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ISOLATION OF MUTANTS SHOWING TEMPERATURE-SENSITIVE CELL GROWTH FROM EMBRYONAL CARCINOMA CELLS: CONTROL OF STEM CELL DIFFERENTIATION BY INCUBATION TEMPERATURES

Yoshitake Nishimune, Yukio Nishina, Tetsuro Sumi*, Mitsuko Kosaka, Masashi Takeda#, Keishi Matsumoto#, Aizo Matsushiro and Masayoshi Sakuda*

Research Institute for Microbial Diseases, 2nd Department of Oral Surgery, Dental Faculty*, Yamada Oka Suita, Osaka 565, JAPAN

2nd Department of Pathology, Faculty of Medicine#, Osaka University, Kitaku, Osaka 530, JAPAN

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SUMMARY: Embryonal carcinoma(EC) cells, the undifferentiated stem cells of teratocarcinomas, have many properties in common with pluripotent embryonic cells, and thus provide an excellent system for studying the early events involved in embryonic development and stem cell differentiation. We have isolated three novel mutants with temperature-sensitive(ts) cell growth that were able to differentiate at a non-permissive temperature for cell growth. These mutations affect the progression of the cell cycle, leading to the transient accumulation of cells in a specific phase, the S phase, of the cell cycle, which is likely to be the primary cause of stem cell differentiation of EC cells at non-permissive temperature. Isolation of these mutants strongly supports the notion that there is a close association between the inhibition of DNA synthesis and EC cell differentiation.

The stem cells of teratocarcinomas are able to differentiate in vitro under certain conditions, and this provides an excellent opportunity for studying the determining processes for cell differentiation as well as the subsequent differentiation steps[1].

Various chemicals that induce the differentiation of embryonal carcinoma cells have been used to study of the mechanisms of stem cell differentiation[2,3]. However, the use of such chemicals could complicate the interpretation of results, since they always have a variety of effects on stem cells. The genetic approach, on the other hand, can be very powerful for clarifying the many complicated steps involved in some biological phenomenon and is the classical approach to such problems. In this context, the development of new genetic systems for stem cell differentiation could be very useful both for elucidating the molecular mechanisms involved in the steps between stem cell proliferation and differentiation and for determining the genes that

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control cell differentiation. Thus, we adopted the latter strategy to study stem cell differentiation.

To date, numerous reports have suggested that there is a relationship between stem cell differentiation and cell cycle progression; however, retardation of cell cycle progression does not always result in cell differentiation[4]. In order to clarify this point, we decided to isolate temperature-sensitive(ts) cell cycle mutants which undergo stem cell differentiation at non-permissive temperature, as isolation of such mutants may reveal the existence of control steps for the cell cycle and stem cell differentiation. Furthermore, cell cycle ts mutants would offer a logical basis for determining which gene is relevant to cell cycle progression and which gene has a key role in triggering the stem cell differentiation.

We chose the screening procedure to isolate ts mutants for cell growth, because our previous observations indicated that differentiation of stem cells could be linked to retardation of the cell cycle in the S phase [2], and because retinoic acid(RA) induced both stem cell differentiation and cessation of cell growth[5,6], which has been attributed specifically to a lengthening of the S phase[7].

Using F9 teratocarcinoma stem cells , we have isolated and characterized several ts growth mutants that undergo differentiation at a temperature which arrests cell growth. These mutants showed a unique characteristic of temperature-sensitivity that affects the stem cell replication at the S phase, resulting in differentiation of EC cells.

