

## Cell surface markers to monitor the process of visceral endoderm differentiation from embryonal carcinoma cells: identification of the stage sensitive to high concentration of retinoic acid

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### SUMMARY

Two cell surface antigens, brushin and FT-1 were effective in analysis of the process of visceral endoderm differentiation. Brushin was detected on both primitive and visceral endoderm, while FT-1 was detected only on visceral endoderm. When aggregates of N4-1 embryonal carcinoma cells were exposed to  $10^{-8}$  M-retinoic acid for more than 2 days, external cells differentiated to multilayered and vacuolized visceral endoderm. However, aggregates treated with  $10^{-6}$  M-retinoic acid developed an endoderm layer, which remained one cell thick and was not vacuolized. Cell surface properties of the endoderm cells indicated that the high concentration of retinoic acid inhibited the differentiation pathway at the stage between primitive endoderm cells and visceral endoderm cells. By pulsed exposure to  $10^{-6}$  M-retinoic acid, the period sensitive to the high concentration of retinoic acid was shown to be around day 4 after the initial exposure to retinoic acid.

### INTRODUCTION

Embryonal carcinoma (EC) cells are stem cells of teratocarcinoma, and their differentiation systems are used as the model to analyse early stages of mammalian embryogenesis (Martin, 1980). The most commonly used method to induce their differentiation is to treat the cell with certain concentrations of retinoic acid (RA) (Strickland & Mahdavi, 1978). When F9 cells, a clonal line of EC cells, are grown as cell aggregates and exposed to RA, the external cells differentiate to visceral endoderm cells (Hogan, Tayler & Adamson, 1981; Grover, Oshima & Adamson, 1983). However, sparsely grown F9 cells can be induced to differentiate to parietal endoderm cells by treating with RA and dibutyryl cyclic AMP (Strickland, Smith & Marotti, 1980). In the case of another EC cell line, P19, the concentration of RA influenced the types of differentiated cells formed: endoderm cells were

*Key words:* embryonal carcinoma cells, visceral endoderm, retinoic acid, cell surface antigens.

optimumly formed at  $10^{-9}$  M-RA, while nerve cells formed at  $10^{-7}$  M and higher (Edwards & McBurney, 1983). Thus, RA-induced differentiation of EC cells is a suitable system to analyse the mechanism regulating the direction of cell differentiation. In this line of work, it is essential to define intermediate stages in a cell lineage and to reveal the stage sensitive to external manipulation. Accordingly, we have been interested in differentiation of visceral endoderm, since this differentiation system is dependent on cell interaction (Hogan *et al.* 1981; Grover, Andrews & Adamson, 1983) and is inhibited by a glycosylation inhibitor, tunicamycin (Grabel & Martin, 1983). In this communication, we describe cell surface properties of intermediate cells leading to visceral endoderm from a line of EC cells. Furthermore, we report that high concentrations of RA inhibited the differentiation pathway, and indicate the stage sensitive to this inhibitory effect.

#### MATERIALS AND METHODS

##### Reagents

Anti-brushin serum (Ozawa, Yonezawa, Sato & Muramatsu, 1982) and monoclonal anti-FT-1 antibody (Kasai, Takashi, Takahashi & Tokunaga, 1984) were prepared as described previously. Anti-SSEA-1 (Solter & Knowles, 1978) was given by Dr D. Solter, and affinity-purified rabbit anti-mouse  $\alpha$ -fetoprotein by Prof. S. Nishi. RA and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., foetal calf serum (FCS) from Grand Island Biological Co., Dulbecco-modified Eagle's minimum essential medium (DME) from Flow Laboratories, fluorescent isothiocyanate (FITC)-conjugated *Sophora japonica* agglutinin (FITC-SJA, Lot No. 0813D) and FITC-conjugated *Dolichos biflorus* agglutinin (FITC-DBA) from E·Y Laboratories, guinea-pig complement and horse-radish peroxidase-conjugated goat anti-mouse IgM (HRP-GAM) from Cappel Laboratories, FITC-conjugated goat anti-mouse IgG (FITC-GAM) and FITC-conjugated goat anti-rabbit IgG (FITC-GAR) from Miles Yeda.

##### Mouse embryos

Mouse embryos were obtained from naturally mated Jcl:ICR mice (CLEA JAPAN). The day when vaginal plug was observed was taken as day 0 of pregnancy. Early blastocysts (day 3·6) were obtained by flushing the uterus by HEPES-buffered Whitten's medium (HWM-BSA) pH 7·4 (Whitten, 1971). Late blastocysts were obtained by culturing early blastocysts in DME containing heat-inactivated (56°C, 30 min) 10 % FCS for a day. Inner cell mass (ICM) was isolated by immunosurgery from early and late blastocysts according to Solter & Knowles (1975). Rabbit antiserum against DBA receptors (Ozawa *et al.* 1982) was used for the purpose. For prolonged culture of ICM, ICMs immunosurgically isolated from the late blastocysts were cultured for one day in a drop of DME-FCS under liquid paraffin in a Terasaki microtest plate. Immunosurgery of cultured ICMs was performed as described above.

Egg cylinders on day 6·5 were dissected from decidua. When Reichert's membrane with parietal endoderm was stripped away from the egg cylinders with watchmaker's forceps, visceral endoderm was exposed to the medium. In order to expose ectoderm, visceral endoderm was then removed in HWM-BSA by watchmaker's forceps after soaking the embryos in Dulbecco's phosphate-buffered saline lacking  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  [PBS (-)] containing 1 M-glycine and 0·2 % EDTA at room temperature for 15 min to loosen the junction between visceral endoderm and ectoderm according to Dziadek (1981).

##### Teratocarcinoma cells

N4-1 is an EC cell line (Sato *et al.* 1983) cloned from spontaneous testicular teratocarcinoma STT-2 originated in 129/Sv mice. STT-2 teratocarcinoma was kindly given by the late Dr T.

Noguchi. N4-1 cells were grown in DME-FCS and could be maintained by subculturing every 3–4 days using 0.25 % trypsin and 0.2 % EDTA in PBS (–). A parietal yolk sac cell line, PYS-2 (Lehman, Speers, Swartzendruber & Pierce, 1974) and a visceral yolk sac cell line END-C-2 (Sato & Muramatsu, 1985) were cultured similarly except that in case of culturing PYS-2, the concentration of FCS was lowered to 5 %.

### *Induction of differentiation of N4-1 embryonal carcinoma cells by RA*

Aggregates of N4-1 cells were formed by culturing  $2 \times 10^5$  cells in a bacteriological dish of 30 mm diameter (Falcon) for 1 day. Aggregates comprising approximately 100 cells were collected by a capillary and transferred to 96-well microtiter plates (Falcon) containing 0.1 ml of DME-FCS with various concentrations of RA. Each well contained about five aggregates. Under these conditions, aggregates loosely adhered to the dish, while differentiation of visceral endoderm was not affected by the adhesion. This method is preferable, since adhesion of the aggregates to each other was inhibited under these conditions.

