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Conversion of Human Colonic Adenoma Cells to Adenocarcinoma Cells Through Inflammation in Nude Mice

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SUMMARY: The roles of inflammation in the malignant progression of tumors during multistep carcinogenesis have been much discussed but remain to be elucidated. To determine the direct contribution of inflammation to colon carcinogenesis, we established a new model of progression of human colonic adenoma cells using a nude mouse; the progression is accelerated by coimplantation of a plastic plate. The FPCK-1–1 cell line, derived from a colonic polyp in a patient with familial adenomatous polyposis, is nontumorigenic when injected subcutaneously into nude mice in a cell suspension of up to 5×10^6 cells per mouse. However implantation of 1×10^5 FPCK-1–1 cells attached to a plastic plate induced first acute and then chronic inflammation, and formed progressively growing tumors that were histologically determined as moderately differentiated adenocarcinoma in 65% of mice. Moreover cell lines established from the growing tumors were found to be tumorigenic when injected into mice even without a plastic plate. The tumor arising from the adenoma cells implanted attached to a plastic plate was surrounded by highly proliferating fibrous stroma. This fibrous tissue was considered essential for malignant progression, rather than for attachment to the plastic plate substrate, because the tumors were formed after injection of FPCK-1–1 cells into the fibrous tissue from which the plastic plate had been removed before the cell injection. The conditioned medium (CM) obtained from the fibroblasts derived from a plastic plate-associated stromal tissue was found to contain factors that stimulated growth of FPCK-1–1 cells, but not of the derivative progressor cell lines. The factor was stable to heating and neuraminidase treatment, but labile to trypsin treatment. The main growth-potentiating activity was contained in the fraction larger than 100 kDa. In contrast, the activity to promote FPCK-1–1 cell growth was not present in the CM of subcutaneous fibroblasts from untreated nude mice or the fibroblast cell lines C3H10T 1/2 and NIH3T3. These results demonstrated that inflammation-associated stroma promoted the conversion of colonic adenoma cells to adenocarcinoma cells. (*Lab Invest 2000, 80:1617–1628*).

Carcinogenesis is a complex multistep process that involves many genetic and epigenetic events. Colorectal carcinogenesis is an excellent example in which at least seven genetic alterations have been identified, including activation of oncogenes and loss of tumor suppressor genes (Kinzler and Vogelstein, 1996). Stepwise progression in association with genetic abnormality in the so-called adenoma-carcinoma sequence is frequently observed in patients with familial adenomatous polyposis (FAP), which is characterized by a germline mutation of the APC gene and development of hundreds to thousands of colorectal polyps (Sugarbaker et al, 1985; Townsend and Beauchamp, 1995). Apart from or in conjunction with the genetic insult, epigenetic mechanisms relating to an inflammatory process seem to be important for carcinogenesis, as evidenced by the regression of adenomas and prevention of carcinoma formation in FAP patients by treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) (DuBois et al, 1996; Giardiello et al, 1993; Nugent et al, 1993; Takayama et al, 1998). A close correlation between chronic inflammation and colorectal carcinogenesis has been reported in human (Choi and Zelig, 1994; Lyda et al, 1998; Podolsky, 1991) as well as animal models (Berg et al, 1996; DuBois et al, 1996).

Despite these lines of evidence, the exact mechanism of how inflammation promotes tumor progression remains obscure. To elucidate the interactions, we developed a nude mouse model in which inflammation-promoted malignant conversion of human adenoma cells could be observed consistently. An epithelial cell line designated as FPCK-1 was established by Kawaguchi and colleagues from a colonic tubular adenoma formed in a male FAP patient (Kawaguchi et al, 1991). One of the sublines of

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FPCK-1, FPCK-1–1, does not form tumors in nude mice when up to 5×10^6 cells are injected.

We have previously found that foreign body-induced inflammation not only promotes the local growth of rodent tumors but also converts them into more aggressive tumors; that is, they acquire enhanced tumorigenicity and metastatic ability (Hamada et al, 1992; Okada et al, 1992, 1993). We have reported that inflammation-derived active oxygen species and cytokine/growth factors played major roles in the tumor progression (Hamada et al, 1992; Okada et al, 1992, 1994, 1999).

We investigated whether the FPCK-1–1 cells could be converted to malignant cells by contact with inflammatory cells. The inflammation was evoked by implantation of a foreign body such as gelatin sponge or plastic plate. Those foreign bodies differ in absorbability in vivo; therefore duration of the foreign-body-induced inflammation varied. We here demonstrate for the first time that the late phase of inflammation and its associated stromal reaction facilitated malignant conversion of human colonic adenoma cells, and suggest that a soluble factor or factors secreted by inflammation-associated fibroblasts might be involved in this mechanism.

Results

Conversion of Human Colonic Adenoma Cells to Adenocarcinoma Cells by Coimplantation with Plastic Plate

Table 1 shows the tumorigenicity of the human colonic adenoma cell line implanted with or without foreign bodies, either a plastic plate or gelatin sponge. FPCK-1–1 cells were nontumorigenic in KSN nude mice when up to 5×10^6 cells were injected subcutaneously (sc) in saline suspension. However, the cells that had been implanted attached to a piece of plastic plate grew progressively in 7 of 10 mice in Experiment 1 (70%) and 6 of 10 mice in Experiment 2 (60%), overall 13 of 20 (65%). On the other hand when 5×10^6 FPCK-1–1 cells were injected into preinserted gelatin sponge at the subcutaneous site, they formed tumor in only 1 of 12 mice (8%). However no tumor was observed in any of the nude mice implanted with either plastic plate or gelatin sponge alone up to 20 months after implantation.