MATERIALS AND METHODS

Cell line and isolation of temperature-sensitive mutants

The parental cell was a mouse EC cell line, F9. Temperature-sensitive cells were isolated as follows: Exponentially growing F9 cells were cultured on gelatin-coated culture dishes in Eagle's minimal essential medium(MEM) containing 5mM glutamine, 1mM Na-pyruvate and 10% fetal calf serum(FCS) at 32.5°C and treated with 1μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) at 32.5°C for 16hr. After washing out the MNNG,the cells were incubated at 32.5°C for 3 days and then shifted to 37.5°C. Selection at this temperature is reported to be effective for isolating many ts mutants especially those with a mutation at the S phase[8]. After 1-2hr at this temperature, 5-fluorodeoxyuridine (FUdR,25μg/ml) and uridine(125μg/ml) were added to the culture; and the cells were further incubated at 37.5°C for 10-16hr, followed by washing for three times with phosphate-buffered saline(PBS). The cells were then cultured in growth medium containing 25μg/ml of thymidine for 2-3 days at 32.5°C. After this first selection, the cells were subjected to a second cycle of selection with FUdR. After 10-15 cycles of such selections, the cells were replated and cultured at 32.5°C for about 1-2 weeks. The surviving colonies were recloned 3 times and tested for their temperature-sensitivity.

Assay for plasminogen activator(PA)

Cells($1x10^5$) were plated in 35-mm diameter dishes and incubated for about 12h at 32.5°C. The plates were then transferred to an incubator at 39°C or RA(10^{-7}M) was added to the culture medium at 32.5°C as a positive control of cell differentiation. For the PA assay, 24 hours before harvesting, all plates were washed with serum-free medium, and the cells were incubated in 1 ml of

MEM containing 0.2% bovine serum albumin Fr.V. The harvested media were stored at -30°C until the PA assay. The cell number was also determined at each harvesting by a Coulter counter. After the cells were treated with PBS containing 0.125% trypsin and 0.5mM EDTA (trypsin:EDTA) at 32.5°C for 20 min, PA activity in the culture medium was assayed by the [^{125}I]-fibrin degradation procedure[9] and normalized to the cell number.

Detection of SSEA-1 antigen: Immunocytochemical staining of cells by the avidin-biotin complex method

Aliquots of cells harvested under each culture condition, each suspended in culture medium, were incubated on a poly-L-lysine coated glass slide for about 1 hour at 32.5°C, washed with PBS and then, fixed with 0.4% paraformaldehyde for 30 min. After incubation with SSEA-1 monoclonal antibody(1.6×10^{-3} dilution of ascites fluid of the hybridoma) or with normal mouse IgM as a control, binding of this antibody was demonstrated with VECTASTAIN ABC reagent(Vector Laboratories, Burlingame, CA) using horseradish peroxidase developed diaminobezidine as a marker. After the enzyme reaction was terminated, all cells(stained and non-stained cells) were counted. The results were expressed as the percentage of brown colored cells to the total cells counted.

Flow cytometric analysis

Cells in the logarithmic growth phase were harvested with trypsin:EDTA and then fixed with ethanol. After that, the cells were successively treated with 0.5mg/ml ribonuclease A (from bovine pancreas(Sigma), pretreated at 80°C, 5 min) in PBS at 37°C for 30 min., washed with PBS, stained with ethidium bromide(20μg/ml) and analyzed with a cytofluorograph(FACS type 11, Becton Dickinson).

RESULTS

Isolation of temperature-sensitive mutants of teratocarcinoma F9 cells

Several ts mutants retaining EC-like morphology at permissive temperature were isolated from F9 cells after mutagenesis with MNNG followed by selection at the non-permissive temperature(39°C) with FUdR. All of the mutant cell lines grew normally at 32.5°C, with a doubling time of 18h-19h(Fig.1). Because doubling times for the wild-type cells at 32.5°C was 18-19h, growth rates of the mutant cell lines at 32.5°C were almost identical with that of the wild type cells. The growth rates of the mutants at high temperature, however, were much lower than those of F9 cells(doubling time of F9 is 11-12h at 39°C)(Fig 1). Behaviors at non-permissive temperature varied among mutants:the increase in cell number before cessation of cell growth was almost about 5 times in the case of one mutant (M30) and 2-3 times in the cases of others(B14.231 and C11.222); M30 cells had a tendency to detach from the plate after cessation of growth, whereas the others did not show such a tendency for several days.

The mutant cells kept at 39°C were transferred to 32.5° C at various times to examine the reversibility of altered cell growth. As seen in Fig 1,upon shift-down the cells resumed growth after a small delay,although the recovery of cell growth did not reach the control level if the periods of heat exposure were prolonged. Thus, all of the mutant cells retained their capacity for cell division even after exposure to 39°C for as long as 2 days, and the temperature effect was reversible to a degree dependent upon the periods of incubation at high temperature.

