Jpn. J. Cancer Res. 84, 625–632, June 1993

Changes of Proliferative Activity and Phenotypes in Spontaneous Differentiation of a Colon Cancer Cell Line

Ayumi Hara,[1] Toshifumi Hibi,[1,2,5] Masahiro Yoshioka,[3] Kyoko Toda,[4] Noriaki Watanabe,[4] Atsushi Hayashi,[1] Yasushi Iwao,[1] Hidetsugu Saito,[1] Tetsu Watanabe[1] and Masaharu Tsuchiya[1]

[1]*Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160,* [2]*Keio Cancer Center, 35 Shinanomachi, Shinjuku-ku, Tokyo 160,* [3]*Department of Internal Medicine, Minamitama Hospital, 3-10-1 Sandamachi, Hachiohji 193 and* [4]*Department of Internal Medicine, Kitasato Institute Hospital, 5-9-1 Shirogane, Minato-ku, Tokyo 108*

We examined the alterations of proliferative activity and c-*myc* expression of a colon cancer cell line (Caco-2) during its spontaneous differentiation. Caco-2 cells were cultured in various types of media and the degree of differentiation was monitored in terms of dome formation in cell monolayers and expression of alkaline phosphatase (ALP) activity. In Caco-2 cells cultured with Eagle's minimum essential medium (EMEM) containing 10% fetal calf serum (FCS), dome formation was demonstrated and ALP activity was markedly increased after the cells reached confluence. Five-fold reduction of c-*myc* mRNA and a marked decrease in S-phase cells were observed in the differentiated cells. These changes were not induced in FCS-free EMEM. The addition of insulin and transferrin to FCS-free EMEM did not induce cell differentiation or reduction of c-*myc* mRNA expression. When Caco-2 cells were cultured with three different serum-free media, the induction of dome formation and the increase of ALP activity were observed to varying degrees. Expression of c-*myc* mRNA in the cells cultured with one serum-free medium decreased to a level similar to that in fully differentiated cells cultured with EMEM containing 10% FCS. These results suggest that a spontaneous switch from a proliferative state with high c-*myc* expression to differentiated phenotype occurs after cells reach confluence and depends on the culture conditions.

Key words: Colon cancer — Caco-2 — Cell differentiation — c-*myc*

The induction of differentiation in cancer cells has been recognized as a key area in studies on carcinogenesis and cancer treatment. For such studies, an *in vitro* model of differentiation is required. Many reports have claimed that *in vitro* differentiation could be induced by various kinds of differentiation inducers, such as N,N-dimethylformamide,[1] sodium butyrate[2] and retinoic acid.[3] However, these inducers might be harmful to cancer cells and act lethally on some population of the cells. It is possible that the selected population resistant to the inducers may proliferate and differentiate. The inducers could also cause direct changes in the oncogenes which must play a key role in the differentiation. To avoid these undesirable effects of the inducers, a spontaneous differentiation model would be preferable. The colon cancer cell line, Caco-2, established by Fogh *et al.*,[4] has been accepted as a model of polarized intestinal epithelial cells because it exhibits spontaneous enterocytic differentiation under the standard culture conditions.[5] The characteristic markers of differentiation, including an increase in brush border membrane alkaline phosphatase (ALP) activity and dome formation in cell monolayers, are expressed after cells reach confluence without any exogenous inducing agents. Thus, we can exclude the possibility that some special population grows selectively during differentiation. Therefore, this model provides a useful means of tracing molecular alterations during spontaneous differentiation of colon cancer cells, such as oncogene activation and suppressor gene inactivation.[6,7]

The *myc* proto-oncogene (c-*myc*) is expressed in many types of normal and tumor-derived cells.[8–10] The expression of c-*myc* is closely related to the regulation of cell proliferation.[11,12] There are several reports that colon cancer cells show high expression of c-*myc* mRNA, compared with that of normal colon epithelial cells.[13–15] In other systems, the expression of c-*myc* appears to play either an obligatory or auxiliary role in growth arrest or differentiation.[16–18] Recent clinical observation indicates that the expression of c-*myc* oncogene might prove to be a useful marker to evaluate the malignant potential of colorectal polyps,[19] and *in vitro* studies using chemical inducers revealed that the level of c-*myc* mRNA expression is reduced in differentiated colon cancer cell lines.[20] There has been no direct evidence that the level of c-*myc* mRNA expression has an inverse relationship to spontaneous differentiation. Thus, in the present study, we investigated the changes in proliferative activity and

[5] To whom correspondence should be addressed: Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160.

c-*myc* mRNA level during the spontaneous differentiation of Caco-2 cells, and attempted to identify some of the factors inducing differentiation.

MATERIALS AND METHODS

Cell culture and dome formation Caco-2 cells (1×10^6) obtained from the American Type Culture Collection (Rockville, MD) were seeded in 25-cm^2 plastic flasks (Gibco Laboratories, Grand Island, NY) and grown in Eagle's minimum essential medium (EMEM, Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 1% non-essential amino acids (NEAA, Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) in an atmosphere of 5% CO_2/95% air at 37°C. The medium was changed every two days. Cells between 30 to 50 passages were used for the experiments.

