for 10 min at 37°C.

### Cell Culture

After fibroblasts were removed by successive transfer onto a new petri dish, 25 islets per well were embedded in collagen gels. The islets were cultured at 37°C for 10 days in the humidified atmosphere of 5% $CO_2$. During the test period (7 days), various concentrations of fetal bovine serum (FBS) were added into the basal medium, and epidermal growth factor (EGF) or retinoic acid (RA) was added into the basal medium supplemented with 20% (v/v) FBS. The culture medium was exchanged everyday.

### Quantification of Total DNA Contents

On the last day of culture, media were completely removed by extensive washing with PBS. The collagen gels containing islets were collected by aspiration and mixed with 1 ml of PBS. The islets were homogenized using a homogenizer (IKA, Labortechnik, Germany) for 30 sec on ice. After homogenates were centrifuged for 10 min at 4°C, the supernatant was collected and stored at -20°C until assayed. Total DNA contents were measured by the fluorometric method [9]. After the standard curve for DNA (calf thymus DNA) were drawn, the concentration of unknown samples was obtained by intrapolation.

### Whole Mount Immunocytochemistry

The whole mount immunocytochemical localization of β- and α-cells was accomplished by a slightly modified method of Sternberger's [29]. Briefly, fresh islets or cultured islets were fixed in 4% paraformaldehyde for 3 h and washed with PBST for 3 h on ice. The plasma membranes were permeabilized with 0.01% $H_2O_2$-methanol for 30 min, followed by blocking with 1% BSA-PBST solution. Primary antibody (prediluted, for 20 min at room temperature) or nonimmunized rabbit serum as a negative control, and peroxidase-conjugated secondary antibody (1:75, 1% BSA-PBST) were sequentially incubated for 15 min at room temperature. Following three washings with PBST and TBS, the islets were examined under a transmission microscope (Nikon, Japan) in the presence of AEC substrate-chromogen.

## RESULTS

### Islet Isolation and Embedding in Collagen Gels

The islets were isolated from 10 week old male Sprague-Dawley rats by a minor modification of the collagenase digestion method. Following collagenase digestion and filtration using a 500-μm screen, which retained undigested acinar tissues, but not islets, the filtrate was appropriately diluted with RPMI 1640 supplemented with 10% (v/v) NCS. When viewed against a black background, the islets could be recognized easily under a dissecting microscope. They appeared as free, round, or ovoid structures with a grayish-white to brownish-red color (Fig. 1). A micropipette

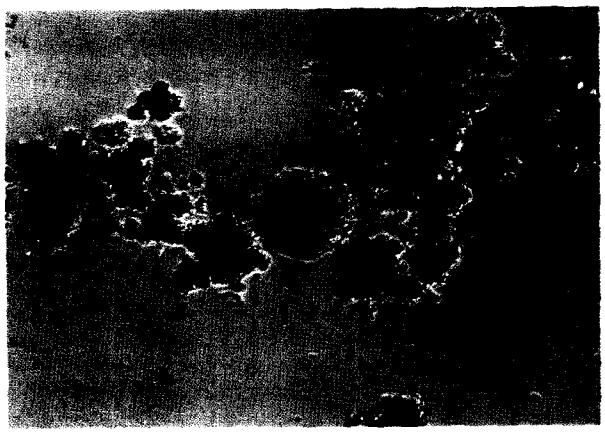

**Fig. 1.** Photomicrograph showing an islet (I) surrounded with digested acinar tissues (A).

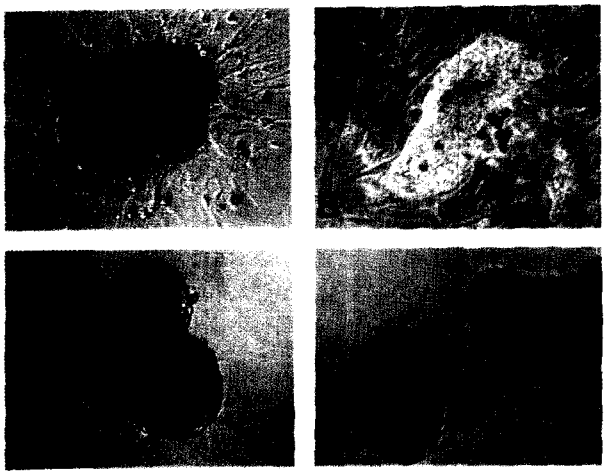

**Fig. 2.** The islets embedded in collagen gels after removal of fibroblasts.
As fibroblastic outgrowth prevents normal islet proliferation (A, B), the fibroblast-free islets were embedded in collagen gels (C, D).

of which the diameter is slightly larger than that of the islets, was used to transfer individual islets. Approximately 200~250 islets could be transferred during 2 h.