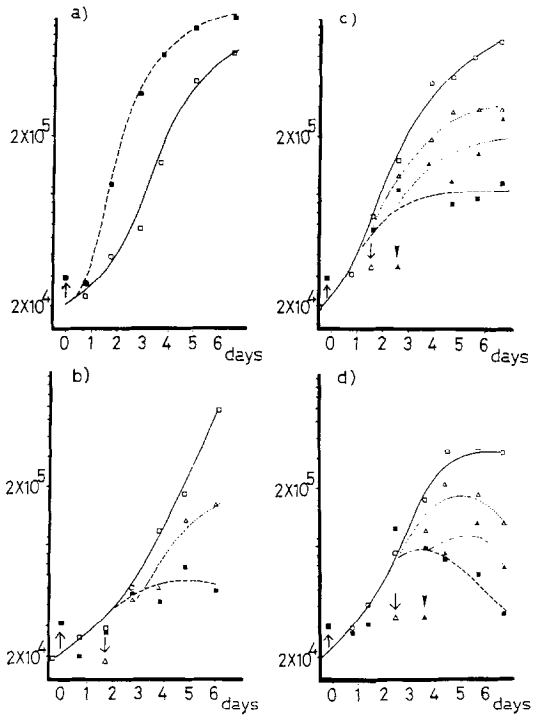

Figure 1.
Growth profiles of the temperature-sensitive mutants.
$2x10^4$ cells of the parental F9 (a) and ts mutants, B14.231(b), C11.222(c) and M30(d), were seeded in plastic Petri dishes coated with gelatin and incubated in culture medium at 32.5°C(solid line:open squares)or shifted up to 39°C(broken line:closed squares) at the time indicated by the upward arrow(12h after seeding). In order to check the reversibility of growth, cultures of ts mutants at 39°C were shifted down to 32.5°C at the time indicated by the downward arrow (open triangle) or arrowhead(closed triangle) positions(dotted line). Cell number (vertical axis) was determined for each plot in duplicate.

Mutant cell lines shifted to 39°C continued to proliferate for about 2-3 days(Fig 1). Thereafter, as their growth ceased, their cell morphology became altered to that of differentiated cells: they became heterogeneous in appearance, some of the cells in the populations increased in size, and some others showed morphology typical of endodermal cells.

To assess the differentiation of the mutants after cessation of cell growth at non-permissive temperature, we examined plasminogen activator(PA) secretion and cell surface antigenic changes such as the disappearance of SSEA-1, which have been used for monitoring teratocarcinoma stem cell differentiation[10,11].

Production of PA at 39°C

Levels of PA were estimated by measuring radioactivities of solubilized [^{125}I]-fibrin in the presence of plasminogen[9]. Activity of PA in the media secreted from each mutant was determined for each cell line grown at 39°, 32.5° and at 32.5°C in the presence of retinoic acid. Figure 2 illustrates the

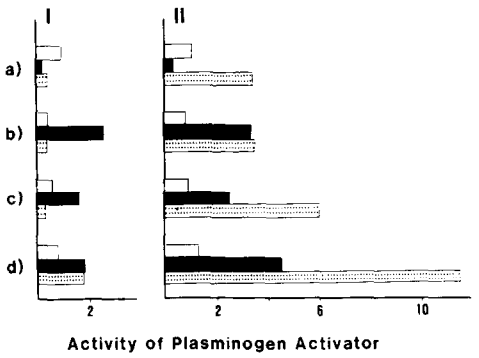

Figure 2.
Activity of plasminogen activator(PA) secreted into culture medium at different temperatures

All assays were performed in triplicate. Control incubations without cells or plasminogen were always assayed and these values were subtracted from the experimental values. Data are presented as the fraction solubilized from the total available substrate on a per cell basis(% solubilized [^{125}I] per 10^5 cells). Open, closed and dotted bars are PA activities found at 32.5°C, 39°C and after RA induction at 32.5°C, respectively. Twelve hours after cell inoculation at 32.5°C,the cells were exposed to a temperature shift or to retinoic acid. After 18h(I) or 36h(II), the media were changed to PA assay media and incubated with cells for 24hr. Media were then collected and stored at -30°C until the PA assay. a)parental F9 cells, b)B14.231, c)C11.222, d)M30.