Figure 1 shows growth curves of the tumors in 7 individual mice implanted with the adenoma cells attached to plastic plates. The mean latency period (ie, estimated time lapse after implantation till the mean tumor diameter reaching over 3 mm) was 107 ± 7 days. Around 200 days after implantation, we established 5 and 1 cell lines from the tumors arising in mice implanted with FPCK-1–1 cells with the plastic plate and with gelatin sponge, respectively.

Histopathologic Findings of the Arising Tumors after Coimplantation with Plastic Plate

Abundant neovascularization was macroscopically observed at the implantation site in the mice im-

Table 1. Tumorigenicity of FPCK-1–1 Cells Implanted in Suspension, Attached to a Plastic Plate, or into a Preinserted Gelatin Sponge in KSN Nude Mice

Cells	Tumorigenicity (no. of mice with tumor/no. of mice injected)[a]											
	5×10^6 cells in saline suspension			1×10^5 cells attached to plastic plate				5×10^6 cells into preinserted gelatin sponge				
	Exp. 1	Exp. 2	Total	Exp. 1	Exp. 2	Total	Mean latency period (days)[b]	Plate alone	Exp. 1	Exp. 2	Total	Mean latency period (days)[b]	Sponge alone
FPCK-1–1	0/5	0/7	0/12	7/10	6/10	13/20	107 ± 7	—	1/4	0/8	1/12	149	—
None	—	—	—	—	—	—	—	0/8[c]	—	—	—	—	0/4[c]

[a] Adenoma cells were implanted in PBS suspension or attached to plastic plates sc in 6- to 8-week-old KSN nude mice; and 5×10^6 cells were implanted sc into preinserted gelatin sponge. The mice were observed for 12 months.
[b] Latency period = days before mean tumor diameter reached >3 mm.
[c] After plastic plate or gelatin sponge was implanted sc without adenoma cells, none of the mice ($n = 8$ and $n = 4$, respectively) developed tumors during observation. The nontumor-bearing mice were examined for an additional 8 months, during which no other tumors were observed.

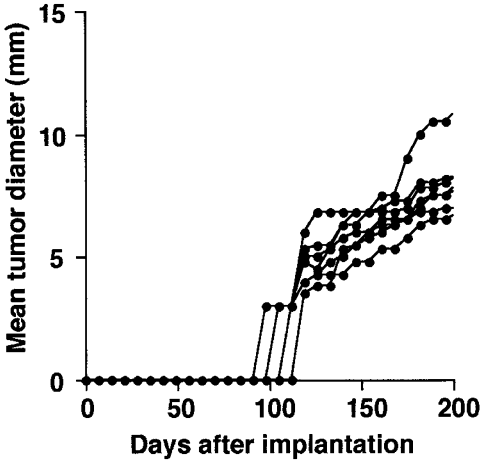

Figure 1.
In vivo growth curves of human colonic adenoma cell line, FPCK-1–1 (1×10^5) cells after implantation attached to a plastic plate in KSN nude mice ($n = 7$).

planted with FPCK-1–1 cells attached to the plastic plate around 120 days after the implantation (Fig. 2A). However, neovascularization was not observed in mice with plastic plates alone (Fig. 2B). Histologic examination revealed that the arising tumors were moderately differentiated adenocarcinoma, and the tumor mass was surrounded by fibrous stroma (Fig. 2, C and D). Azan staining also showed that the surrounding stroma consisted of collagen fiber (Fig. 2, D and E).

Morphologic changes of newly established tumor cells were also observed in culture. FPCK-1–1 adenoma cells consistently showed epithelial growth patterns similar to that of the parental FPCK-1 (Kawaguchi et al, 1991). They contacted each other and formed island-like patterns in culture (Fig. 3, A to C). On the other hand, adenocarcinoma cell lines converted from FPCK-1–1 adenoma cells spread diffusely and did not form island-like patterns. Typical features of an adenocarcinoma line are shown in Fig. 3, D to F.

In Vivo Progressive Growth of the Cultured Cell Lines Obtained from the Arising Tumors

Table 2 shows the tumorigenicity of $FPCKpP_1$ and $FPCKsP_1$ tumor lines compared with their original FPCK-1–1 cells. All of the adenoma-derived tumor lines acquired progressive-growth properties in nude mice without further requiring foreign body implantation, but exhibited no detectable changes in their in vitro growth properties such as doubling time, plating efficiency, and colony formation in soft agar (Table 2). We observed that the adenocarcinoma cell lines produced significantly larger amounts of potent angiogenic factor, vascular endothelial growth factor (VEGF) compared with FPCK-1–1 cells (Table 2). We also observed that the tumorigenicity of adenoma cells and derivative adenocarcinoma lines remained stable for more than 2 years while they were maintained under the regular culture conditions (data not shown).