Dome formation in cell monolayers, which was attributed to the ion and water transport across polarized epithelial cells,[5] was observed by using a phase-contrast light microscope (Olympus, Tokyo).

Enzyme and protein assays Caco-2 cells were harvested and homogenized in 2 m*M* Tris-HCl and 50 m*M* mannitol, pH 7.2, containing 1 m*M* phenylmethylsulfonyl fluoride. Brush border membrane hydrolase (ALP) activity was assayed at 37°C using *p*-nitrophenyl phosphate as the substrate. Enzyme activity was expressed in units per mg protein. One unit is equivalent to 1 m*M* substrate hydrolyzed per minute. Protein was measured by a modification of the method of Lowry *et al.*[21]

Cell growth curve Cells were harvested using 0.25% trypsin in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (Gibco) and counted with a hemocytometer. Viability was determined by the Trypan blue dye exclusion test.

Cell cycle analysis The cell cycle was analyzed by flow cytometry as described by Dolbeare *et al.*[22] Caco-2 cells were pulse-labeled with 5-bromo-2′-deoxyuridine (BrdU, Sigma, St. Louis, MO) for 30 min, fixed with 70% ethanol and stained with FITC-conjugated anti-BrdU monoclonal antibody (Beckton-Dickinson, MA). Cells were further stained with propidium iodide (PI, Sigma) and then analyzed by flow cytometry (FACStar, Beckton-Dickinson).

Purification of mRNA and dot blotting Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method described by Chomczynski and Sacchi.[23] Twenty micrograms of total RNA was placed in each dot of a 1% agarose slab, electrophoresed, and then blotted onto nitrocellulose (NC) filters (Schleicher and Schuell, Dassel, Germany), using a dot-blotting apparatus (MilliBlot-s, Millipore, Bedford, MA). The NC filter was then sequentially probed with ^{32}P-labeled probes specific for human c-*myc* 3′ exon and β-actin.

Effects of culture conditions on the differentiation Caco-2 cells were cultured for 24 h in the standard EMEM medium described above. The cells were washed with PBS twice, and transferred to various kinds of culture media as follows; EMEM containing different concentrations of FCS (0%, 0.1%, 1%, 5%, 10%, 20%), Cosmedium 001 (Cosmo Bio Co. Ltd., Tokyo), S-Clone SFO (Sanko Junyaku Co. Ltd., Tokyo), S-Clone SFB (Sanko Junyaku Co. Ltd.), and EMEM containing transferrin (10 μg/ml) and/or insulin (10 μg/ml) and/or sodium selenite (25 n*M*). Cosmedium 001, S-Clone SFO and S-Clone SFB are serum-free media. Both Cosmedium 001 and S-Clone SFO contain transferrin and insulin in complete serum-free media, and S-Clone SFB contains transferrin, insulin and bovine serum albumin. Medium was changed every two days, and the number of domes and the alkaline phosphatase activity in the cells were examined on the 18th day of culture.

RESULTS

Morphological and phenotypic changes in Caco-2 cells cultured with 10% FCS-containing media Caco-2 cells grew to form a cobblestone-like monolayer and reached confluence on the 5th day of culture in EMEM containing 10% FCS (standard medium). On the 4th or 5th day of culture, the shape of each cell changed from flat (broad base) type to columnar (narrow base) type. Dome (or hemi-cyst) formation appeared on the 6th day of culture and the size of domes increased gradually during the culture period. On the 18th day of culture, large domes appeared in the cell monolayer (Fig. 1). The cells showed a logarithmic growth curve even after they had reached confluence and the cell number reached a plateau on the 9th day of culture (Fig. 2A). Caco-2 cells spontaneously exhibited differentiation phenotypes as reported previously.[5] In this study, we investigated the number of domes in cell monolayers and the activity of brush border hydrolase, ALP, in the cell homogenate as differentiation markers. The number of domes increased gradually and reached a plateau on the 15th day of culture. ALP activity in the cell homogenate increased during the culture period as shown in Fig. 2B.

Cell cycle analysis of Caco-2 cells cultured with EMEM containing 10% FCS When undifferentiated Caco-2 cells were preconfluent and in logarithmic growth (on the 3rd day), the cells in G_0-G_1 phase, S phase and G_2-M phase amounted to 67.1%, 24.4% and 8.5%, respectively. When the cells were cultured with EMEM containing 10% FCS for 18 days, the proliferative activity was reduced. Increase in G_0-G_1 phase cells (91.3%) and marked decrease in S phase cells (4.0%) were observed (Fig. 3A, 3B).

c-*myc* mRNA levels in Caco-2 cells cultured with EMEM containing 10% FCS The expression of c-*myc* mRNA of preconfluent, undifferentiated (the 3rd day of culture) cells and that of differentiated (the 18th day of culture) cells were examined by RNA dot blotting. More than 5-fold decrease in c-*myc* mRNA level was seen in the differentiated Caco-2 cells, compared with the preconfluent, undifferentiated cells (Fig. 4A). These results suggest that the induction of differentiated phenotypes is associated with the reduction of c-*myc* mRNA expression.