Differentiation was allowed to occur for 10 days, and the day when the aggregates were transferred to the RA-containing medium was designed as day 0. The medium was changed on days 2, 4, 6, 7 and 8. In some cases, a cell aggregate cultured for a certain period in the presence of RA was washed once with DME-FCS and transferred into a well of 24-well plate (Nunc) containing 1 ml of DME-FCS, and was further cultured.

### *Cell counting*

Cell aggregates were washed with PBS (–) and incubated with 0.125 % trypsin in PBS (–) containing 0.5 M-glycine and 0.2 % EDTA at 37°C for 10 min. After vigorous pipetting, the cell suspension was further incubated at 37°C for 10 min. The cells were recovered by centrifugation and counted by a haemocytometer.

### *Immunohistochemistry*

Unfixed samples were incubated with first antibodies, washed with HWM-BSA and then incubated with second antibodies. Incubations with antibodies were performed at 4°C for 1 h. The following dilutions of the antibodies were used: anti-brushin, 30-fold; anti-FT-1, 100-fold; anti-SSEA-1, 100-fold; anti- $\alpha$ -fetoprotein, 40-fold. As second antibodies, FITC-GAR (for anti-brushin or anti- $\alpha$ -fetoprotein) and FITC-GAM (for anti-FT-1 or anti-SSEA-1) were used at a dilution of 20-fold. For control staining, the first antibodies were replaced with normal mouse serum (in case of FT-1 and SSEA-1) or with normal rabbit serum (in case of brushin and  $\alpha$ -fetoprotein). No positive reactions were observed in the control stainings. FITC-SJA was used at a concentration of  $500 \mu\text{g ml}^{-1}$  and the incubation was performed at 4°C for 20 min. For control staining, FITC-SJA was preincubated with 0.1 M-GalNAc solution. No positive staining was observed in the control run. That the result was not affected by possible internalization of the lectin–ligand complex was confirmed previously (Sato & Muramatsu, 1985). The specimen was observed by Olympus fluorescent microscope (model BH-2) equipped with an epilluminator.

For fixation, cell aggregates were soaked in 10 % buffered formalin, pH 7.4 or ethanol/acetic acid (95:5, v/v) at 4°C for 30 min, thoroughly washed with PBS (+), and were soaked in eosin for several minutes. After washing in PBS (+), the specimen was embedded in 2.5 % agar (Difco), and after standard histological procedures, the block of agar was embedded in paraffin. Then the specimen was serially sectioned into slices of  $3 \mu\text{m}$  thickness. For cryostat sectioning, cell aggregates were fixed by ethanol/acetic acid at 4°C for 30 min, washed with PBS (+), marked by eosin, embedded in agar and sectioned with a cryostat. Postimplantation embryos were treated in the same way except that they were not marked by eosin nor embedded in agar. The sections were reacted with antibodies or lectins under the conditions described for staining the unfixed specimens except that room temperature was employed. HRP-GAM was used as the second antibody to analyse the distribution of SSEA-1. Some sections were also stained with haematoxylin–eosin.

### Scanning electron microscopy (SEM)

Cell aggregates were adhered to glass cover slips which had been treated with 0.01 % poly-L-lysine in PBS (+). The aggregates or cells grown on cover slips were fixed in 4 % glutaraldehyde in PBS (+), postfixed with 2 % osmium tetroxide, dehydrated through graded ethanols and critical-point dried. After gold coating on a sputter coater, the specimen was observed with a Hitachi scanning electron microscope (model HFS-2).