Confirmation of Human Origin of the Adenocarcinoma Cell Lines

We wished to confirm that the adenocarcinoma lines were derived from FPCK-1–1 adenoma cells (human cells) and not from mouse cells. An RT-PCR assay was performed to distinguish the origins of the arising tumors by using primer sets specific to human- and mouse-thymosin $\beta 4$, one of the ubiquitous polypeptides regulating actin polymerization (Gomez-Marquez et al, 1989). cDNA was reverse-transcribed from mRNA obtained from FPCK-1–1 adenoma, adenoma-derived adenocarcinoma lines, human colorectal tumor cell lines, and mouse cell lines. PCR amplification of those cDNA by human primers resulted in PCR products, which were observed at 217 bp in adenoma cells, adenoma-derived adenocarcinoma lines, and also in human tumors (Fig. 4 upper, lanes 2–6 and lanes 7–8, respectively). However PCR products were not observed in either the plastic plate-reactive mouse fibroblast or the fibrosarcoma cell line (Fig. 4 upper, lanes 9 and 10, respectively). On the other hand, when mouse primers were used, no bands were seen in the lanes loaded with PCR-amplified samples from the human-originated cells; however bands were observed at 295 bp in the lanes loaded with samples from mouse-originated cells (Fig. 4 lower, lanes 9 and 10).

Effects of Plastic Plate-Reactive Fibrous Stroma on the Growth of FPCK-1-1 Cells

To determine whether progression of FPCK-1–1 cells was due to direct attachment to the plastic plate or interaction with stroma cells reactive to the plate, we injected FPCK-1–1 cells directly at the site of stromal tissues where the plastic plate had been implanted and removed 120 days after implantation. Histologic examination revealed that stromal tissues were highly proliferating fibrous stroma (Fig. 2F). The growth of FPCK-1–1 cells was observed in all six mice, whereas no tumor development was observed in nontreated or sham-operated mice (Table 3).

Figure 5 shows growth curves of the tumors in six mice implanted with the adenoma cells into stromal tissue. The mean latency period was 30 ± 6 days, which was shorter than that in adenoma cells after implantation attached to plastic plate (Fig. 1).

Factors Derived from Plastic Plate-Reactive Fibroblasts Stimulate Adenoma Cells but not the Derivative Adenocarcinoma Cell Growth

To further analyze the influence of plastic plate-reactive stroma on adenoma cell growth, we established fibroblast cell lines from the same stromal tissues as described above. We then harvested the supernatant as conditioned medium (CM) from the fibroblasts and assessed its effect on the growth of adenoma cells. Compared with control medium, the CM promoted the growth of FPCK-1–1 adenoma cells

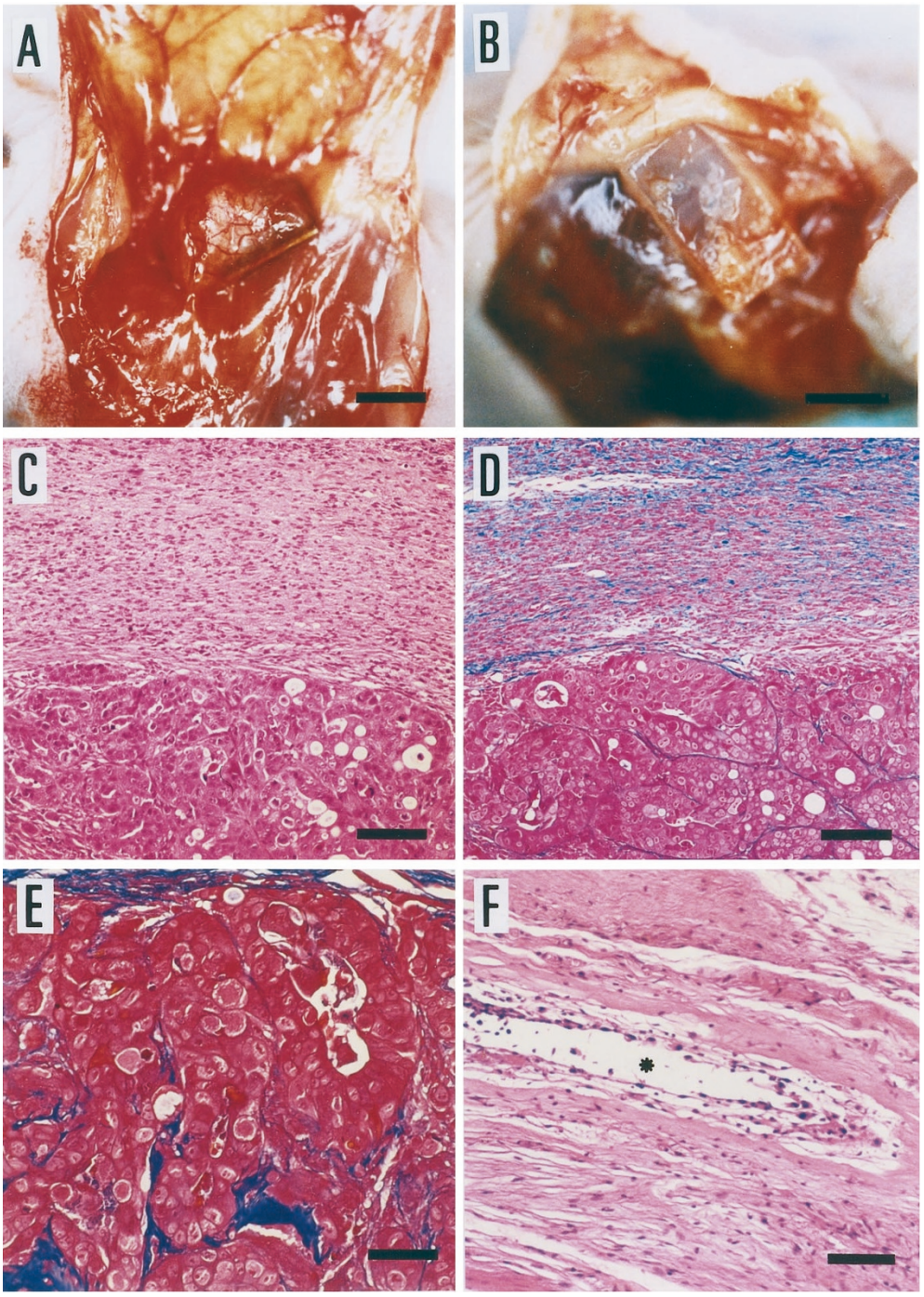

Figure 2.