Effect of FCS in culture media on the differentiation In order to examine the mechanisms of differentiation of Caco-2 cells, we first studied the effect of FCS in the culture media on the differentiation. Caco-2 cells cultured in serum-free EMEM grew in cobblestone-like monolayers and were almost confluent by the 7th day of

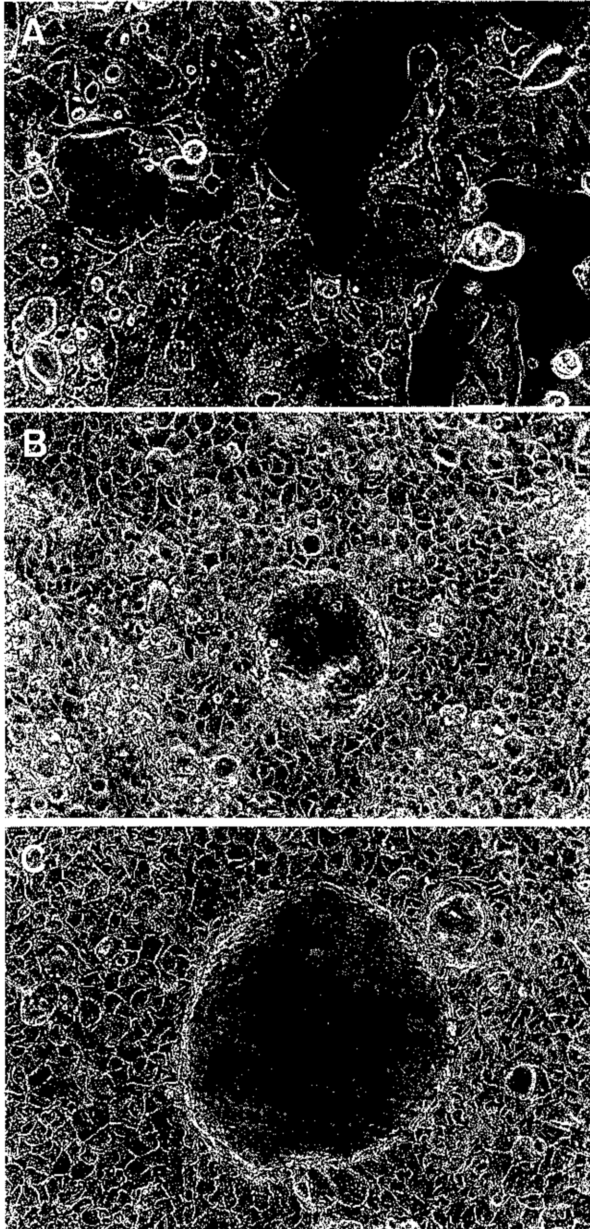

Fig. 1. Caco-2 cell monolayer observed by phase-contrast light microscopy (×200). (A) Cobblestone-like cells growing to form a monolayer on the 3rd day. (B) On the 6th day, domes (or hemi-cysts) appeared on the cell monolayer. (C) On the 18th day, the domes increased in size and fused with each other.

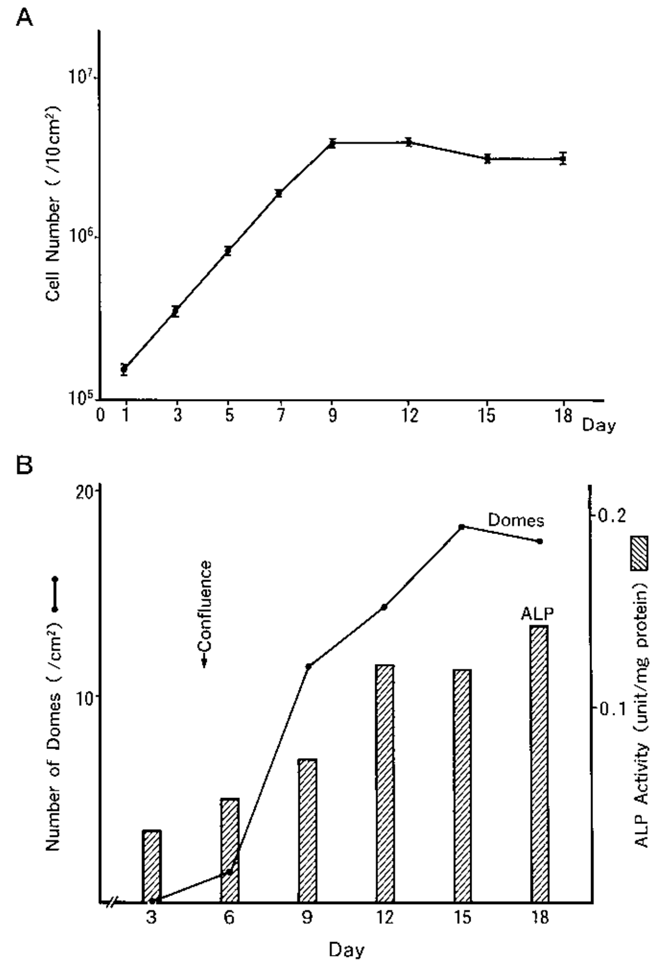

Fig. 2. (A) Cell growth curve of Caco-2 cells. The cell number increased logarithmically up to the 9th day of culture and reached a plateau. (B) The changes in number of domes and alkaline phosphatase activity in cell homogenates during Caco-2 cell differentiation. ALP activity gradually increased up to the 12th day and the number of domes increased up to the 15th day.