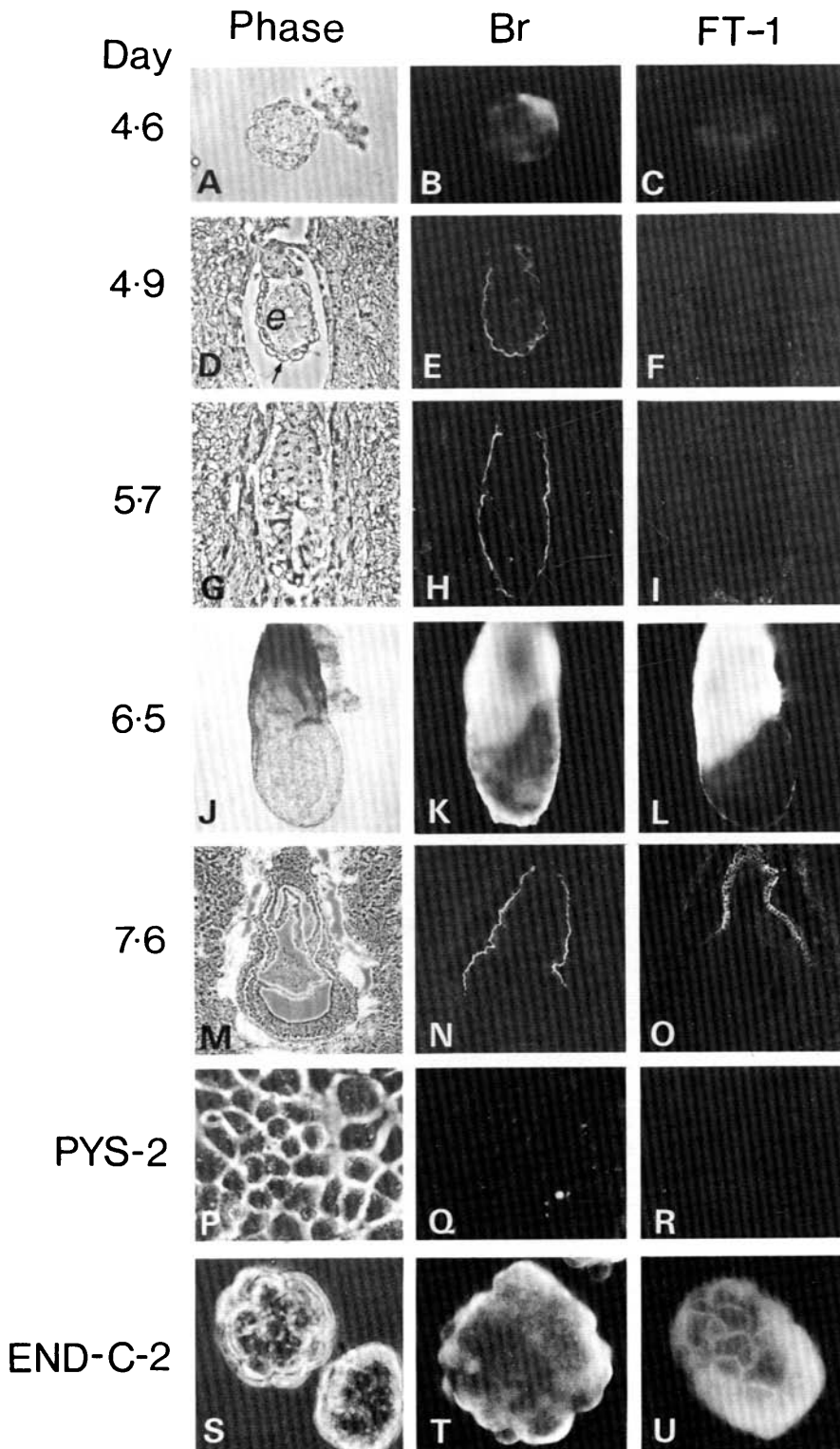
## RESULTS

### Cell surface markers to monitor visceral endoderm formation

Brushin is an antigen defined by a multiabsorbed rabbit antiserum against DBA receptors of teratocarcinoma OTT6050 (Ozawa *et al.* 1982). Brushin was not expressed in all preimplantation-stage embryos (data not shown). The antigen was first detected on the outer surface of ICM of a late blastocyst (day 4.6) (Fig. 1A, B). The brushin-positive cells appeared to be primitive endoderm cells. In order to confirm that brushin is expressed in primitive endoderm cells, the following experiments were performed. ICMs were immunosurgically isolated from late blastocysts, and were cultured for 1 day. After culture, a monolayer of endoderm cells covered the entire surface of ICMs. These endoderm cells were considered to be still at the stage of primitive endoderm because of their immature morphology (Hogan & Tilly, 1978) and non-expression of  $\alpha$ -fetoprotein (Dziadek, 1979) and of receptors for SJA (Sato & Muramatsu, 1985), both of which are markers of mature visceral endoderm. Brushin was expressed on the surface of the primitive endoderm-like cells (data not shown). Since these endoderm cells cover the entire surface of the cultured ICMs, the endoderm cells could be removed by a second immunosurgery without impairing epiblasts. The immunosurgically isolated epiblasts from the cultured ICMs were not stained by anti-brushin (data not shown). In order to avoid possible alteration of cell surface due to *in vitro* culturing of ICMs, sections of implanting embryos were also examined. On sections of a day 4.9 mouse embryo, epiblasts were surrounded by a monolayer of endoderm cells

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Fig. 1. Immunofluorescent microphotographs of living and sectioned early mouse embryos and teratocarcinoma cells, which were reacted with the anti-brushin (B,E,H, K,N,Q and T) and the anti-FT-1 (C,F,I,L,O,R and U) antibody. (A–C) ICMs isolated by immunosurgery from late blastocysts. At this time, primitive endoderm cells had appeared on a part of the surface of ICM. A part of ICM, which was considered to be primitive endoderm cells, was reactive with anti-brushin antibody (B), while did not react with anti-FT-1 antibody (C). (D–F) Sagittal sections of late blastocysts (day 4.9) just implanted into the maternal tissues. At this stage, a primitive endoderm cell layer (arrow) could be clearly seen on the epiblast (*e*). Ethanol/acetic acid-fixed materials. (G–I) Sagittal sections of early egg cylinders (day 5.7). Formalin-fixed materials. (J–L) Living egg cylinders deprived of Reichert's membrane. (M–O) Sagittal sections of 7-day embryos (day 7.6). Formalin-fixed materials. (P–R) Sheet of a parietal yolk sac cell line, PYS-2. (S–U) Aggregates of a visceral yolk sac cell line, END-C-2. Phase, phase contrast microphotograph; Br, immunofluorescent staining for brushin; FT-1, immunofluorescent staining for FT-1. Figs A,D,G,J,M and P represent the same area as seen in Figs B,E,H,L,N and Q, respectively. (A–C, P–U)  $\times 250$ ; (D–I)  $\times 150$ ; (J–L)  $\times 53$ ; (M–O)  $\times 40$ .



(Fig. 1D). These endoderm cells were regarded as primitive endoderm cells because their morphological appearance was immature (rather small and round shaped). Absence of parietal endoderm cells along the uterine wall, in spite of thorough inspection of serial sections, was consistent with the view that the endoderm cells were still in the immature state and did not segregate into parietal and visceral endoderm cells. The primitive endoderm cells of day 4.9 embryos expressed brushin, while other cells at that stage did not, except for trace (probably non-specific) staining of cells facing proamniotic cavity (Fig. 1E). From all these results, we considered that presence of brushin in primitive endoderm cells is well established. In paraffin sections of day 5.7 (Fig. 1G,H) and day 6.5 mouse embryos, the antigen was detected only in visceral endoderm: the result is consistent with that of Ozawa *et al.* (1982). Restricted distribution of brushin on visceral endoderm at these stages was confirmed by using dissected pieces of the embryos (Fig. 1J,K). Furthermore, localization of brushin in the visceral endoderm cells was also seen in a day 7.6 embryo (Fig. 1M,N). The antigen was not expressed in F9 and N4-1 cells nor in PYS-2 parietal endoderm cells (Fig. 1P,Q), but was expressed in a visceral endoderm cell line, END-C-2 (Fig. 1S,T). Thus, we concluded that brushin is a marker of both primitive and visceral endoderm.

FT-1 is an antigen defined by a monoclonal antibody against GRSL leukemia cells, and is a marker of embryonic thymocytes (Kasai *et al.* 1984). The antigen was not detected in any pre- and peri-implantation-stage embryos, including primitive endoderm (Fig. 1C,F). Using both sections and dissected embryos, we found that FT-1 was first expressed in visceral endoderm of 6.5-day embryos (Fig. 1L), but not in 5.7-day embryos (Fig. 1I). On day 7.6, expression of FT-1 became localized on the extraembryonic visceral endoderm cells (Fig. 1O). In cultured cell lines, the antigen was not detected in F9, N4-1 and PYS-2 cells (Fig. 1R), but was detected in END-C-2 visceral endoderm cell line (Fig. 1U). Thus, FT-1 could be regarded as a marker of visceral endoderm.

In addition, we have utilized the following markers to follow the differentiation of visceral endoderm: SSEA-1 which is a cell surface antigen defined by a monoclonal IgM antibody and is expressed in EC cells but not in many of their differentiated derivatives (Solter & Knowles, 1978; Solter, Shevinsky, Knowles & Strickland, 1979), receptors for SJA which can be used as a marker of visceral endoderm in early postimplantation mouse embryos and in teratocarcinoma cells (Sato & Muramatsu, 1985), and  $\alpha$ -fetoprotein which is an established marker of visceral endoderm in early postimplantation embryos (Dziadek & Adamson, 1978).

#### *General properties and differentiation capabilities of N4-1 cells*

N4-1 is a clonal line of EC cells established from a spontaneous testicular teratocarcinoma STT-2. The cell grew as cell aggregates when propagated without feeder layers. They adhered to substratum 1–2 days after subculturing, but began to round up and often detach from the dish on the 3rd day. After 1 week, most of the aggregates were floating in the medium. At the time 'hollow buds' of

endoderm cells could be sometimes observed at the surface of floating N4-1 aggregates just as in the case of F9 cells (Sherman & Miller, 1978). After 2 weeks in culture, the central portion of the aggregates became necrotic and hollow, and the outer surface was partially covered by endoderm cells. The endoderm cells appeared to be at the initial stage of differentiation. No new cell types other than endoderm cells appeared, even after 2 months in culture without subculturing. Solid tumours formed by subcutaneous injection of N4-1 cells into 129/Sv mice were mostly composed of EC cells, and no differentiated cells other than endoderm cells were observed. Thus, the differentiation capability of N4-1 cells in the absence of RA resembled that of F9 cells.