Pathological findings of the arising tumors after coimplantation with a plastic plate. Macroscopic features of FPCK-1–1 (1×10^5 cells) implanted with a plastic plate attached (*A*) and the plastic plate alone (*B*) on day 121 (*bar*, 5 mm). Histologic sections were obtained from the arising tumor after sc implantation of FPCK-1–1 cells attached to the plastic plate. Hematoxylin and eosin (HE) staining (*C*) and Azan staining (*D*) (*bar*, 100 μm). *E*, High magnification of the same tumor with Azan staining (*bar*, 50 μm). *F*, HE staining of the stromal tissues where the plastic plate had been implanted and removed 120 days after implantation. * indicates the space caused by plastic plate implantation (*bar,* 100 μm).

by more than 200% (Fig. 6A). The active entity detected in the CM was stable against 95° C heating for 10 minutes, 56° C heating for 2 hours, or neuraminidase treatment for 24 hours, but labile to treatment with trypsin for 24 hours (Fig. 6A). The CM was fractionated by a YM 100 membrane for a molecular mass exclusion of larger than 100 kDa (Fig. 6B). The growth-potentiating activity was contained in the fraction of molecules larger than 100 kDa.

We examined the effects on the adenoma cell growth of medium conditioned with mouse fibroblasts of different origins. Growth-promoting activity of the fractionated CM of larger than 100 kDa was secreted by plastic plate-reactive fibroblasts but not by normal

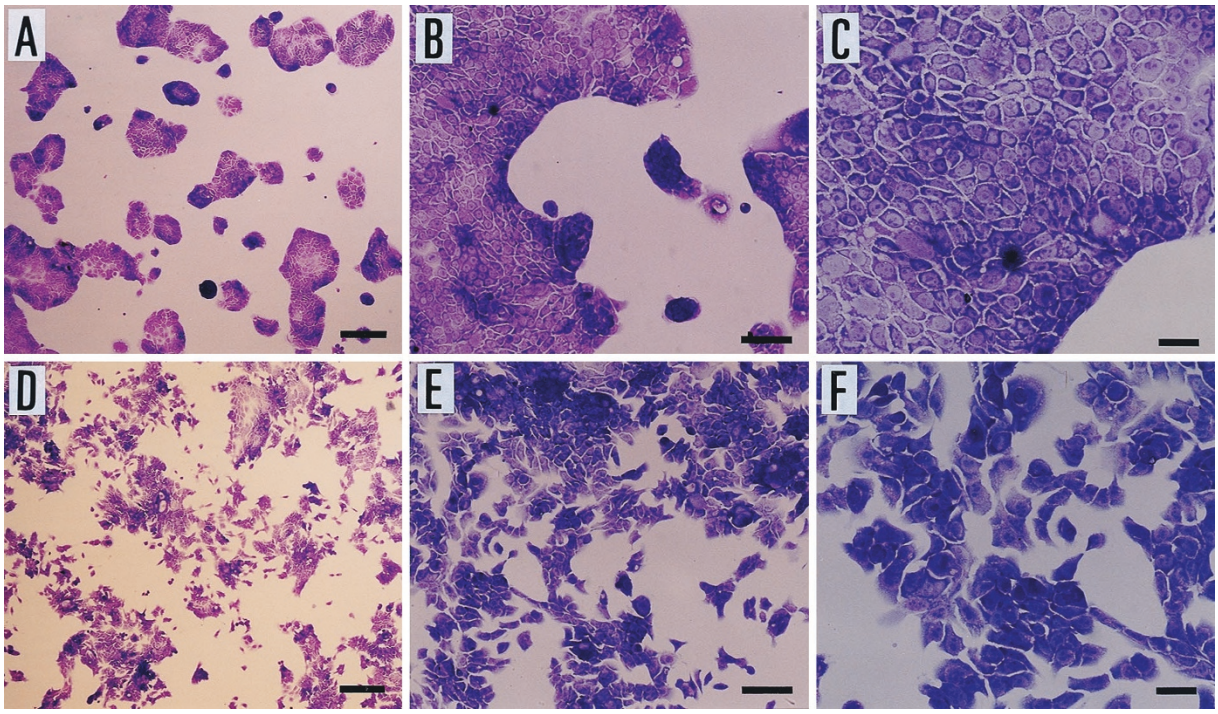

Figure 3.

Morphologic changes associated with the progression of adenoma cells grown in culture. FPCK-1–1 adenoma and the derived adenocarcinoma cells being attached to microcover glass in culture and strained with May-Gruenwald's and Giemsa solutions. *A* to *C*, FPCK-1–1 adenoma; D–F, FPCKpP_1-4. Original magnification: *A* and *D*, ×40 (*bar*, 250 μm). *B* and *E*, ×200 (*bar,* 50 μm). *C* and *D*, ×400 magnification of each cell line (*bar*, 25 μm).