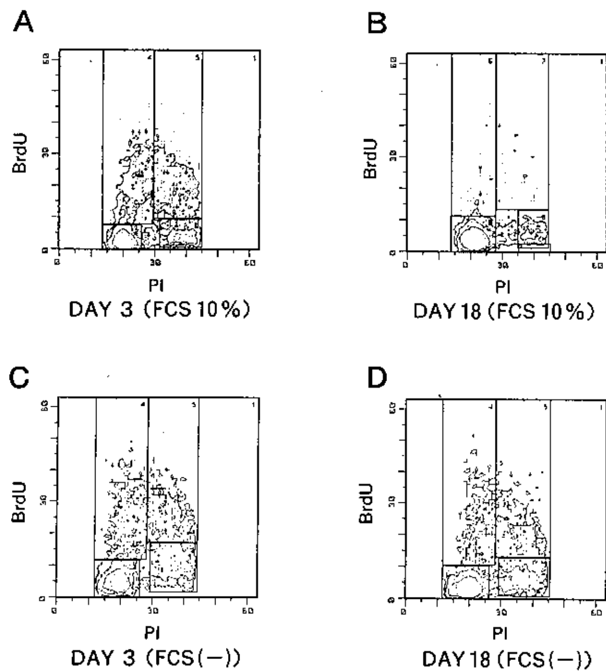

Fig. 3. Cell cycle analysis on the 3rd day (A, C) and 18th day (B, D) of culture with or without FCS by two-color flow cytometry using FITC-conjugated anti-BrdU monoclonal antibody and PI staining. Cells in S phase were identified as BrdU (+) PI (+) cells. Cells in G_0-G_1 and G_2-M were BrdU (−) PI (−) and BrdU (−) PI (+), respectively. (A), (B) with 10% FCS and (C), (D) without FCS. Increase in G_0-G_1 phase cells and decrease in S phase cells were observed on the 18th day of culture with 10% FCS (B). In contrast, these changes were not seen on the 18th day of culture without FCS (D).

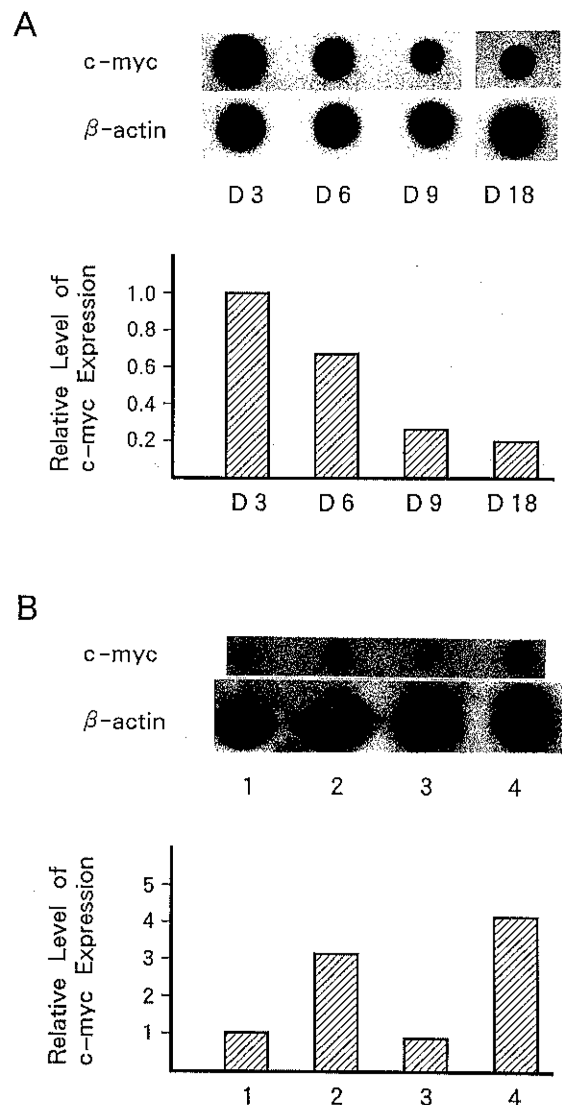

Fig. 4. (A) Expression of c-*myc* RNA in Caco-2 cells during Caco-2 cell differentiation. The level of c-*myc* expression decreased gradually up to the 9th day of culture. (B) Changes in c-*myc* RNA expression in Caco-2 cells cultured under various conditions. Lane 1; EMEM containing 10% FCS; lane 2, EMEM; lane 3, Cosmedium 001; lane 4, EMEM containing transferrin and insulin. A reduction of c-*myc* expression was observed in the culture with EMEM containing 10% FCS and Cosmedium 001.