When aggregates of N4-1 cells were treated with various concentration of RA, endodermal differentiation at the outer surface of aggregates was promoted. The degree of differentiation of endoderm depended on the concentration of RA as shown below.

#### *Endoderm differentiation at low concentrations of RA*

The process of differentiation of visceral endoderm in the presence of  $10^{-8}$  M-RA was analysed by morphological criteria (Figs 2, 3) as well as by immuno-histochemical markers (Figs 2, 4). Percent of the area expressing a marker to the total observable surface of the aggregate is shown in Fig. 5. The change of cell numbers during the process is summarized in Fig. 6: the pattern of the growth curve is similar to that of RA-treated aggregates of F9 cells (Grover *et al.* 1983). Events occurring in each differentiation stage are described below.

One or two days after RA treatment, phase-contrast microscopic observation revealed no gross morphological changes except for the appearance of 'hollow buds' at the surface of a part of the cell aggregates. These cells expressed brushin (arrow in Fig. 2C), but not SSEA-1, SJA receptors nor FT-1 (Fig. 2D). SEM observation revealed that cells of 'hollow buds' showed relatively smooth surfaces and had small amounts of villi (data not shown). Thus, it is concluded that they are primitive endoderm-like cells. SSEA-1 was expressed on most of the cells at the surface of aggregates (Fig. 5) and in the interior. The aggregate at this undifferentiated stage is classified as type I.

On the 3rd day, a group of small cells with round or triangular shapes appeared at several points of the surface of the cell aggregate (Figs 2E,G, 3A). These cells had few vacuoles in the cytoplasm (Fig. 2F). SEM revealed that cells of 7–10  $\mu$ m diameter possessed sparsely distributed microvilli (arrows in Fig. 3A) and could be distinguished from the neighbouring EC cells (arrowheads in Fig. 3A). These cells expressed brushin (Fig. 2G) but not SSEA-1, SJA receptors nor FT-1 (Fig. 2H) and were classified as primitive endoderm-like (PrE) cells. At the end of the period, cells expressing brushin comprised 10–25 % of the entire surface of the aggregates (Fig. 5). EC cells adjacent to PrE cells still expressed SSEA-1. The number of PrE cells increased in the next stage. Intercellular spaces were observed in the interior of the aggregates (Fig. 2F). SSEA-1 was expressed both on the surface and in the interior of the aggregates just as on day 1–2.

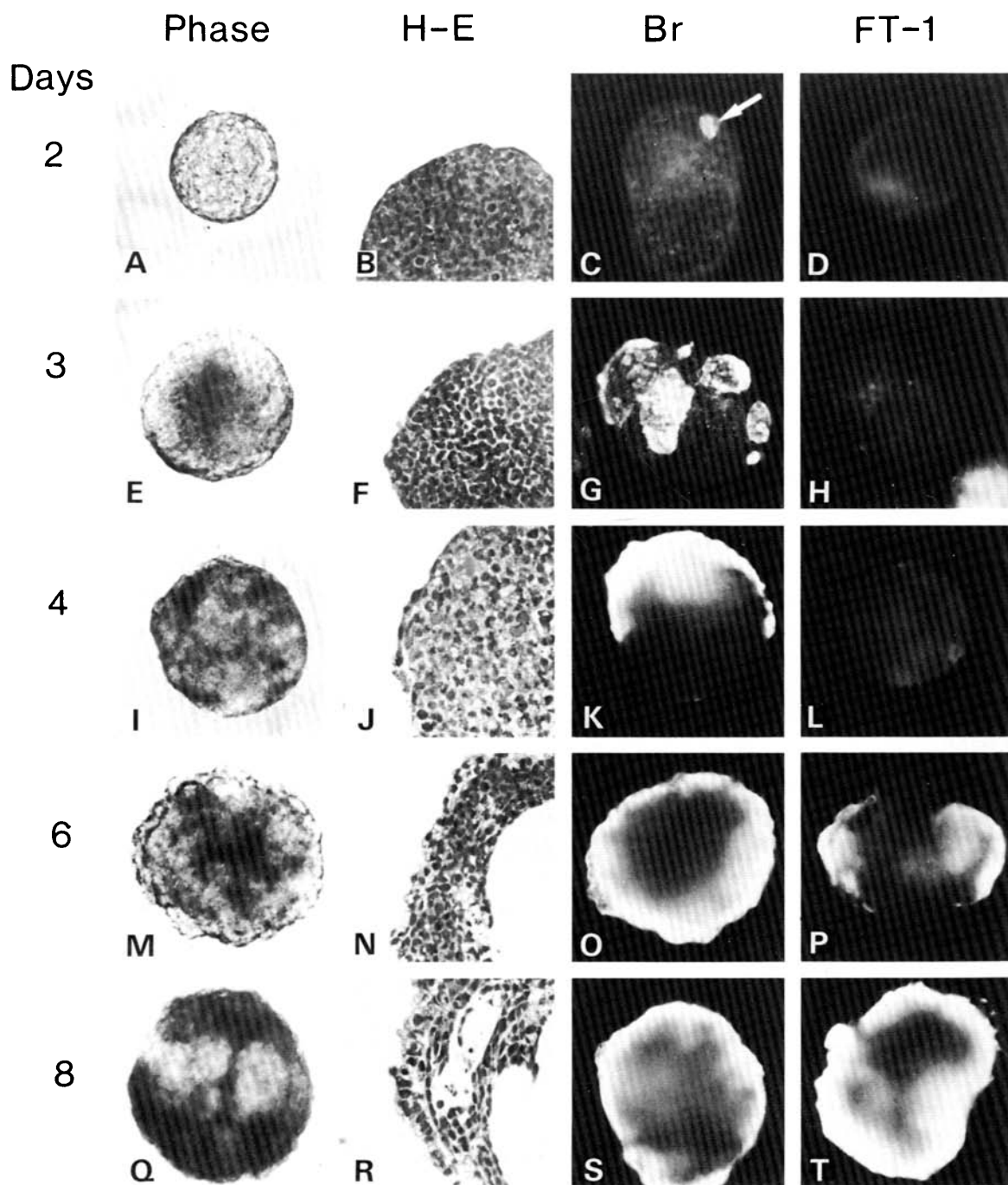


Fig. 2. Histological and immunohistochemical examination of N4-1 aggregates treated with  $10^{-8}$  M-RA. RA treatment was carried out for 2 (A-D), 3 (E-H), 4 (I-L), 6 (M-P) and 8 (Q-T) days. Phase, phase contrast microphotograph; H-E, haematoxylin-eosin staining of formalin-fixed materials; Br, immunofluorescent staining of living materials for brushin; FT-1, immunofluorescent staining of living materials for FT-1. (A,C,D,E,G-I,K-M)  $\times 95$ ; (B,F,J,N,R)  $\times 235$ ; (O,P,S,T)  $\times 75$ ; (Q)  $\times 50$ .

