The tumorigenic, invasive and metastatic potential of epithelial and round subpopulations of the SW480 human colon cancer cell line

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Abstract. It has been reported that the SW480 human colon cancer cell line consists of E-type and R-type cells. The long-term tumorigenic potential, invasive and metastatic properties of these subclones have not been characterized. E-type and R-type cells were subcloned using limiting dilution methods from parental SW480 cells. The cell growth rate was determined by MTT colorimetric assay, and colony forming efficiency was analyzed using Matrigel-coated plates. The activity of matrix metalloproteinase (MMP) and of urokinase plasminogen activator (uPA) was assessed by zymography. Invasive and locomotive ability was analyzed using transwell chambers. *In situ* apoptosis detection of these subclones was also performed. *In vivo* long-term tumorigenicity and nodal metastasis were evaluated using nude mice. E-type cells produced spontaneously regressive tumors in spite of invasion and lymph node metastasis. In contrast, R-type cells revealed progressively growing tumors without invasion or metastasis. E-type cells exhibited increased apoptosis and invasive and motile ability, as well as strong MMP-9 and -2 activity. Although phorbol 12-myristate 13-acetate treatment induced MMP-9 activity in E-type cells, it had no effect on R-type cells. These findings suggest that E- and R-type cells may have different biological properties in terms of colon cancer progression, regression, invasion and nodal metastasis, and might serve as a useful model for these studies.

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Introduction

SW480 human colon cancer cells (ATCC CCL 228) isolated from a Dukes' B colon cancer by Leibovitz *et al* (1) in 1976 are one of the best characterized colon cancer cell lines (2-10). Tomita *et al* (11) reported that SW480 cells consist of two distinct subpopulations, which have been designated as E-type (epithelial) and R-type (round). According to the study, R-type cells showed decreased doubling time, loss of contact inhibition, less adhesiveness to culture plates, higher anchorage-independent growth in soft agar, much more aneuploid karyotype, and much larger tumors in nude mice. Subsequently, the authors concluded that R-type cells represent a more malignant variant of the SW480 cells. These findings motivated us to investigate the long-term tumorigenic potential and invasive and metastatic properties of SW480 subclones.

In this study, we demonstrated that E-type cells produce spontaneously regressive primary tumors in nude mice and show frequent capsular invasion and metastasis to axillary lymph nodes. In contrast, R-type cells produce much larger primary tumors in mice without invasion or nodal metastasis, and very weak apoptotic staining of the primary tumor. In addition, these subclones were revealed to have different *in vitro* invasive potential, motile activity and MMP profiles. These properties of E- and R-type cells may be useful for the study of spontaneous tumor regression, invasion, and lymph node metastasis in human colon cancer.

Materials and methods

Cell culture and isolation of subpopulations. The SW480 human colon cancer cell line was obtained from American Type Culture Collection (ATCC CCL 228) and maintained as described elsewhere (12). For cell cloning, SW480 cells

were plated onto 96-well plates at a density of 1 cell/well. After the selection of a single cell in the well, the clones were expanded. The morphology of individual colonies was examined under a phase contrast microscope and two distinct types of colonies were identified and expanded. Finally, three independent colonies of E-type (E_1, E_2 and E_3) cells and three of R-type (R_2, R_3 and R_5) were obtained. Of these, the E_3 and R_2 subclones were used.

Growth studies on monolayer cultures and Matrigel. To analyze the growth rates of E_3 and R_2 cells, 10^4 cells were plated onto each well of 96-well culture plates. From day 2, cells from one set of plates were counted by MTT assay up to day 5 of culture. The time taken for the culture to increase 2-fold in the middle of the exponential phase of growth was determined as the population doubling time.

To evaluate colony forming efficiency on Matrigel (BD Bioscience, Bedford, MA), 200 μl Matrigel was transferred into each well of 24-well plates on ice. After the polymerization of Matrigel, cell suspensions (10^4 cells/well) were plated and incubated. After 8 days of culture, the number of colonies in 200 mm^2 areas of the plate larger than 0.25 mm in diameter was counted under a phase contrast microscope. The percent colony forming efficiency (CFE) was determined by the formula as CFE = Number of colonies/Number of cells seeded x 100 (12,13).