Before islets were embedded in collagen gels, it was confirmed that collagen gels could be formed by mixing with an appropriate ratio of collagen solution of 10X RPMI 1640 and sodium bicarbonate solution. After fibroblasts were removed by successive transfer onto new petri dishes, 25 islets per well were embedded in collagen gels (Fig. 2).

### Effects of Serum, EGF, and RA on Islet Proliferation

After 25 islets per well were embedded in collagen gels, heterologous serum, EGF, and RA were added to the culture medium and examined for their effects on islet proliferation with respect to morphology and total DNA contents. While FBS had stimulatory effects on islet proliferation in a dose-dependent manner in terms of both

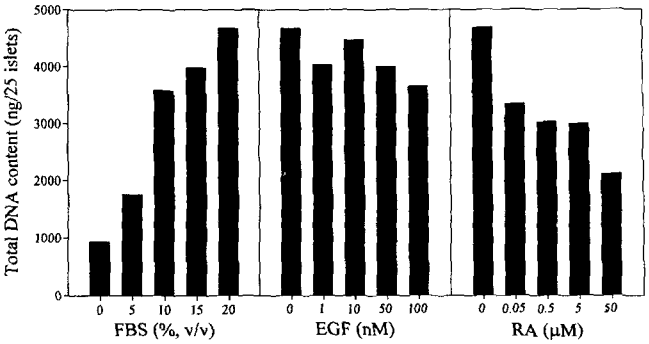


**Fig. 3.** The effects of FBS, EGF, and RA on islet proliferation with respect to total DNA contents.
During the test period (7 days), various concentrations of fetal bovine serum (FBS) were added into the basal medium, and epidermal growth factor (EGF) or retinoic acid (RA) was added into the basal medium supplemented with 20% (v/v) FBS. Values are the mean of three separate experiments.

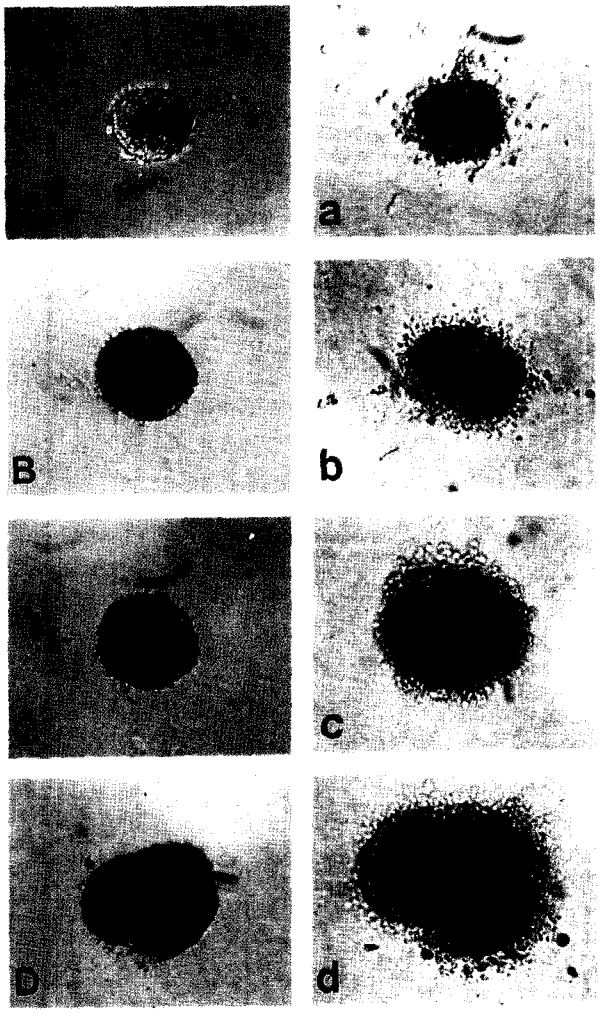


**Fig. 4.** Morphological changes by heterologous serum (FBS).
The islets embedded in collagen gels shortly after embedding (left panel) and after the test period of 7 days (right panel). (A, a: 5%; B, b: 10%; C, c: 15%; D, d: 20% FBS (v/v)).