culture. However, the morphological changes as seen in the cells cultured with EMEM containing 10% FCS, were not induced in FCS-free EMEM (Fig. 5). In the FCS-free condition, the cell number showed a logarithmic growth curve until the cells reached confluence, as reported previously.[24] The induction of dome formation or the increase of ALP activity was not observed while more than 20% of the cell population remained in S phase (Fig. 3C, 3D). To investigate the effect of FCS on the induction of the differentiation phenotypes in detail, Caco-2 cells were cultured in EMEM containing various concentrations of FCS. Dome formation in the cell monolayer was induced in the cells cultured with over 5% FCS-containing EMEM, and ALP activity in the cells cultured with over 1% FCS-containing media was significantly higher than that of the cells cultured with FCS free EMEM on the 18th day (Table I).

Phenotypic changes of Caco-2 cells in serum-free media In order to establish whether the events of spontaneous differentiation were induced by inherent factor(s) in FCS, we next used three kinds of serum-free media (Cosmedium 001, S-Clone SFO and S-Clone SFB). These serum-free media were prepared for the culture of various types of cells or purification of monoclonal antibodies. Although the details of the components are not

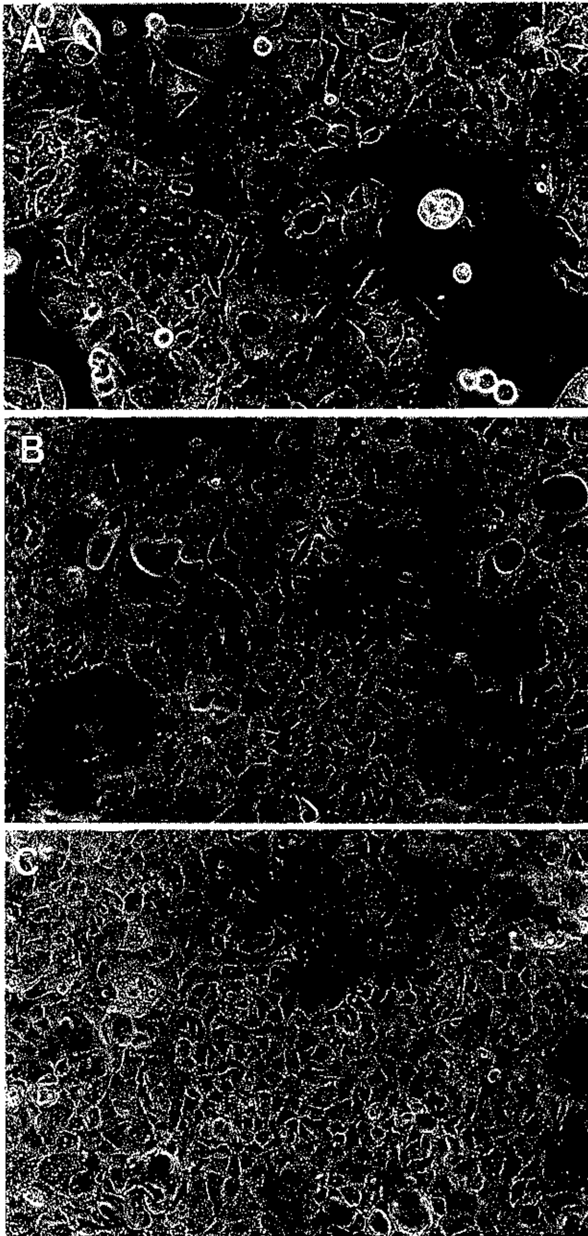

Fig. 5. Monolayer of Caco-2 cells cultured with FCS-free media. (A) on the 3rd day (B) on the 6th day (C) on the 18th day. The cells grew and increased the number in FCS-free media. However, dome formation was not observed during the culture period.

Table I. Effects of FCS on Induction of ALP Activity and Dome Formation in Caco-2 Cells

Addition to EMEM	ALP activity (unit/mg protein)	No. of domes (/cm²)
None	0.035±0.003	none
FCS 20%	0.104±0.006*	15.4±2.8*
10%	0.102±0.006*	17.3±2.2*
5%	0.093±0.007*	8.0±1.0*
1%	0.085±0.008*	none
0.1%	0.047±0.007	none

Values are expressed as the mean±SE of six experiments.
* $P<0.005$ versus none.