Table 2. Comparison of Tumorigenicity in Nude Mice and In Vitro Characteristics of the Parental FPCK-1–1 Cell Line and the Tumors Arisen from FPCK-1–1 Cells Implanted Attached to a Plastic Plate or Injected into a Preinserted Gelatin Sponge

		In vivo		In vitro			
		Tumorigenicity (No. of mice with tumor/no. of mice injected)[b]					
Cell lines[a]	Cell line established from FPCK-1–1 cells implanted with	5×10^6	1×10^6	VEGF production pg/ml[c]	Doubling time (hours)	Plating efficiency (%)[d]	Number of colonies per 4×10^4 cells in soft agar[e]
FPCK-1–1	—	0/11	0/9	3246 ± 293	38.5	20.8	0, 1, 2
FPCKpP_1–1	Plastic plate	2/2*	0/2[g]	7833 ± 471	27.1	6.4	>250, >250, >250
FPCKpP_1–2	Plastic plate	2/2*	2/2*	7082 ± 1350	31.3	13.5	1, 1, 2
FPCKpP_1–3	Plastic plate	2/2*	6/6*	5303 ± 78	31.5	13.2	6, 10, 11
FPCKpP_1–4	Plastic plate	NT[f]	2/2*	6075 ± 642	43.6	17.6	0, 3, 5
FPCKpP_1–6	Plastic plate	3/3*	1/2**	8007 ± 552	NT[f]	18.3	6, 15, 15
FPCKsP_1–1	Gelatin sponge	4/4*	NT[f]	7465 ± 35	23.2	3.1	3, 3, 5

[a] FPCK-1–1 cells (1×10^5) were attached to a plastic plate in culture and implanted sc, or 5×10^6 cells were injected sc into preinserted gelatin sponge in KSN nude mice. The arising tumors were cultured separately and named FPCKpP_1–1 serially to FPCKpP_1–6 and FPCKsP_1–1.

[b] KSN nude mice were injected sc with FPCK-1–1 cells or FPCKpP_1 cells without plastic plate.

[c] VEGF levels in supernatants were measured by using 5×10^5 tumor cells cultured in 24-well plates for 48 hours.

[d] Cells (1×10^3) of each cell line were plated into 60-mm dishes and incubated for 8 days.

[e] Cells (4×10^4) were suspended in 1 ml of 0.3% agar and placed on a presolidified 0.6% agar in 6-well plates and incubated for 3 weeks.

[f] Not tested.

[g] Not significant vs implanted FPCK-1-1 cells alone.

* $p < 0.001$, ** $p < 0.05$.

subcutaneous fibroblasts or the immortalized fibroblast cell lines C3H10T 1/2 or NIH3T3 (Fig. 6C). We further confirmed that all six independently established plastic plate-associated fibroblasts secreted such a growth-potentiating factor of larger than 100 kDa, whereas normal fibroblasts did not (not shown).

We next examined growth of the adenoma cell-derived adenocarcinoma lines in response to the factor

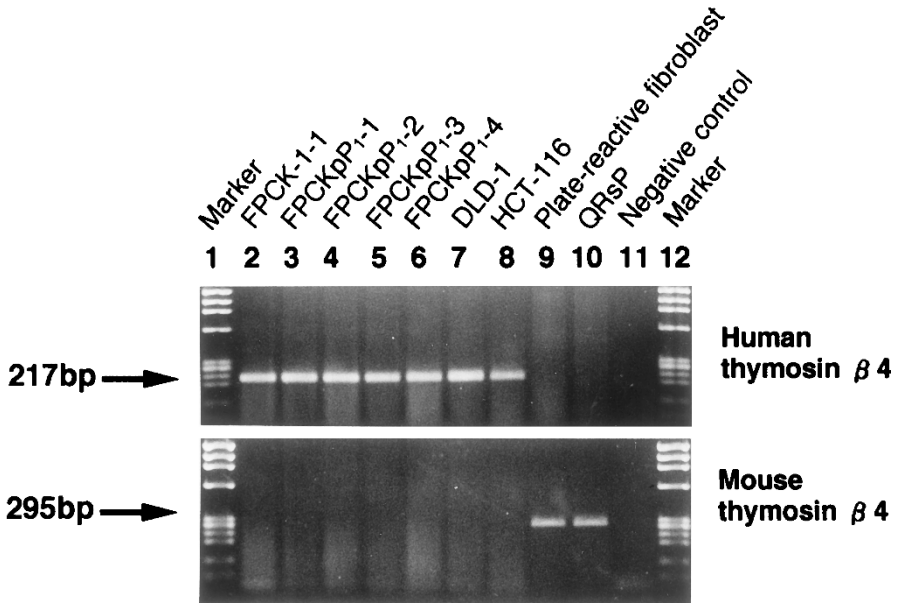

Figure 4.

Confirmation of human origin of the adenocarcinoma cell lines by RT-PCR analysis. cDNA was synthesized from mRNA samples extracted from parental FPCK-1–1, FPCK-1–1-derived adenocarcinoma lines, human colorectal tumor cell lines, mouse plastic plate-reactive fibroblast, and mouse fibrosarcoma cell line. Each cDNA was amplified with specific primer sets for human or mouse thymosin β4. Lane 1, øX174-*Hae*III digest DNA marker; Lane 2, parental FPCK-1–1 cells; Lanes 3–6, FPCK-1–1-derived cell lines (FPCKpP$_1$-1, FPCKpP$_1$-2, FPCKpP$_1$-3 and FPCKpP$_1$-4 cell line, respectively; Lane 7, DLD-1 cell line; Lane 8, HCT-116 cell line; Lane 9, plastic plate-reactive mouse fibroblast; Lane 10, mouse fibrosarcoma cell line; Lane 11, negative control containing all the reaction mixtures except cDNA; Lane 12, øX174-*Hae*III digest DNA marker.