Nude mice tumorigenicity. As previously described (14), exponentially growing cells were harvested by brief trypsinization, washed three times with calcium and magnesium-free PBS, and resuspended at a final concentration of $5x10^7$ cells/ml in serum-free DMEM (SFM). Pathogen-free female BALB/cAnNCrj-nu athymic nude mice (4 weeks old, Charles River Laboratories, Kanazawa, Japan) were anesthetized with diethyl ether by inhalation, and 10^7 cells in 200 μl of SFM were inoculated subcutaneously into the right flank. Mice were surveyed regularly, tumors were measured with a caliper, and tumor volumes were determined using the following formula: Volume = 0.5 x $(Width)^2$ x Length.

In situ apoptosis staining. Tissue sections of E_3 and R_2 primary tumors were stained using the *In situ* Apoptosis Detection Kit (Oncor; Gaithersburg, MD) according to the manufacturer's instructions.

In vitro invasion and motility assay. Transwell cell culture chambers containing 6.5 mm diameter polycarbonate filters with 8-μm pores (Costar, Cambridge, MA) were used for the previously described assay (14). For the invasion assay, filters coated with basement membrane Matrigel (100 μg/filter) were used. Cells were seeded at a density of $2x10^5$ cells/200 μl into the upper chambers. After 72 h of incubation, cells on the top of the filter generated by non-invasive cells were removed with cotton swabs. The invasive cells beneath the filter were stained with hematoxylin and counted under a microscope (x100). For the motility assay, the same system was used without Matrigel.

Protease analysis by substrate-embedded gel. Proteins in conditioned medium (CM) were separated by electrophoresis in 10% polyacrylamide gel impregnated with 1 mg/ml of gelatin (Fisher Chemical Co., Fair Lawn, NJ) or 1 mg/ml of casein (Sigma Chemical Co., St. Louis, MO) and 13 μg/ml of plasminogen (Sigma Chemical Co.) under non-reducing conditions (14). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min, proteolysed with reaction buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, and 0.02% NaN_3, pH 8.0) for 72 h at 37˚C, and stained with Coomassie Blue G-250. To further characterize the proteases, 5 mM 1,10-phenanthroline (Sigma Chemical Co.) as a metalloproteinase (MMP) inhibitor or 5 μg/ml aprotinin (Sigma Chemical Co.) as a serine protease inhibitor was added to the incubation buffer. For the characterization of urokinase plasminogen activator (uPA), 1 mM of amiloride was added to the incubation buffer.

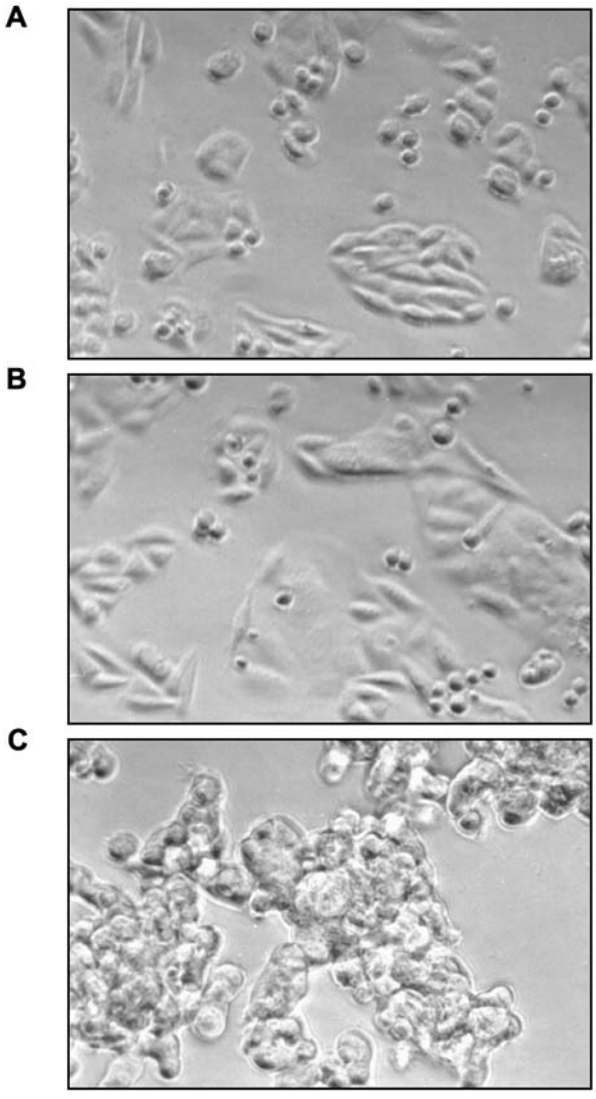

Figure 1. Morphologies of the SW480 cell line and its subclone E_3 and R_2 cells established with limiting dilution. (A) Parental SW480 cells (x200). (B) E_3 cells (x200) forming flat epithelial-like cuboidal morphology. (C) R_2 cells (x200) with a rounded shape growing in clusters of piled-up cells.