On day 4, the cells, which are brushin-positive and FT-1-negative, comprised 20–60 % of the surface of the cell aggregates (Figs 2I,K,L, 5). Furthermore, SSEA-1 became hardly detectable even in the morphologically undifferentiated cells present on the surface of the aggregates (Fig. 5). The aggregates completely lost adhesiveness to a plastic dish, and their shape became irregular (Fig. 2I). The interior cells expressed SSEA-1 weakly and uniformly. At this point, around 80 % of the cell aggregates began to form several small cysts (Fig. 2J). As above, the characteristic event on day 3–4 is the development of PrE. We call aggregates at this stage type II.

On day 5–6, the small PrE cells were gradually transformed into cells of cuboidal shape, which covered the entire surface of the aggregates (Fig. 2M). Thus, the aggregates took the form of simple embryoid bodies. The endoderm cells of cuboidal shape had numerous microvilli at the free surface (Fig. 3B) and had several vacuoles in the cytoplasm (Fig. 2N). Upon immunohistochemical staining, 80–100 % of the cells were shown to express brushin (Figs 2O, 5). However, the cells positive in FT-1, SJA receptors and  $\alpha$ -fetoprotein were still 10–30 % of the entire surface of aggregates (Figs 2P, 5). Thus, endoderm cells at this stage were more advanced than PrE cells, but still did not differentiate into mature visceral endoderm cells. We call these cells pre-visceral endoderm cells. In the

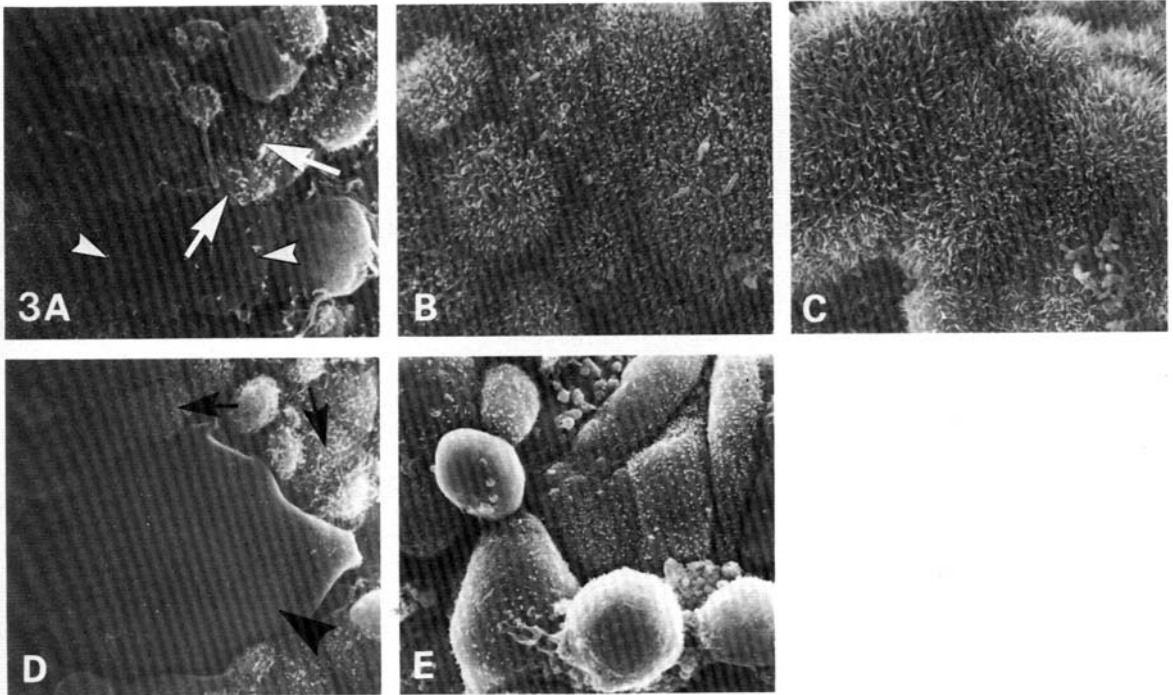


Fig. 3. Cell surface architectures of N4-1 aggregates treated with  $10^{-8}$  M- (A–C) or  $10^{-6}$  M- (D,E) RA were observed by SEM. (A,D) Day-3 aggregate. (B) Day-6 aggregate. (C,E) Day-8 aggregate. (A)  $\times 1445$ ; (B)  $\times 1840$ ; (C)  $\times 1970$ ; (D)  $\times 1315$ ; (E)  $\times 2500$ .

interior, cells expressing SSEA-1 started to be confined to the area near the developing cyst (Fig. 4A). The characteristic event on day 5–6 is the development of previsceral endoderm cells. The aggregate at this stage is called type III.

On day 7, endodermal cells underwent further dramatic alterations. They took on a columnar shape, and the cytoplasm was highly vacuolated (Fig. 2Q,R). Thus, they resembled extraembryonic visceral endoderm of normal mouse embryos at the egg cylinder stage (Snell & Stevens, 1966). The entire surface of the endoderm was covered with numerous microvilli (Fig. 3C). At the period, all the cells at the surface expressed brushin (Figs 2S, 5). Cells expressing SJA receptors comprised 40–70 % of the external cells; those expressing FT-1 and  $\alpha$ -fetoprotein comprised 10–60 % (Fig. 5). On day 8, FT-1 and  $\alpha$ -fetoprotein were expressed in nearly all endoderm cells (Figs 2T, 5). Thus, endoderm cells at this stage were becoming mature visceral endoderm. In the aggregates, several small cysts, which started to appear on day 3 or 4, were enlarged, fused to each other and formed a large cyst. Thus, almost all the aggregates took the form of cystic embryoid bodies (Fig. 2Q). The cyst was filled with amorphous substances resembling those found in the proamniotic cavity of 7-day-old mouse embryos, whereas cellular debris was rarely found in it (Fig. 2N,R). Two or three layers of cells were frequently observed under the visceral endoderm. SSEA-1 was expressed in the innermost layer, especially when the layer faced the cyst. The cells expressing SSEA-1 were considered to be EC cells, although they developed an elongated form (Fig. 4B). Indeed, EC cells were observed in addition to endoderm cells, when the cell aggregates at the stage were trypsinized and plated to a tissue culture dish. The characteristic event between day 7 and day 10 is the maturation of visceral endoderm. The aggregate at this period is classified as type IV.

On day 8 and thereafter, the cyst became larger, and maturation of visceral endoderm proceeded further with respect to the antigenic properties (Fig. 5).