Table II. Effects of Serum-free Media no Induction of ALP Activity and Dome Formation in Caco-2 Cells

Medium	ALP activity (unit/mg protein)	No. of domes (/cm²)
EMEM	0.025±0.005	none
EMEM+10% FCS	0.103±0.004***	21.5±1.4***
Cosmedium 001	0.172±0.011***	25.7±3.2***
S-Clone SFO	0.060±0.005**	4.5±1.4*
S-Clone SFB	0.048±0.005*	3.0±0.6**

Values are expressed as the mean±SE of six experiments.
* $P<0.05$, ** $P<0.01$ and *** $P<0.005$ versus EMEM alone.

available, it is stated that they do not contain any protein or peptide except for transferrin, insulin and bovine serum albumin (only S-Clone SFB contains bovine serum albumin). All of these serum-free media significantly induced differentiation phenotypes in Caco-2 cells in comparison with FCS-free EMEM, but there were some differences in the induction of differentiation among them. Cosmedium 001 induced dome formation and ALP activity more effectively than EMEM containing 10% FCS. On the other hand, S-Clone SFO and S-Clone SFB did not induce differentiated phenotypes comparable to those obtained with EMEM containing 10% FCS (Table II). These results indicated that spontaneous differentiation of Caco-2 cells was not induced by inherent factor(s) in FCS.

Effects of transferrin, insulin and sodium selenite on the spontaneous differentiation Next, we examined the effects of transferrin, insulin and sodium selenite on the differentiation of Caco-2 cells, since they have been recognized as important components for proliferation in serum-free media.[25] Dome formation was not induced in the cells cultured with EMEM supplemented with transferrin or sodium selenite. A small number of domes (mean$=1.3/cm^2$) was seen in the case of cells cultured with EMEM supplemented with insulin, but it was not significantly different from that seen in the cells cultured with FCS-free EMEM. ALP activity in the cells cultured with EMEM supplemented with transferrin, insulin or sodium selenite was not increased. Caco-2 cells cultured with EMEM supplemented with the combination of

Table III. Effects of Transferrin (Tf), Insulin (Ins) and Selenous Acid (Se) on Induction of ALP Activity and Dome Formation in Caco-2 Cells

Addition to EMEM	ALP activity (unit/mg protein)	No. of domes (/cm^2)
None	0.035±0.005	none
FCS 10%	0.101±0.007*	20.3±1.5*
Tf 10 μg/ml	0.026±0.005	none
Ins 10 μg/ml	0.030±0.005	1.3±0.5
Se 25 n*M*	0.043±0.006	none
Tf+Ins	0.038±0.005	none
Tf+Ins+Se	0.045±0.001	none

Values are expressed as the mean±SE of six experiments.
* $P<0.005$ versus none.

transferrin and insulin or the combination of transferrin, insulin and selenium did not show any differentiation phenotypes (Table III).

Levels of c-*myc* mRNA in Caco-2 cells cultured with media which induced or did not induce enterocytic differentiation Caco-2 cells cultured under four kinds of culture conditions were harvested on the 18th day, and the levels of c-*myc* mRNA were estimated by RNA dot blotting. The cells cultured with Cosmedium 001, in which differentiation phenotypes were induced more effectively than in the cells cultured with EMEM containing 10% FCS, showed lower levels of c-*myc* expression than the cells cultured with EMEM containing 10% FCS. The cells cultured with FCS-free EMEM or EMEM containing transferrin and insulin, in which no differentiation phenotypes were induced, showed high levels of c-*myc* expression (Fig. 4B).

DISCUSSION

Since the first report by Pinto *et al.*,[5] the Caco-2 cell line has been used as a unique model for investigation of the structural and functional properties of differentiated enterocytes. This cell line acquires enterocytic characteristics during spontaneous differentiation.[26,27] Caco-2 cells grew into cobblestone-like monolayers and underwent enterocytic differentiation after they had reached confluence, as reported previously.[5] However, the mechanisms of cell differentiation during the culture period have not been clarified yet. In the present study, we found that Caco-2 cells showed a decrease in S-phase cell population and a decrease in c-*myc* mRNA expression after they had reached confluence and acquired differentiation markers. Several lines of evidence suggest that c-*myc* proto-oncogene is involved in the process of cell proliferation and differentiation in various cell systems. A decrease in c-*myc* mRNA level during chemically induced differentiation of the HL-60 leukemic cell line,[28] F9 teratocarcinoma cells[17,18] and mouse erythroleukemia cells[29,30] has been reported. In these cells, the differentiation is also associated with the decline of proliferative activity. Since these studies utilized chemical inducers for differentiation, direct effects of the inducers on cell proliferation and c-*myc* expression could not be excluded. In contrast, the present observation that the spontaneous differentiation was associated with the reduction of proliferation and c-*myc* mRNA levels, indicates more obviously that a switch from an unlimitedly proliferative state to a set of terminal cell divisions occurs while the cell expresses the differentiation phenotypes.