Statistical analysis. Significance of difference between values was assessed using the Kruskal-Wallis test, and all values were expressed as the mean ± SD. Statistical significance was assigned as $P < 0.05$.

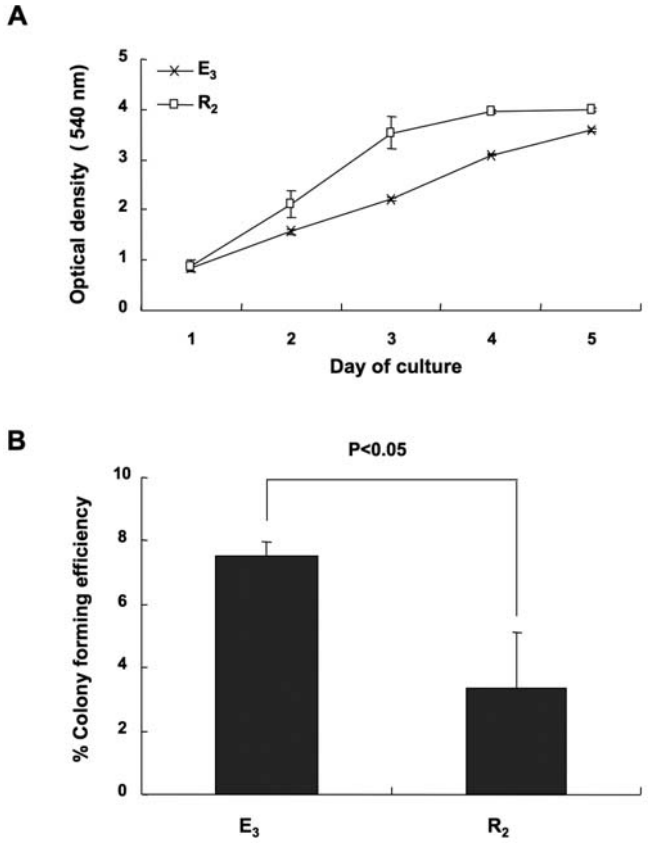

Figure 2. Growth studies on monolayer cultures and Matrigel. (A) Proliferation of E_3 and R_2 cells. Cells were plated at a density of 10^4 cells/well on 96-well plates and cultured for up to 5 days. After the indicated time of culture, cell numbers were determined by MTT colorimetric assay. Data are the mean ± SD of one representative of three repeat experiments. (B) Colony forming efficiency (CFE) on Matrigel. After 8 days of culture, the number of colonies in 200 mm^2 areas >0.25 mm in diameter was counted in five randomly selected areas per well, and the total number of colonies in a well was determined. All assays were performed in triplicate, and the CFE (%) was calculated. Data represent the mean ± SD of the CFE.

Results and Discussion

Using a limiting dilution of parental SW480 cells (Fig. 1A), we obtained three independent colonies of E-type subpopulations and three of R-type. Of these, we used E_3 and R_2 cells as representative subclones of E- and R-type cells because of their similarity with the E- and R-type cells of Tomita *et al*. E_3 cells exhibited epithelial colonies composed of cells with a cuboidal shape (Fig. 1B). In contrast, R_2 cells had a rounded and refractile morphology and were piled up on each other and attached loosely to the plates (Fig. 1C). In the cytogenetic analysis, more complex structural abnormalities were observed in E_3 cells than in R_2 cells. However, tetraploidy cells were predominant in R_2 tumors (data not shown). These chromosomal characteristics of our subclones were identical to the E- and R-type cells originally described by Tomita *et al* (11).

It was reported that the doubling times of E- and R-type cells were 31.6 and 15.6 h, respectively, and that the CFE was increased almost 10-fold in R-type cells compared to E-type cells. As shown in Fig. 2A, R_2 cells grew faster than E_3 cells. The doubling times of E_3 and R_2 cells, calculated from their respective growth curves, were 25.7 and 22.6 h, respectively. In addition, the % CFE of E_3 cells was 2.2-fold higher than that of R_2 cells (Fig. 2B; 7.5±0.4 versus 3.4±1.7%; $P<0.05$). It was therefore suggested that E-type cells have a greater ability to colonize than R-type cells. This CFE value differs from that of a previous report (11). We performed the CFE on matrix protein Matrigel, which seems to be more physiological compared to the 0.3% soft agar used in the previous study. This may have caused the disparate values of CFE.