Table 3. Enhanced Tumorigenicity of FPCK-1–1 Cells Injected into the Stromal Fibrous Tissues Surrounding Plastic Plate

5×10^6 cells of adenoma cells injected into nude mice with	Incidence: No. of mice with tumor/no. of mice tested
Fibrous stroma[a]	6/6[d]
Nontreated[b]	0/6[d]
Sham operated[c]	0/6[d]

[a] FPCK-1–1 cells (5×10^6 cells/0.1 ml) in PBS suspension were injected into the stromal fibrous tissues surrounding the plastic plate previously implanted sc without FPCK-1–1 cells and removed after 120 days.

[b] FPCK-1–1 cells (5×10^6 cells/0.1 ml) in PBS suspension were injected into nontreated KSN nude mice.

[c] FPCK-1–1 cells (5×10^6 cells/0.1 ml) in PBS suspension were injected into sham-operated KSN nude mice.

[d] $p < 0.001$ versus adenoma cells injected into nontreated or sham-operated nude mice.

larger than 100 kDa. The growth stimulatory activity was observed relative to the adenoma cell line but not to the adenocarcinoma lines (Fig. 7).

Discussion

In this study we demonstrated that human colonic adenoma cells could be converted to adenocarcinoma cells by foreign body-induced inflammation in nude mice, substantiated by histopathologic examination. Once the adenoma cells acquired the tumorigenic phenotype, they maintained the promoted tumorigenicity when transplanted into other nude mice without a foreign body.

Establishment of the model is based on our previous studies. We earlier established rodent tumor progression models by using poorly tumorigenic or weakly metastatic tumors in normal syngeneic hosts coimplanted with a plastic plate or gelatin sponge (Hamada et al, 1992; Okada et al, 1992, 1993, 1999). Regressive mouse fibrosarcoma cells were converted to more malignant cells after contacting inflammatory cells caused by the plastic plate (Okada et al, 1993) or gelatin sponge (Okada et al, 1992) in the early phase.

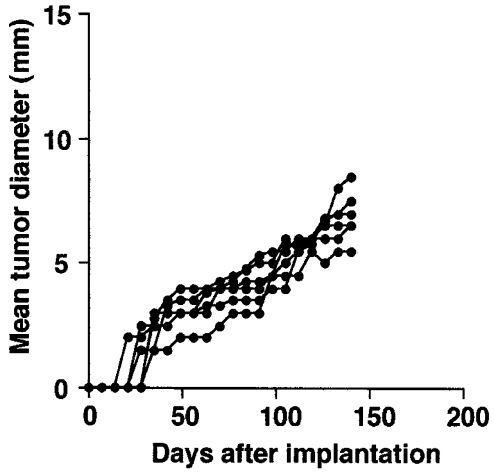

Figure 5.

In vivo growth curves of 5×10^6 FPCK-1–1 cells injected into fibrous tissue that had been induced by preinsertion of a plastic plate for 120 days, and the plate removed before cell injection (n = 6).

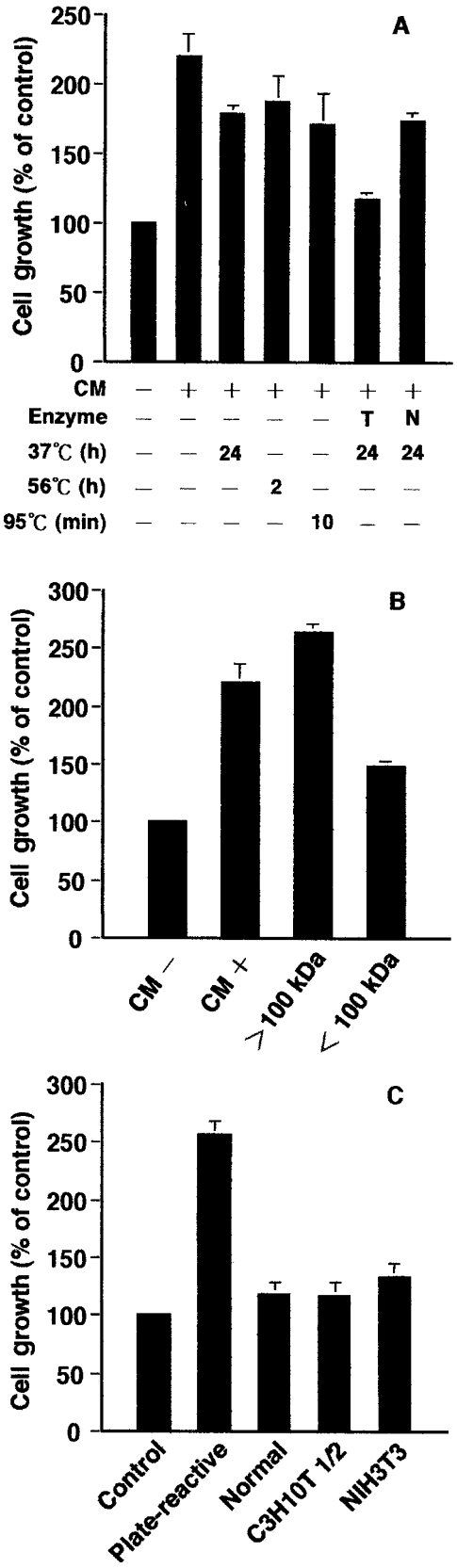

Figure 6.