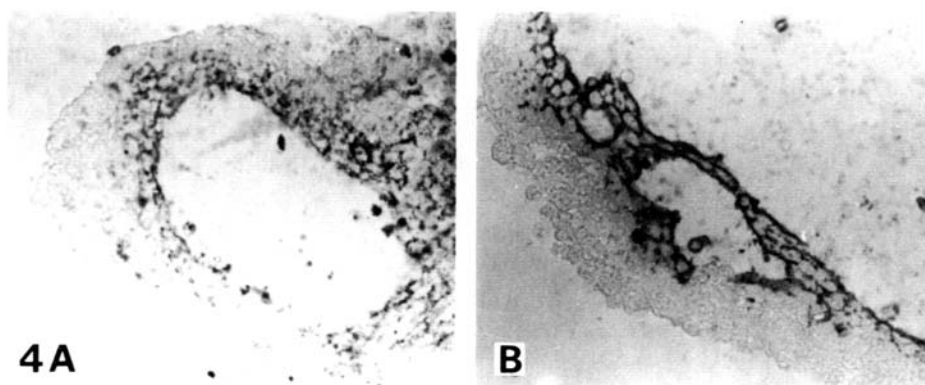


Fig. 4. RA-treated N4-1 aggregates, which were stained with the anti-SSEA-1 antibody by the immunoperoxidase method. (A) An aggregate on day 6. (B) An aggregate on day 8. Note that in both cases outer endodermal cells were completely negative for SSEA-1 and the positive cells were localized around the cyst. Formalin-fixed materials.  $\times 250$ .

As above, visceral endoderm formation proceeded through intermediate cells, two of which could be identified by cell surface properties and morphology: namely primitive endoderm-type cells and previsceral endoderm cells.

#### *Endoderm differentiation of N4-1 cells at high concentration of RA*

When aggregates of N4-1 cells were continuously treated with  $10^{-6}$  M-RA, endoderm differentiation proceeded till certain stages. Under these conditions cell growth was partly inhibited as compared to cells treated with  $10^{-8}$  M-RA (Fig. 6). Extensive cell death, however, was not observed during the incubation period. Until day 2, the aggregates did not undergo significant changes except that the entire aggregates became slightly darkened.

On day 3, small and rather flat cells appeared at several parts on the surface of the aggregates. These cells were concluded to be PrE cells, since they were brushin (+), FT-1 (-), SJA (-) and  $\alpha$ -fetoprotein (-), and possessed sparsely distributed microvilli (arrows in Fig. 3D). These cells dramatically increased in number on day 4, and covered most of the surface of the aggregates by day 5. The velocity of their

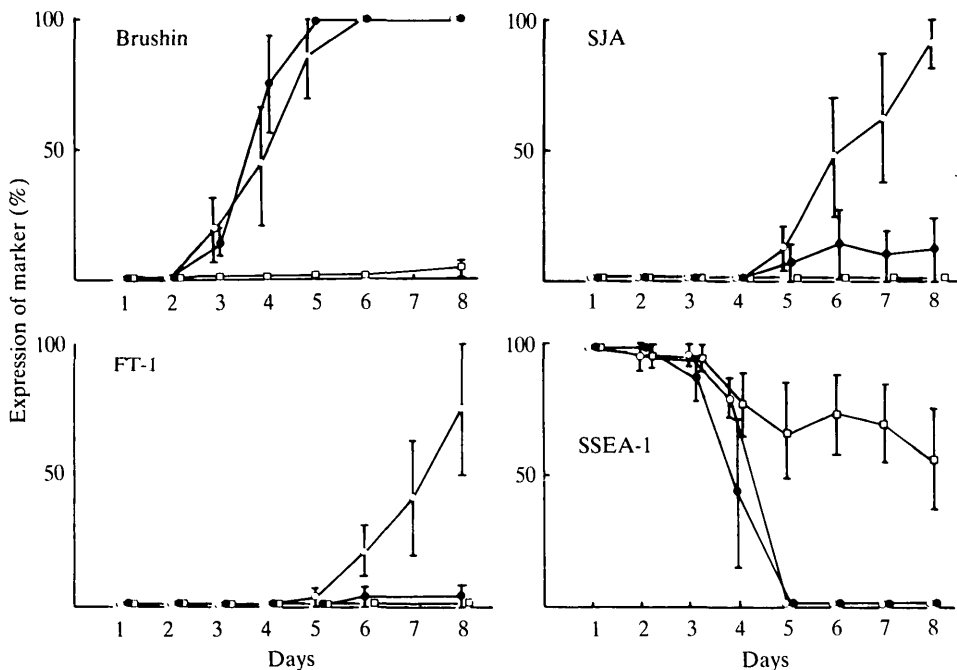


Fig. 5. Monitoring of RA-induced differentiation of outer endoderm layer of N4-1 aggregates by cell surface markers. For each stage, 20–30 aggregates were examined for reactivity to antibodies and lectins. Expression of brushin, SJA, FT-1 and SSEA-1 was examined using unfixed specimen. The result is expressed as the percent of the total observable surface of the aggregates which expresses the markers at a given day of differentiation. Data are presented as the mean determinates from three separate experiments, with the error bar indicating standard error of the mean. □, RA-free (control); ○,  $10^{-8}$  M-RA treatment; ●,  $10^{-6}$  M-RA treatment.

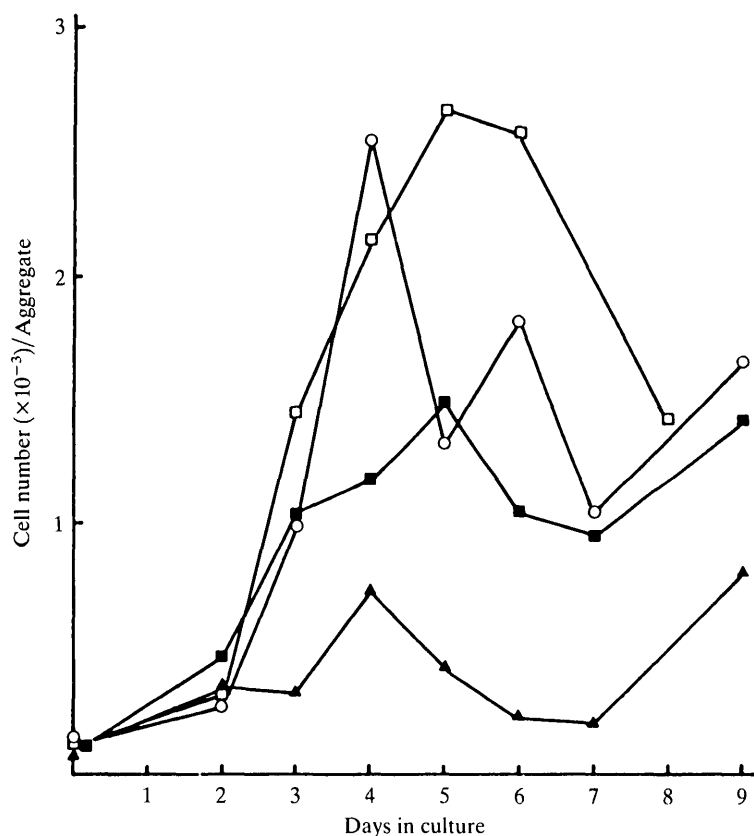


Fig. 6. Growth curve of aggregated N4-1 cells in various concentrations of RA. At the initial stage of culture, each aggregate consisted of about 100 cells. Concentration of RA in the medium; □, RA-free; ○,  $10^{-8}$  M; ■,  $10^{-7}$  M; ▲,  $10^{-6}$  M.

appearance seemed to be even greater compared to their appearance in  $10^{-8}$  M-RA (Fig. 5).