In order to evaluate their tumorigenic potential, parental SW480, E_3 and R_2 cells were injected subcutaneously in nude mice, and tumor volumes were measured. As shown in Table I, R_2 tumors grew very rapidly and continuously up to the time of sacrifice on day 100. At day 20, the mean tumor volume produced by the R_2 cells was 7.0- and 10.6-fold larger than the tumor volumes produced by the parental SW480 and E_3 cells, respectively. These results coincide exactly with those of the original study of Tomita *et al* (11), with the exception of experimental time. All experimental animals in that study were sacrificed on day 25, precluding the long-term evaluation of tumor volume. In our experiments, the tumor volumes of parental SW480 cells increased very slowly with time up to day 100. Notably, E_3 cells produced very slow-growing tumors, similar to parental SW480 cells up to day 20, and then the tumor volumes decreased to a nearly dormant state from

Table I. Tumorigenicity in nude mice.

Days of inoculation	Tumor volume (mm^3)		
	SW480-parental (n=5)	E_3 (n=10)	R_2 (n=10)
10	44.00	35.83±19.21	94.33±14.06
20	63.75	42.17±14.38	445.50±57.28
30	52.85	19.50±8.84	896.00±227.12
40	53.50	2.67±1.89	1,950.83±794.48
60	96.00	5.83±5.66	4,101.67±944.83
80	126.00	5.83±5.66	9,310.00±2,438.72
100	189.55	8.84±9.30	17,797.33±2,258.97

Data represent the mean ± SD. For experiment details, see Materials and methods.

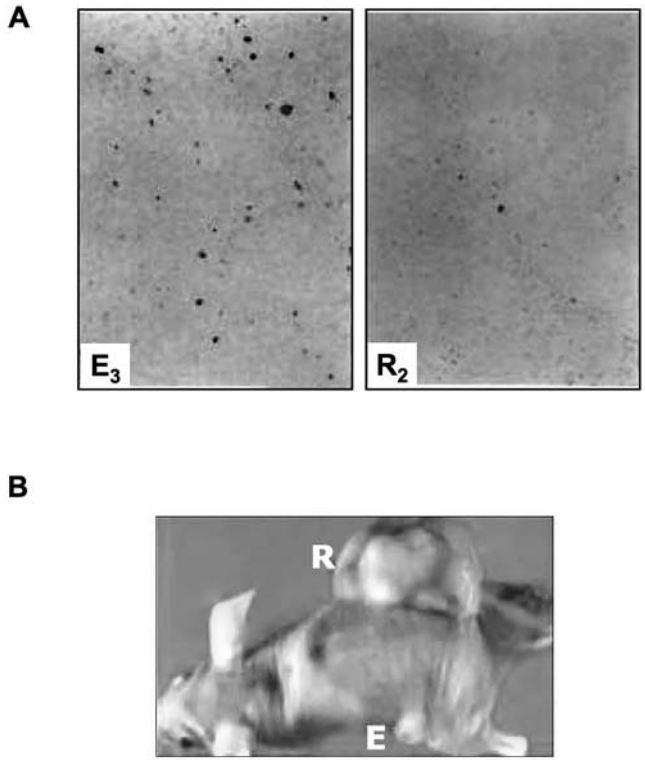

Figure 3. Apoptotic staining of primary tumors produced from E_3 and R_2 cells (x100). (A) R_2 tumors showed very weak and limited staining of apoptotic cells, whereas E_3 tumors showed 4-fold increased staining compared to the R_2 tumors. (B) Tumorigenicity of E_3 and R_2 cells.

day 40 (Fig. 3B). The different tumor growth rates as well as the spontaneous regressive character of these subclones were also observed in severe combined immune deficiency (SCID) mice (data not shown). To determine whether apoptosis plays a role in the regression of E_3 tumors, *in situ* apoptosis detection was performed. The number of apoptotic cells of E_3 tumors was 4.0-fold higher compared to that of R_2 tumors (Fig. 3A). These results suggest that the regression of E_3 tumors may be caused, at least in part, by increased apoptosis in E_3 tumors.

Histologic examination revealed that pathologic complete tumor regression (pCR) of E_3 tumors developed in 50% (5/10) of nude mice. Notably, 80% (8/10) of E_3 tumors, regardless of pCR, revealed ipsilateral axillary lymph node metastasis (Fig. 4B), and 50% (5/10) of the E_3 tumors that did not show pCR revealed peritumoral capsular invasion (Fig. 4A) in spite of primary tumor regression. R_2 tumors did not produce invasive lesions around the primary tumors (Fig. 4C).