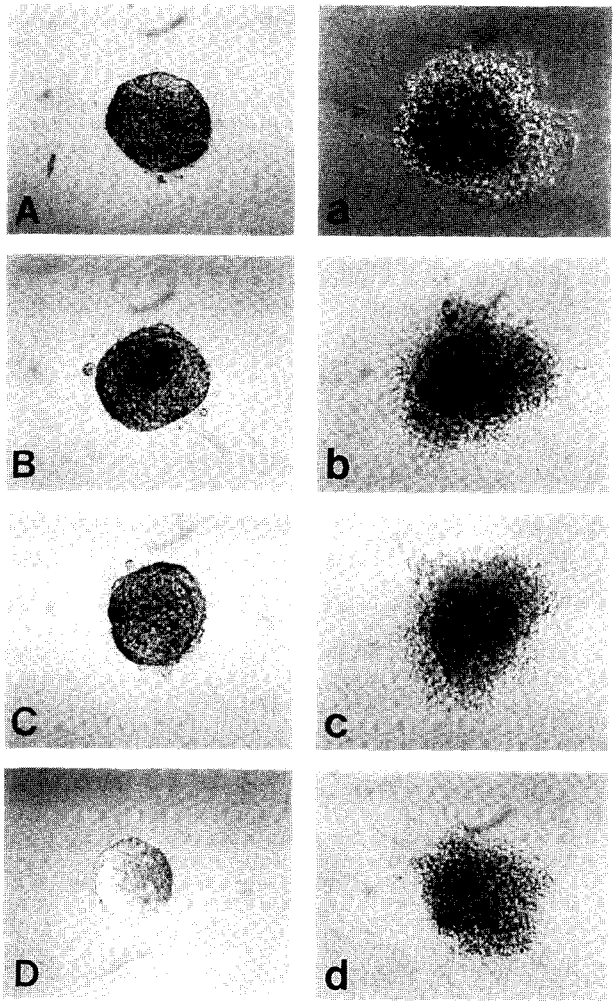


**Fig. 5.** Morphological changes by epidermal growth factor (EGF).
The islets embedded in collagen gels shortly after embedding (left panel) and after the test period of 7 days (right panel). (A, a: 1; B, b: 10; C, c: 50; D, b: 100 EGF (nmol)).

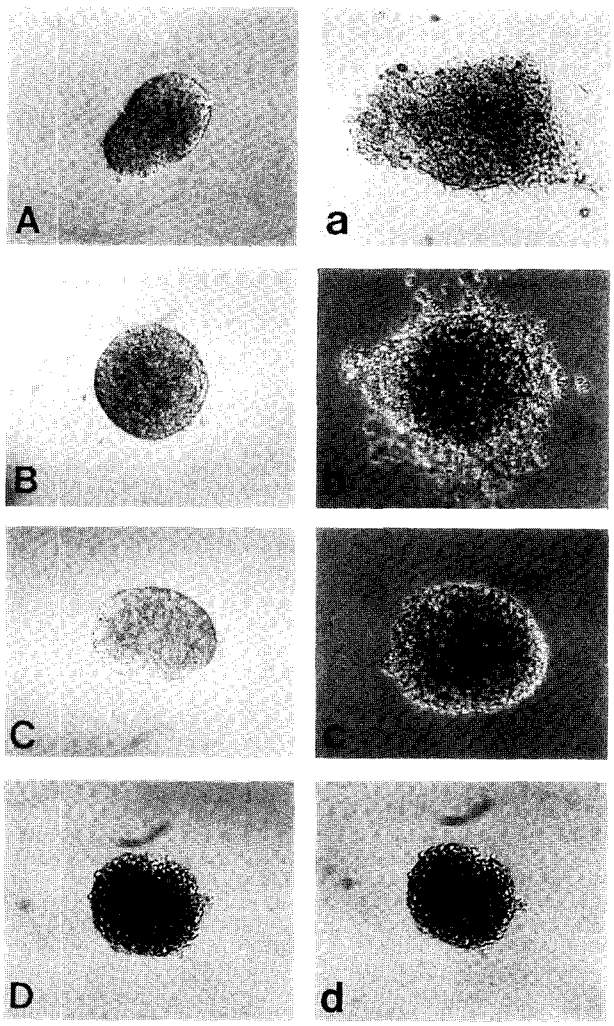


**Fig. 6.** Morphological changes by retinoic acid (RA).
The islets embedded in collagen gels shortly after embedding (left panel) and after the test period of 7 days (right panel). (A, a: 0.05; B, b: 0.5; C, c: 5; D, d: 50 RA (μmol)).