effects of incubation temperature and differentiation induced with retinoic acid on the secretion of PA into the culture media. The ts mutant cells showed relatively low levels of PA at the permissive temperature compared to those of F9 cells observed at either temperature. However, following incubation at the non-permissive temperature, all the mutants showed an increase in the levels of PA, responding more quickly to temperature shift-up than to RA exposure. A significant amount of PA production was not induced with RA within the 42 hr of treatment, while ts mutant cells incubated at 39°C for the same time period showed substantial production of PA(Fig 2). By 60 hr of treatment, however, the levels of PA induced with RA apparently exceeded those produced by heat-induction in the case of two of the mutants (C11.222 and M30). Thus,the levels of PA secreted from the ts mutants at the non-permissive temperature were comparable to those induced with RA, although the levels of activity induced by heat-induction were slightly different among the mutants.

Loss of antigenicity of stem cell culture at 39°C

To further characterize the capacity of ts mutants to differentiate at the non-permissive temperature, cells of each mutant were shifted to 39°C and examined for the cell surface antigen, SSEA-1, a marker for undifferentiated EC cells. To estimate the percentage of ts cells which manifest differentiated phenotypes at the non-permissive temperature, expression of SSEA-1 was immunocytochemically determined using the avidin-biotin complex method. Approximately 30, 60 and 55% of the mutant cells of B14.231, C11.222

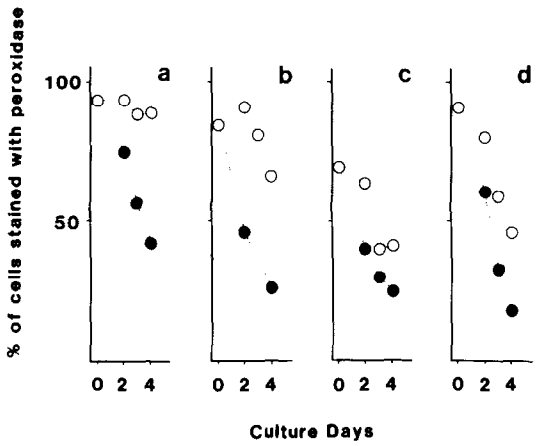

Figure 3.
Loss of cell surface antigen(SSEA-1) in mutant cell lines incubated at non-permissive temperature demonstrated by immunocytochemical staining
Cells stained with the peroxidase reaction, having a brown color, were counted and shown as % of the total number of cells.
Open circles:incubation at 39°C
Closed circles:incubation at 32.5°C with 10^{-7}M of RA.
a) parental F9 cells, b)B14.231, c)C11.222, d)M30

and M30, respectively, showed no staining for SSEA-1 antigen after 4 days of incubation at 39°C, comparable to those in the cells induced with retinoic acid, although the loss of SSEA-1 antigen by heat induction seems to be delayed in comparison with that shown with RA induction(Fig 3).

Cell cycle progression of temperature-sensitive mutants at non-permissive temperature

Cultures of the ts mutants show a gradual cessation of cell growth at the non-permissive temperature(Fig 1). To determine if, under non-permissive conditions, the ts mutants were arrested in a specific phase of the cell cycle, flow cytometric analysis was carried out. As shown in Fig 4, the fraction of cells in the S phase began to increase in the mutant cell lines at 12-24h after shifting up to 39°C, whereas under these conditions, the parental wild type F9 cells underwent one to two cycles of DNA synthesis(Fig 1) and showed no difference in the flow cytometric patterns between 32.5°C and 39°C. In the case of the mutant cell lines, however, the cells retarded in the S phase begin to shift to the G_2-M fraction (Fig 4). Thus, cell cycle progression was not completely arrested at any specific phase even after longer times of incubation(more than 5 days) at the non-permissive temperature(data not shown). These results indicate that the ts mutant cells cultured at 39°C were first arrested at the S phase after the cell cycle proceeds gradually, suggesting that defects in the ts mutant cells primarily affect the S phase of the cycle.