The invasive and locomotive ability of E_3 and R_2 cells has not as yet been studied. The *in vitro* invasive ability of E_3 cells was 26.8-fold higher than that of R_2 cells (26.8±8.1 versus 1.0±0.8 cells/HPF; P=0.0013; Fig. 5). In addition, the *in vitro* motile activity of E_3 cells was 19.3-fold higher than that of R_2 cells (90.2±25.4 versus 4.7±1.6 cells/HPF; P=0.0007; Fig. 5). These findings suggest that E-type cells are much more invasive and motile compared to R-type cells.

The key enzymes that have been shown to be closely associated with invasive and metastatic potential are MMPs and uPA (13-15). However, these protease activities have not

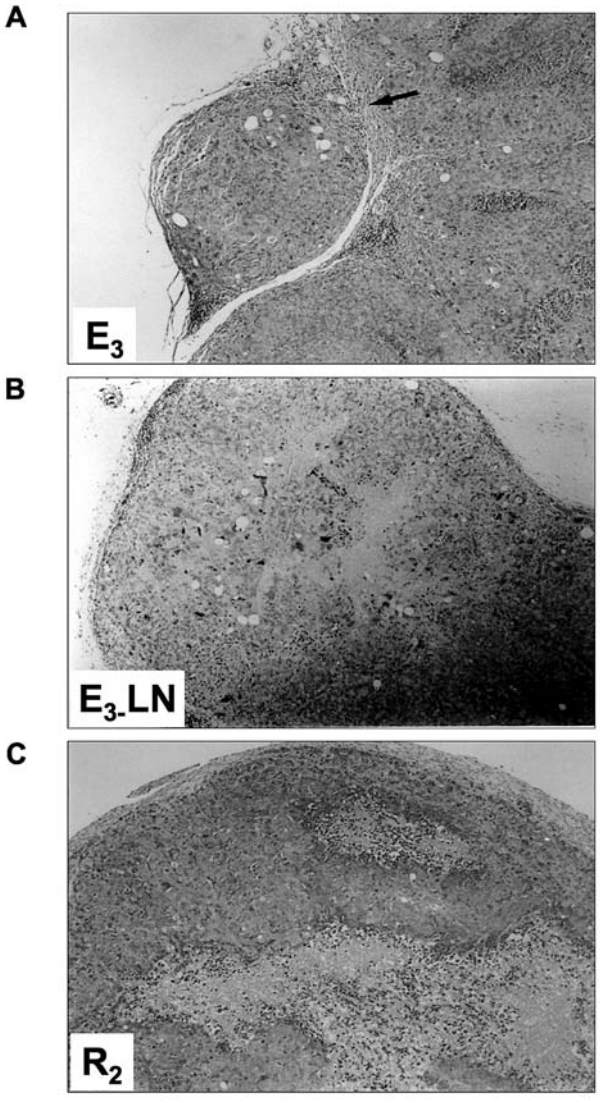

Figure 4. Photomicrographs showing a primary tumor and nodal metastasis of an E_3 and R_2 primary tumor in nude mice (x40). (A) A primary tumor of E_3 cells. The main mass is composed of solid sheets of tumor cells with central necrosis and invades through the capsule, forming a pericapsular satellite tumor nest. (B) Axillary lymph node metastasis observed in nude mice bearing an E_3 tumor. (C) The R_2 tumor, which does not produce an invasive lesion around the primary tumor.

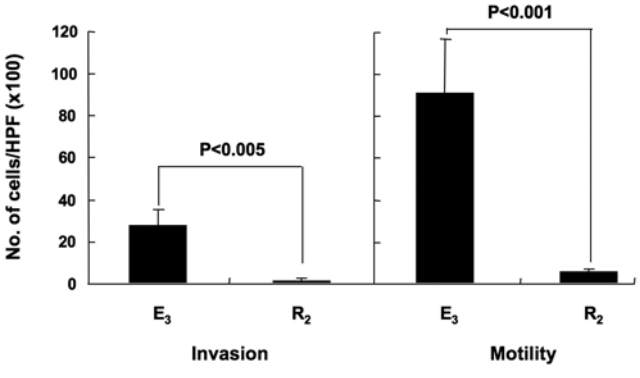

Figure 5. Invasive and motile activities of E_3 and R_2 cells *in vitro*. For the invasion assay, filters of the transwell culture chamber were gelatinized by pre-coating with Matrigel. For the motility assay, the same system without Matrigel was used as described in Materials and methods. After counting invasive or motile cells, percentages were normalized to 100%. Values represent the mean ± SD of three independent experiments.