morphology (Fig. 4) and total DNA contents (Fig. 3), RA had inhibitory effects on islet proliferation induced by serum (Figs. 3, 6). EGF had weak inhibitory effects on islet proliferation except at the concentration of 10 nM where its effect was not significant (Figs. 3, 5).

### Whole Mount Immunocytochemistry

Because adult rat islets proliferated under permissive culture conditions, newly proliferated islet cells were identified using specific antibodies for insulin and glucagon. As a control, fresh rat islets were immunostained with insulin- and glucagon-specific antibodies. Also, nonimmunized rabbit serum was used as a negative control (Fig. 7). The β-cells were predominant and mainly distributed in the center, while the α-cells were distributed on the periphery with some cells in the center. When *in vitro* cultured islets were immunostained as described above, the original distribution pattern of each cell was well preserved and newly proliferated cells were found to be mostly β- and α-cells (Fig. 8).

## DISCUSSION

A number of islet culture systems have been developed since the early 1960s, but most were based on the monolayer method by partially dissociating pancreatic tissues into islet-like organs [3, 4, 22]. Although these systems have provided some valuable informations on replication, growth pattern, and insulin secretion of β-cells [32], they cannot maintain the *in vivo* three-dimensional situation, in which islet cells interact with one another [21]. On the other hand, it is well documented that collagen gels could provide better physiological and isotropic environment

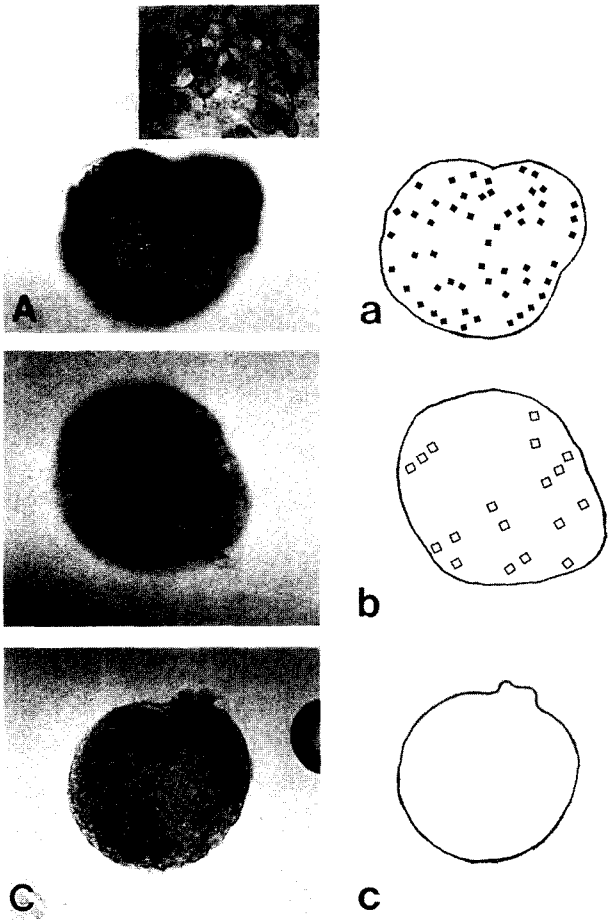


**Fig. 7.** The fresh islets immunostained with insulin- or glucagon-specific antibodies.
The β- and α-cells are shown in (A, a) and (B, b), respectively. The negative control is shown in (C). (I) indicates the magnified image of the boxed portion marked in (A).