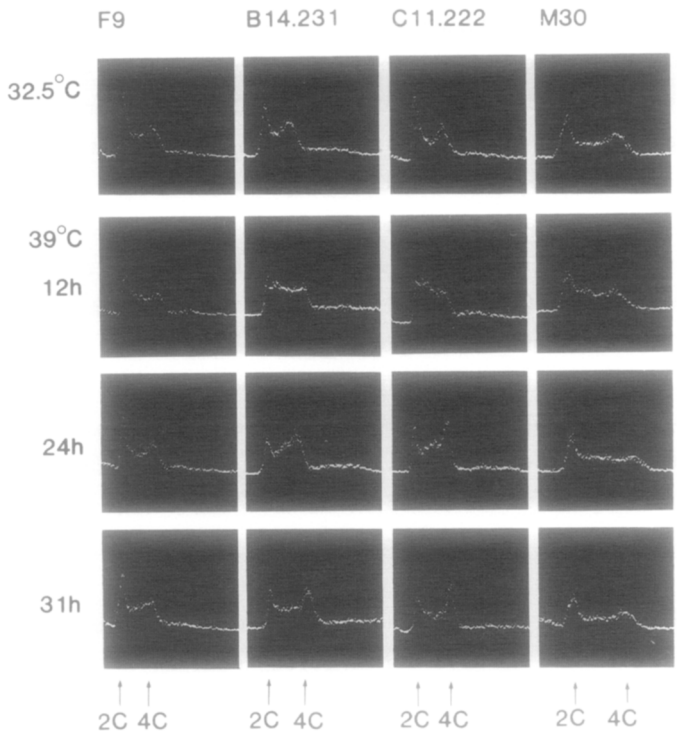

Figure 4.
Flow cytometric analysis of DNA content of F9 and mutant cells.
The vertical axis shows relative cell number; the horizontal axis shows fluorescence,i.e., representing DNA content. Cells having a G_1 DNA content are represented in the first and major peak(2C); cells in G_2 and M are represented in the second smaller peak(4C); cells in the S phase fall between the G_1 and G_2-M peaks. Parental F9 cells and three ts mutants were cultured at 32.5°C and 39°C for 12h, 24h and 31h, respectively.

DISCUSSION: At present, the relationship between cell cycle and differentiation is not firmly established. However, the fact that almost all inhibitors of DNA synthesis induced differentiation[2], indicates a close association between the retardation of cell cycle progression in the S phase and induction of teratocarcinoma stem cell differentiation. Moreover, retinoic acid-induced differentiation of PC13 embryonal carcinoma stem cells into endoderm-like cells accomplished with a reduced growth rate, which is due to an increase in the length of time in the S phase rather than G_1, makes the greatest contribution to the increase in generation time[7]. Thus, this contrasts with the regulation of terminal differentiation where the alteration in growth rate results principally from changes in the length of the G_1 phase[12].

Although the initiation of stem cell differentiation seems to be associated with retardation of cell cycle progression or cessation of cell growth, it remains to be determined whether such retardation and cessation of cell growth are the causes or the results of cell differentiation.

These mutants showing slow cessation of cell growth were further investigated to determine whether this phenotype could be due to defects in S-phase progression or to the results of cell differentiation. Our flow cytometric analysis showed that the ts cells arrested in the S phase, for 12-24h after a temperature-shift up to 39°C, began to show characteristics of differentiation (Figs 2 and 3),suggesting that the retardation of cell growth at the S phase is not a secondary effect on stem cell differentiation but is rather the primary ts effect due to defects in the ts mutant cells.

Thus, isolation of these cell cycle ts mutants, which differentiate at a temperature non-permissive for growth, reinforces the notion that retardation of cell cycle progression at a specific stage or some events during such a retardation may cause the stem cell differentiation. Furthermore, the use of such ts mutants will provide an approach for determining which cell-cycle dependent genes are most relevant to stem cell differentiation.

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