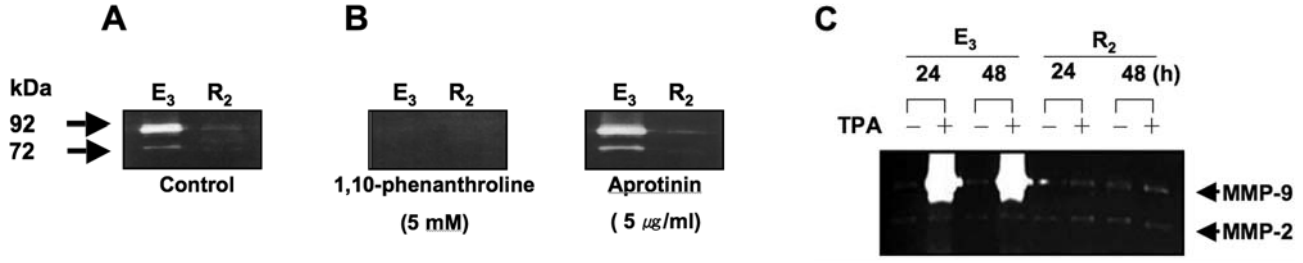

Figure 6. Zymography of MMP-9 and -2 activity in E_2 and R_3 cells. (A) Protease activity in conditioned medium (CM) normalized for 10^7 cells was measured using gelatin zymography as described in Materials and methods. (B) For the characterization of proteases, the MMP inhibitor 1,10- phenanthroline and the serine protease inhibitor aprotinin were used. The CM of E_3 cells showed strong MMP-9 and -2 activity compared to R_2 cells. (C) E_3 and R_2 cells were treated with or without TPA (10^{-7} M) for the indicated times.

been studied in E- and R-type cells. To investigate the differences in MMP production of these subclones, the MMP activity of the conditioned medium (CM) was measured by substrate-embedded SDS-PAGE. The CM of E_3 cells showed strong 92-kDa and relatively weak 72-kDa gelatinolytic activity (Fig. 6A). These gelatinolytic activities were entirely inhibited by the MMP inhibitor 1,10-phenanthroline, but not inhibited by the serine protease inhibitor aprotinin (Fig. 6B). In the present experiment, E_3 and R_2 cells were treated with 10^{-7} M phorbol 12-myristate 13-acetate (TPA) for 24 and 48 h, and MMP-9 activity was measured. Although TPA induced MMP-9 activity in E-type cells, it had no effect on R-type cells (Fig. 6C). It can therefore be concluded that E_3 cells secrete large amounts of MMP-9 and -2 compared to R_2 cells, and that E- and R-type cells may have different TPA signaling pathways. We also tested the activity of the plasminogen activator of these cells. The CM of both E_3 and R_2 cells showed identical intensity of 55-kDa caseinolytic bands, which was completely inhibited by uPA inhibitor (1 mM amiloride; data not shown). Consequently, there was no difference in the uPA of these subclones.

Tumor progression is an evolutionary process determined by the generation of heterogeneity and the selection of the variants most suited to survival, growth and invasion (16). It has been demonstrated that genetic and phenotypic instability in cell lines results in heterogeneous cell populations (17,18). This heterogeneity is the basis of malignant potential and contributes to the development of variant cells with different abilities (19). Recently, the presence of high rates of structural chromosomal instability was observed in the SW480 cell line (20). Higher instability rates in subclones compared to parental cells were also demonstrated (21,22). These subclones were obtained from the parental SW480 cells by the limiting dilution technique, regardless of any morphological selection criteria (20-22). Accordingly, our findings are unique in that they indicate that spontaneous regression occurs in E_3 tumors *in vivo* at a high frequency, despite invasion and nodal metastasis. On the other hand, R_2 tumors were revealed to have progressive growing properties without invasion or metastasis. We hope that future studies using these subclones may explain the process of clonal selection for progression, regression, invasion and nodal metastasis in colon cancer, and are currently investigating the differential regulation of MMP-9 and -2 by TPA and TNF-α in E_2 and R_3 cells.

To summarize, the present study demonstrated that E_3 and R_2 cells have different biological properties in terms of their tumorigenicity, invasion and nodal metastasis. These subclones may be useful in the study of colon cancer progression.

Acknowledgements

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