The present study also demonstrated that the growth and differentiation of Caco-2 cells could be modified by the alteration of culture conditions. Our previous study has shown that Caco-2 cells cultured in FCS-free EMEM have the ability to grow into a cobblestone-like monolayer and to reach confluence by the 7th day of culture.[24] Under this culture condition (FCS-free), however, dome formation or ALP expression is not induced. Another brush border hydrolase, dipeptidyl aminopeptidase IV, was not induced either (data not shown). A relatively high c-*myc* mRNA level was observed in the cells cultured with FCS-free medium. When FCS was added to culture media, the enterocytic differentiation associated with the reduction of c-*myc* mRNA expression was induced dose-dependently. This fact suggests that FCS contains some serum factor which influences the process of Caco-2 cell differentiation. In order to search for the serum factor, the effect of commercially available serum-free media (Cosmedium 001, S-Clone SFO and S-Clone SFB) on the Caco-2 cell differentiation was examined. These media could induce differentiation to varying degrees. Thus, differentiation factor(s) of Caco-2 cells might not be inherent in FCS itself. It is possible that human transforming growth factor-β (hTGF-β) is a differentiation factor in Caco-2 cells, since hTGF-β is contained in FCS and has been reported to have a potential to differentiate colon cancer cell line.[20] However, TGF-β does not induce the differentiation of Caco-2 cells.[24]

Since those serum-free media do not contain protein except for transferrin and insulin, we next added transferrin and insulin to serum-free EMEM in the presence or absence of sodium selenite, which has been reported as an important element in culture media to control cell proliferation[31] and to have an inhibitory effect on the proliferation of neoplastic tissue in animal experiments.[32] However, these components, even in combination, did not affect the cell phenotypes. From these results, it is possible that non-protein elements such as carbohydrates, amino acids or fatty acids may be responsible for Caco-2 cell differentiation.

Although the precise mechanisms of Caco-2 cell differentiation induced by FCS or serum-free media remain to be elucidated, several kinds of cells which exhibited various degrees of differentiation phenotypes were obtained in the present study. Interestingly, these cells expressed various degrees of proliferative activity and showed c-*myc* mRNA expression which dependent on the degree of differentiation. Indeed, highly differentiated cells cultured with EMEM containing 10% FCS or Cosmedium 001 showed relatively lower expression of c-*myc* mRNA when compared with that in partially differentiated cells cultured with EMEM containing 1% FCS or S-Clone. These results suggest that the spontaneous enterocytic differentiation of a colon cancer cell line was concomitant with the decrease in S phase cells and c-*myc* mRNA level. Consequently, c-*myc* mRNA level might be a new marker of the differentiation of Caco-2 cells for further study, since c-*myc* mRNA level has been used as a differentiation marker in other cell lines.[11, 12, 28] These results also support the clinical observation that an increase in c-*myc* expression occurs during multistep carcinogenesis from adenoma to cancer in the colon.[19]

In conclusion, Caco-2 cells are committed to differentiated and less proliferative phenotypes. A spontaneous switch from an unlimitedly proliferative state to the onset of a differentiation program occurs after the cells reach confluence and depends on the culture conditions. Therefore, this cell line should provide a unique experimental model for investigation of the inter-relationship between cell proliferation and differentiation in the absence of chemical inducers.

(Received December 15, 1992/Accepted March 10, 1993)

REFERENCES

1) Dexter, D. L. N,N-Dimethylformamide-induced morphological differentiation and reduction of tumorigenicity in cultured mouse rhabdomyosarcoma cells. *Cancer Res.*, **7**, 3136–3140 (1977).
2) Kim, Y. S., Tsao, D., Suddiqui, B., Whitehead, J. S., Arnstein, P., Bennet, J. and Hicks, J. Effects of sodium butyrate and dimethyl-sulfoxide on biochemical properties of human colon cancer cells. *Cancer*, **45**, 1185–1192 (1980).
3) Strickland, S. and Mahdavi, V. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell*, **15**, 393–403 (1978).
4) Fogh, J., Fogh, J. M. and Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.*, **59**, 221–226 (1977).
5) Pinto, M., Robine-Leon, S., Appay, M. D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P. and Haffen, K. Enterocyte-like differentiation and polarization of the colon cancer cell line Caco-2 in culture. *Biol. Cell*, **47**, 323–330 (1983).
6) Vogelstein, B., Fearon, E. R., Hamilton, S. E., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525–532 (1988).
7) Stanbridge, E. J. Identifying tumor suppressor genes in human colorectal cancer. *Science*, **247**, 12–13 (1990).
8) Westin, E. H., Wong-Staal, F., Gelmann, R., Favera, R. D., Papas, T. S., Lautenberger, J. A., Eva, A., Reddy, E. P., Tronic, S. R., Aaronson, S. A. and Gallo, R. C. Expression of cellular homologues of retroviral oncogenes in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA*, **79**, 2490–2494 (1982).
9) Bishop, J. M. Cellular oncogene and retroviruses. *Annu. Rev. Biochem.*, **52**, 301–354 (1983).
10) Little, C. D., Naus, M. N., Carney, D. N., Gadzar, D. F. and Minna, J. D. Amplification and expression of c-*myc* oncogene in human lung cancer cell line. *Nature*, **306**, 194–196 (1983).
11) Armelin, H. A., Armelin, M. C. S., Kelly, K., Stewart, T., Leder, P., Cochran, B. H. and Stiles, C. D. Functional role for c-*myc* in mitogenic response to platelet-derived growth factor. *Nature*, **310**, 655–660 (1984).
12) Kelly, K. and Siebenlist, U. The role of c-*myc* in the proliferation of normal and neoplastic cells. *J. Clin. Immunol.*, **5**, 65–77 (1985).
13) Alitaro, K., Schwab, M., Lin, C. C., Varmus, H. E. and Bishop, J. M. Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. *Proc. Natl. Acad. Sci. USA*, **80**, 1707–1711 (1983).
14) Slamon, D. J., deKernion, J. B., Verma, I. M. and Cline, J. J. Expression of cellular oncogenes in human malignancies. *Science*, **224**, 256–262 (1984).
15) Erisman, M. D., Rothberg, P. G., Diehl, R. E., Morse, C. C., Spandorfer, J. M. and Astrin, S. M. Deregulation of c-*myc* gene expression in human colon carcinoma is not accompanied by amplification or rearrangement of the gene. *Mol. Cell. Biol.*, **5**, 1969–1976 (1985).
16) Lachman, H. M., Hatton, K. S., Skoultchi, A. I. and Schildkraut, C. L. c-*myc* mRNA levels in the cell cycle change in mouse erythroleukemia cells following inducer treatment. *Proc. Natl. Acad. Sci. USA*, **82**, 5323–5327 (1985).
17) Dean, M., Levine, R. A. and Campisi, J. c-*myc* regulation during retinoic acid-induced differentiation of F9 cells is post-transcriptional and associated with growth arrest. *Mol. Cell. Biol.*, **6**, 518–524 (1986).