On day 5–6, the majority of endoderm cells remained PrE cells with sparse microvilli (Fig. 3E) and only a few cells showed numerous microvilli. Cyst formation and the appearance of highly vacuolated, columnar-shaped endoderm were not observed till day 10 (Fig. 7D,E). The endoderm layer was still composed of only one cell layer (Fig. 7E). Furthermore SJA receptors, FT-1 and  $\alpha$ -fetoprotein were expressed in less than 5 % of the external cells even after day 10 (Fig. 5). Thus, in the presence of the high concentration of RA, PrE differentiation took place, but the process did not proceed far enough to form mature visceral endoderm. The arrested cells were a mixture of PrE cells and previsceral endoderm cells.

In addition to the endoderm cells described above,  $10^{-6}$  M-RA induced another cell type. Thus, large and flat cells appeared on various parts of the surface of the aggregates after day 2. SEM observation revealed cells of 20–40  $\mu$ m diameter with

irregular shape, few microvilli and smooth surface (arrowhead in Fig. 3D). When the aggregates treated with  $10^{-6}$  M-RA for 3–4 days were transferred to tissue culture dishes, the aggregates tightly bound to the dish, and after a day, the large and flat cells migrated out (Fig. 7A). The cells had surface architectures identical to the large and flat cells in the aggregates (data not shown). They did not proliferate, and after a week became thin and extended dendrites (Fig. 7B). The cells did not react with DBA (arrows in Fig. 7C), which reacts with both parietal and visceral endoderm cells in postimplantation mouse embryos (Noguchi, Noguchi, Watanabe & Muramatsu, 1982). The cell also did not express SSEA-1 or brushin (data not shown). The migratory cells are probably intermediate cells leading to cells of mesoderm lineage. These cells were not observed in case of treatment with  $10^{-8}$  to  $10^{-9}$  M-RA.

#### *Effects of duration of exposure to RA*

In the above experiments, RA was added throughout the culture period. Then, we examined the effect of the duration of exposure to RA.

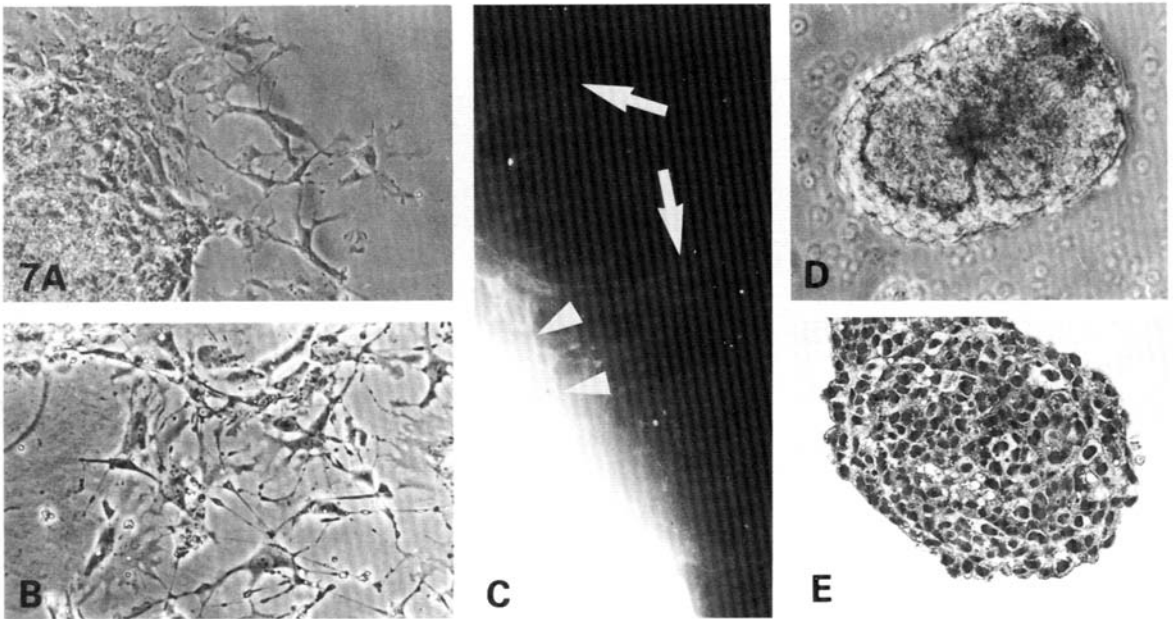


Fig. 7. Outgrowth of non-endoderm cells and simple embryoid bodies from N4-1 aggregates treated with  $10^{-6}$  M-RA. (A,B) Non-endoderm cells migrated from day 4 aggregates 2 days (A) and 7 days (B) after transfer into RA-free medium. Flat cells with some dendrites outgrew and became thin during the culture period. (C) Outgrowth reacted with FITC-labelled DBA. The cells migrating from aggregates did not react with FITC-DBA (arrows), while endoderm cells on the aggregates reacted strongly with DBA (arrowheads). (D,E) Phase-contrast microphotograph (D) and section (E) of a formalin-fixed day 8 aggregate. (A,B)  $\times 120$ ; (C)  $\times 150$ ; (D)  $\times 85$ ; (E)  $\times 120$ .

Treatment with  $10^{-8}$  M-RA for 2 days was sufficient to induce mature visceral endoderm cells, while the aggregates treated with the reagent for 10 h or less behaved identically to the untreated ones (Table 1). Treatment between 10 to 28 h resulted in partial differentiation. These aggregates differentiated to stage II in the same way as the aggregates continuously treated with  $10^{-8}$  M-RA, while further differentiation of the external layer was inhibited, and mature visceral endoderm was scarcely observed even on day 8. The outer cell surface of these aggregates was brushin (+) and SSEA-1 (-) in most of the area (data not shown). Cyst formation appeared to proceed normally in these aggregates.