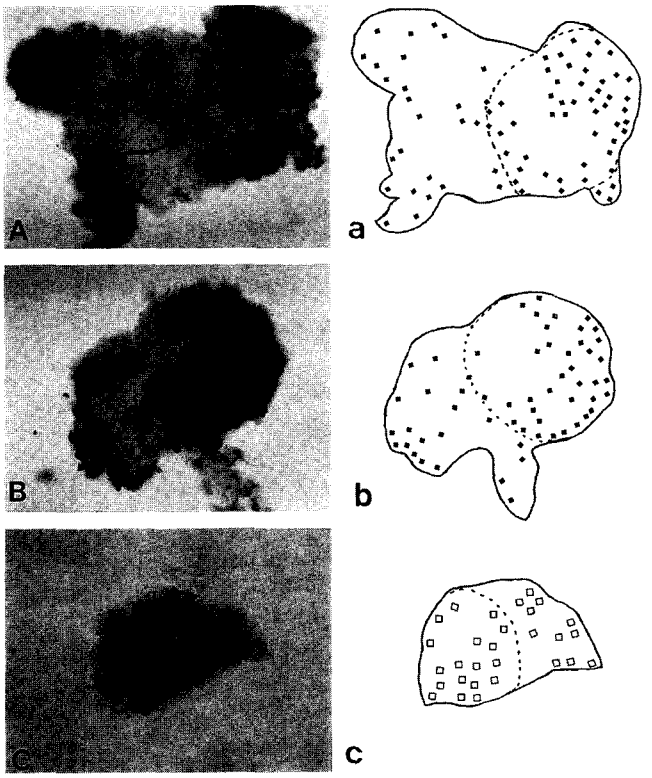


**Fig. 8.** The cultured islets immunostained with insulin- or glucagon-specific antibodies.
The β- and α -cells are shown in (A, B, a, b) and (C, c), repectively. The dotted line indicates the original size of islets.

which has been shown to promote the organization of different epithelial cell types into three-dimensional, tissue-like structures [20, 26, 33]. Thus, our culture system not only maintains the *in vivo* interaction between each cell type in a three-dimensional situation, but also provides permissive conditions for islets to proliferate.

As the islets are scattered in exocrine acini, parenchymal substrata which are primarily composed of collagen, fibronectin, elastin, etc. were digested by the action of collagenase at 37°C. Careful control was exerted on the incubation time, because islets themselves may be fragmented or underdigested depending on the incubation time. The mean islet yield was 200–250 islets/rat and the diameter of islets ranged from 50 μm to 300 μm, which is consistent with previous data [12]. Although the Ficoll, the Percoll or the dextran gradient centrifugation methods are already available for higher yields [7, 27], these methods could not be used in this study due to possible damage to islets by osmotic pressure.

Mammalian pancreatic islets are mainly composed of β-cells, but α-cells, D-cells, PP-cells, endothelial cells, nerve cells, and fibroblasts are also present. In particular, fibroblasts are known to move through collagen gels *in vitro* and through the extracellular matrix *in vivo*, at a rate of about 1 μm/min while maintaining their bipolar form [6]. As fibroblastic outgrowth prevents the proper islet proliferation (Fig. 2), they were removed by a rather coarse, but effective procedure used in this study. After removal of fibroblasts, 25 islets per well were embedded in collagen gels whose intensity was adjusted to allow islets to proliferate in more flexible surroundings (0.06% (w/v) vs 0.23% (w/v) in the original paper [21]). In these conditions, heterologous serum, EGF, and RA were added to the culture medium and their effects on islet proliferation were examined. Although a great number of agents involved in insulin secretion of β-cells have been examined in previous literature [2, 13, 15, 19], the effects of heterologous serum, EGF, and RA on adult islets had not yet been tested.

The results demonstrated that FBS had stimulatory effects on islet proliferation in a dose-dependent manner, while RA had inhibitory effects on islet proliferation induced by serum. EGF had weak inhibitory effects on islet proliferation except at the concentration of 10 nM where its effect was not significant. In the case of heterologous serum, its effect

was consistent with previous results by other groups, although their systems were somewhat different from ours [10].

Normal islets of Langerhans from rats have a DNA content of 50±0.05 ng/islet and the cell number per islet has been estimated to be about 5,700 [12]. If these estimations are taken into account, the total DNA contents of cultured islets may have been underestimated. But the values from this study are still meaningful in comparing cell proliferation because sample preparations were performed under the same conditions.

The whole mount immunocytochemical localization of β- and α-cells confirmed that the original distribution in islets was well preserved (Fig. 7) and newly proliferated islet cells were mainly β- and α-cells (Fig. 8). Further study is underway on whether these cells were from the division of preexisting cells or from differentiation from precursor cells [23].

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