18) Griep, A. E. and DeLuca, H. F. Decreased c-*myc* expression is an early event in retinoic acid-induced differentiation of F9 teratocarcinoma cells. *Proc. Natl. Acad. Sci. USA*, **83**, 5539–5543 (1986).
19) Imaseki, H., Hayashi, H., Taira, M., Ito, Y., Tabata, Y., Onoda, S., Isono, K. and Tatibana, M. Expression of c-*myc* oncogene in colorectal polyps as a biological marker for monitoring malignant potential. *Cancer*, **64**, 704–709 (1989).
20) Mulder, K. M. and Brattain, M. G. Alterations in c-*myc* expression in relation to maturational status of human colon carcinoma cells. *Int. J. Cancer*, **42**, 64–70 (1988).
21) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
22) Dolbeare, F., Grantzer, H., Pallavicini, M. G. and Gray, J. W. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA*, **80**, 5573–5577 (1983).
23) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
24) Hara, A., Hibi, T., Yoshioka, M., Toda, K., Watanabe, N., Morita, M., Suzuki, T., Serizawa, H., Saito, H., Iwao, Y., Watanabe, T. and Tsuchiya, M. Effect of serum factors on the differentiation of a colon cancer cell line Caco-2. *Jpn. J. Gastroenterol.*, **89**, 588–595 (1992) (in Japanese).
25) Murakami, H. and Masui, H. Hormonal control of human colon carcinoma cell growth in serum-free medium. *Proc. Natl. Acad. Sci. USA*, **77**, 3464–3468 (1980).
26) Hauri, H., Sterchi, E., Bienz, D., Fransen, J. A. and Marxer, A. Expression and intercellular transport of microvillus-membrane hydrolases in human intestinal epithelial cells. *J. Cell. Biol.*, **101**, 838–851 (1985).
27) Rousset, M., Laburthe, M., Pinto, M., Chevalier, G., Rouyer-Fessard, C., Dussaulx, E., Trugnan, G., Boige, N., Brun, J. L. and Zweibaum, A. Enterocytic differentiation and glucose utilization in human colon tumor cell line Caco-2: modulation by Forskolin. *J. Cell Physiol.*, **123**, 377–385 (1985).
28) Watanabe, T., Sariban, E., Mitchell, T. and Kufe, D. Human c-*myc* and N-*ras* expression during induction of HL-60 cellular differentiation. *Biochem. Biophys. Res. Commun.*, **126**, 999–1005 (1985).
29) Coppola, J. A. and Cole, M. D. Constitutive c-*myc* oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. *Nature*, **320**, 760–763 (1986).
30) Dmitrovsky, E., Kuehl, W. M., Hollis, G. F., Kirsch, I. R., Bender, T. P. and Segal, S. Expression of a transfected human c-*myc* oncogene inhibits differentiation of a mouse erythroleukemia cell line. *Nature*, **322**, 748–750 (1986).
31) Nano, J. L., Czerucka, D., Menguy, F. and Pampal, P. Effect of selenium on the growth of three human colon cancer cell lines. *Biol. Trace Elem. Res.*, **20**, 31–43 (1989).
32) Rampal, P., Nano, J. L., Veyres, A., Rampal, E. and Francois, E. Prevention du cancer du colon chimio induit par un apport oral de selenium (Se) chez le rat. *Gastroenterol. Clin. Biol.*, **10**, 81A (1986).