When the aggregates were first treated with  $10^{-6}$  M-RA for 2 days and transferred to the medium containing  $10^{-8}$  M-RA, visceral endoderm differentiation occurred normally (Table 2). However, treatment with  $10^{-6}$  M-RA for more than 3 days delayed the visceral endoderm differentiation in  $10^{-8}$  M-RA (Table 2). On the other hand, visceral endoderm differentiation was inhibited in aggregates treated with  $10^{-8}$  M-RA for 4 days and then transferred to the medium containing  $10^{-6}$  M-RA (Table 3). Exposure to  $10^{-8}$  M-RA for 5 days allowed the differentiation of mature visceral endoderm, even after the transfer to the medium with  $10^{-6}$  M-RA (Table 3). From these results, we concluded that around day 4 is the stage when visceral endoderm differentiation is sensitive to the inhibitory effect of high concentration of RA.

## DISCUSSION

We have analysed the process of visceral endoderm differentiation by applying cell surface markers and SEM. Among the markers used, brushin was found to be a specific marker of primitive and visceral endoderm. On the other hand, FT-1 was

Table 1. *Effects of duration of treatment with  $10^{-8}$  M-RA on the visceral endoderm differentiation of N4-1 aggregates*

Period of treatment with RA (h)	Number of aggregates on day 8			
	Total	Type I*	Immature type**	Type IV*
5	11	11	0	0
9	10	10	0	0
13	10	2	8	0
16	9	0	9	0
20	9	0	9	0
24	12	0	12	0
28	10	0	10	0
48	25	0	0	25

After treatment with  $10^{-8}$  M-RA, the aggregates were washed three times with the RA-free medium and cultured till day 8 in the normal medium in 96-well microtiter plates. On day 8, the aggregates were examined morphologically and histochemically.

\* Type I and IV are defined in the text.

\*\* See the text for explanation.

revealed to be the marker of mature visceral endoderm. As described previously (Sato & Muramatsu, 1985), SJA receptors were confirmed to be the marker of visceral endoderm, while SJA receptors emerged a little earlier than FT-1 during differentiation of visceral endoderm. Combining the two cell surface markers, brushin and FT-1, we could distinguish PrE cells and visceral endoderm cells.

Several other markers are useful to monitor visceral endoderm differentiation (Dziadek & Adamson, 1978; Brûlet, Babinet, Kemler & Jacob, 1980; Kapadia, Feizi & Evans, 1981; Oshima *et al.* 1983; Fox, Damjanov, Knowles & Solter, 1984). Among them, two cell surface antigens, SSEA-3 (Fox, *et al.* 1984) and i (Kapadia *et al.* 1981) are also expressed in both primitive endoderm and visceral endoderm, although their modes of expression in other embryonic and adult cells

Table 2. *Visceral endoderm differentiation of N4-1 aggregates treated with  $10^{-6}$  M-RA for a given period and then with  $10^{-8}$  M-RA*

Period of the initial treatment with $10^{-6}$ M-RA (day)	Number of aggregates on day 8			
	Total	Type I*	Type II and III*	Type IV*
0	11	0	0	11
1	23	0	0	23
2	25	0	0	25
3	20	0	14	6
4	18	0	17	1
5	23	0	23	0
6	20	0	20	0

After treatment with  $10^{-6}$  M-RA, the aggregates were washed three times in the RA-free medium and cultured till day 8 with  $10^{-8}$  M-RA. On day 8, the aggregates were examined morphologically and histochemically.

\* The classification of the aggregate is described in the text.

Table 3. *Visceral endoderm differentiation of N4-1 aggregates treated with  $10^{-8}$  M-RA and then with  $10^{-6}$  M-RA*

Period of the initial treatment with $10^{-8}$ M-RA (day)	Number of aggregates on day 8			
	Total	Type I*	Type II and III*	Type IV*
0	7	0	7	0
1	17	0	17	0
2	26	0	26	0
3	25	0	25	0
4	24	0	20	4
5	13	0	0	13
6	14	0	0	14

Experiments were performed as described in the legend of Table 2, except that the aggregates were initially treated with  $10^{-8}$  M-RA, followed by  $10^{-6}$  M-RA.

\* The classification of the aggregate is described in the text.

are different from brushin. So far FT-1 and SJA receptors are the only cell surface markers selectively expressed in visceral endoderm in early postimplantation embryos. Because of the simplicity of their detection, they will be widely used to distinguish mature visceral endoderm cells from other cells.

In addition to cell surface markers, SEM has been effective to monitor the process of differentiation. Especially, with the aid of SEM, we could detect intermediate cells other than PrE cells, namely previsceral endoderm cells. Previsceral endoderm cells were located between PrE cells and visceral endoderm cells in the cell lineage, and were characterized by the presence of numerous microvilli in spite of the non-expression of FT-1 marker. Existence of such an intermediate stage has been noted by Grabel & Martin (1983). They used 'heightening' of the cells with numerous vacuoles, as the character to define the stage.

A newly established cell line, N4-1 was used for the analysis of visceral endoderm differentiation. This was because the F9 cells we have do not differentiate efficiently to mature visceral endoderm. It should be noted that Grover *et al.* (1983) used a subclone B1 of F9 cells to analyse the process of visceral endoderm differentiation. Events occurring during the differentiation of N4-1 cells appear to be similar to those occurring in the differentiation of F9 cells (Grover *et al.* 1983). One difference we noted is that interior cells of the aggregates did not lose SSEA-1 even on day 10 in case of N4-1 cells. SSEA-1 was reported to disappear from the interior cell altogether on day 4-5 in the case of F9 cells (Grover *et al.* 1983).

We found that the high concentration of RA inhibited visceral endoderm differentiation and induced the appearance of migratory cells of non-endoderm properties. This finding is somewhat similar to that of Edwards & McBurney (1983): aggregates of P19 cells treated with  $10^{-7}$  M-RA for 2 days differentiated less endoderm cells and more nerve cells as compared to cells treated with  $10^{-8}$  to  $10^{-9}$  M-RA.

By using cell surface markers, we clarified that the inhibitory point in the differentiation system of N4-1 cells is not at the entrance of endodermal lineage (at the stage of PrE formation), but at more later stages. Furthermore, the period sensitive to the inhibitory action of RA has been determined to be around 4 days. The mechanism behind the inhibition of visceral endoderm differentiation by high concentrations of RA is not known at the present time. Although high concentrations of RA partly inhibited cell growth, deficiency in cell number is not the reason: when 10 or 30 cell aggregates, each composed of about 100 cells, were aggregated together and treated with  $10^{-6}$  M-RA, visceral endoderm did not differentiate (unpublished results). In view of cell death occurring at high concentrations of RA, we should take into account the possibility that the inhibitory effect of RA is not due to RA itself but due to minor impurities in RA preparation, such as decomposition products of RA. In any event, the differentiation system of N4-1 cells to visceral endoderm is an excellent system to analyse the control mechanisms of differentiation, since the stage and the time

sensitive to high concentrations of RA have been revealed by the present investigation.

We thank Dr D. Solter and Prof. S. Nishi for antibodies and Miss K. Sato for her expert secretarial assistance. This work has been supported by Grants from the Ministry of Education, Science and Culture, Japan and Special Coordination Funds for Promoting Science & Technology of the Science and Technology Agency of Japan.

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(Accepted 11 March 1985)