Growth-stimulating effects of the medium conditioned with plastic plate-reactive fibroblasts on adenoma cells. FPCK-1–1 adenoma cells were plated (5 $\times$ 10^3 cells/well) in 96-well plates and incubated with or without the medium containing 50% conditioned medium (CM). After 4 days incubation, the cells were fixed and nuclei were stained with crystal violet, and optical density was measured on a microplate reader. Data are expressed as mean $\pm$ SD of quadruplicate determinations compared with the untreated controls of each cell line as 100%. Cell growth assay was performed as described in "Materials and Methods." Columns and error bars show means and standard deviations of quadruplicate samples. *A*, Growth-stimulating activity of CM with plastic plate-reactive fibroblasts to FPCK-1–1 cell line. The CM was stable to heating or neuraminidase (N), but labile to trypsin (T). *B*, The growth-stimulating activity was detected in the fraction of the CM larger than 100 kDa. *C*, The growth-stimulating activity in the fraction larger than 100 kDa of the medium conditioned with various mouse fibroblasts. Only the fraction larger than 100 kDa secreted from plastic plate-reactive fibroblasts stimulated the adenoma cell growth.

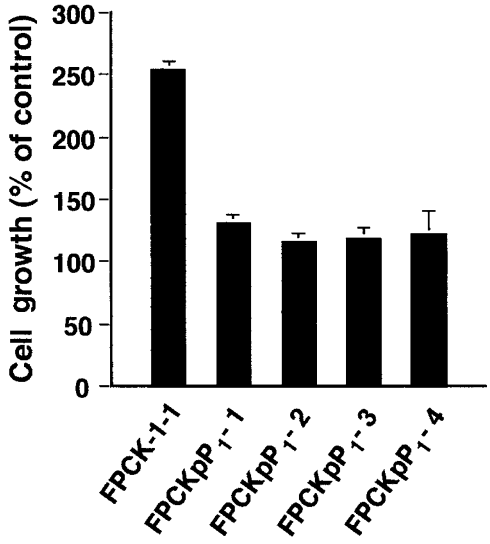

Figure 7.

The factor derived from plastic plate-reactive fibroblasts stimulates the growth of adenoma cells, but not of the derivative adenocarcinoma cells. FPCK-1–1 adenoma or adenoma-derived adenocarcinoma lines were plated (5 $\times$ 10^3 cells/well) in 96-well plates and incubated with or without the medium conditioned with plastic plate-reactive fibroblasts. Data are expressed as mean $\pm$ SD of quadruplicate determinations compared with the untreated controls of each cell line as 100%.

We have found that inflammatory cell-derived active oxygen species and the decreased antioxidative enzyme levels in the tumor cells acted reciprocally to promote tumor progression (Okada et al, 1999). Rat regressive mammary tumors need to be in contact with late-phase inflammatory cells for acceleration of the progression (Hamada et al, 1992). We detected soluble factors secreted from the plastic plate-reactive host cells. The factor also had the potential to promote tumorigenicity and metastatic ability of the tumor cells (Hamada et al, 1992). Those foreign bodies differ in absorbability in vivo; the duration of the foreign-body-induced inflammation varies. Since the plastic plate is not absorbed, its stimulative effect lasts for a long period, causing successive transition from the early to the late phase of inflammation (Hamada et al, 1992). On the other hand, a gelatin sponge is spontaneously absorbed in a short period, and thus induces only the early phase of inflammation (Akporiaye and Kudalore, 1989; Middleton and Campbell, 1989). The fact that the malignant conversion occurred in the adenoma cells with plastic plate but not in those with gelatin

sponge indicates that the conversion requires a long-lasting chronic inflammation. It also confirmed by our evidence that the late phase of plastic plate-surrounding stromal tissue by itself induced the tumorigenic conversion of the adenoma cells (Table 3).

We observed that the fibroblasts obtained from the plastic plate-induced fibrous stroma secreted specific soluble factors, which stimulated growth of only FPCK-1–1 adenoma cells, but not the derived adenocarcinoma cells, and that they were larger than 100 kDa. Switching to nonresponsiveness to this factor may be explained by acquisition of an autocrine growth property by the adenocarcinoma cells. This hypothesis is supported by our findings that all of the adenocarcinoma lines grew progressively in subcutaneous sites of nude mice in the absence of inflammation (Table 2). In fact we found that adenocarcinoma cells secreted much larger amounts of a potent angiogenic factor, namely a vascular endothelial cell growth factor (VEGF), compared with the adenoma cells. It was clear that VEGF was secreted by the tumor cells themselves, because it was detected in culture of the tumor cells. Angiogenesis stimulated by the adenocarcinoma cells was also observed in the macroscopic features, as shown in Fig. 2, A and B. Since we could not find any angiogenesis in the mice implanted with a plastic plate alone, we suspected that the abundant neovascularization was caused mainly by the growth of adenocarcinoma cells.

It has been reported that fibroblasts can promote progression of epithelial tumorigenesis (Champs et al, 1990; Chung et al, 1989; Cornil et al, 1991; Gleave et al, 1992; Skobe and Fusenig, 1998). Soluble growth factor(s) derived from fibroblasts should be one of the factors most responsible for the accelerating malignancy (Skobe and Fusenig, 1998), because those fibroblasts might secrete various growth factors such as transforming growth factor β_1 (TGF-β_1) (Lieubeau et al, 1994; Taipale et al, 1994), interleukin-6 (Lu et al, 1992), platelet-derived growth factor (Ponten et al, 1994), insulin-like growth factors I and II (Ellis et al, 1994; Yee et al, 1991), and hepatocyte growth factor/epithelial scatter factor (Nakamura et al, 1997). The CM obtained from plastic plate-induced stromal fibroblasts contained TGF-β_1, and the growth stimulating activity was reduced by the addition of anti-TGF-β_1, a neutralizing antibody (less than 16% reduction of CM-stimulated adenoma cell growth), but the main component of this factor is still undetermined. We are currently trying to identify the factor or factors other than TGF-β_1.

There is accumulated evidence that the inflammatory reaction promotes colorectal carcinogenesis. For instance, inflammatory colonic diseases, represented by Crohn's disease and ulcerative colitis, hold a high risk of developing into colorectal tumor over time (Choi and Zelig, 1994; Podolsky, 1991). Clinical studies of patients with FAP indicate that administration of nonsteroidal anti-inflammatory drugs (NSAIDs) causes regression of the disease and prevents recurrences of polyp or aberrant crypt foci development that are known to be a premalignant region of colon cancer (DuBois et al, 1996; Giardiello et al, 1993; Nugent et al, 1993; Takayama et al, 1998). Interleukin-10-deficient mice develop enterocolitis and subsequently colonic cancer at a high rate (Berg et al, 1996). However, there has been no evidence that inflammation directly induces conversion of human colonic adenoma cells to adenocarcinoma cells. In this regard our model demonstrated the contribution of inflammation directly to colorectal tumor development and progression.

It takes more than 100 days for implanted adenoma cells to grow to be a palpable tumor; in the meantime they restrain their growth. During such a dormant state, the adenoma cells were continuously affected by the plastic plate-induced inflammation. Because the adenoma cells were injected into the stromal fibrous tissue stimulated by plastic plate, the period of dormancy was shortened. We speculate that the chronic phase of inflammation was necessary for the conversion of the adenoma cells to adenocarcinoma cells and we believe that our model can mimic the natural course of inflammation-promoted carcinogenesis in human.

The emergence of tumors after implantation of human tumor cells into nude mice may be explained as follows. It is unlikely that the mouse host cells were transformed by the oncogenic virus previously infected with adenoma cells because the arising tumors were adenocarcinoma, as proven histologically; it was also confirmed by RT-PCR that the adenocarcinoma cells preserved the human gene but did not contaminate the mouse gene. The contribution of the plastic substrate as a transformation-inducible agent (Brand et al, 1975) has been reported, but it is not convincing because the mice implanted with plastic plates without adenoma cells did not develop tumors during 20 months of observation (Table 1). The spontaneous conversion during cell culture has previously been shown in experiments using other human colon adenoma cell lines (Paraskeva et al, 1992; Markowitz et al, 1994). However, malignant conversion constantly occurred at a high rate (65%) by implantation of the cells with plastic plates, whereas neither emergence of a tumorigenic subpopulation nor morphologic change was observed during in vitro passages of more than 200 times on the same plastic plate substrate (not shown).

The availability of this in vivo model and the resultant tumor lines should provide a valuable source for study of human colonic adenomas accelerated by chronic inflammation and for screening the chemopreventive agents.

Materials and Methods

Human Colonic Adenoma Cells and Culture Conditions

A human colonic adenoma cell line, FPCK-1, was established by Kawaguchi and colleagues from a colonic tubular adenoma lesion developed in a male FAP patient. The clinical and biological characteristics of this cell line have been described elsewhere (Kawaguchi et al, 1991). The FPCK-1 cell line grows

very slowly in culture and does not grow in athymic nude mice. After exposure of FPCK-1 cells to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), several sublines were obtained and one of them, FPCK-1–1, was used in this study (T Kawaguchi and T Kitagawa, unpublished observations). Those sublines grow in culture but are normally nontumorigenic in nude mice. The FPCK-1–1 and its derivative cell lines were maintained with a mixture of 6052 medium and DM-160 medium (Kyokuto, Japan) supplemented with 1% dialyzed fetal bovine serum (FBS; GIBCO BRL, Grand Island, New York), ITSô premix (Becton Dickinson Labware, Maryland; 10 ng/ml of insulin, transferrin, and 10 pg/ml of selenious acid), and 10 ng/ml epidermal growth factor (Takara, Tokyo, Japan). The calcium ion concentration was adjusted to 0.3 mM.

Human colorectal cancer cell lines DLD-1 and HCT-116 were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% FBS (Filtron). A mouse fibrosarcoma cell line, QRsP, was maintained in Eagle's minimum essential medium (MEM; Nissui) containing 8% FBS, sodium pyruvate, nonessential amino acids and L-glutamine.

Procedures of Implantation of Adenoma Cell with Foreign Bodies

Pieces of polystyrene plate (10 × 5 × 1 mm) were cut from a 100-mm culture dish (Corning 430167) and 1×10^5 FPCK-1–1 cells in 50 μl of medium were plated on each piece of plastic plate. After 24 hours incubation, the FPCK-1–1 cells attached to the plastic plate were implanted into 5-week-old female athymic KSN nude mice (Nippon SLC, Hamamatsu, Japan). A subcutaneous pocket reaching up to the thorax was made from a 10-mm incision on the right flank of the pelvic region in each anesthetized mouse. The plastic plate with the adenoma cells attached was implanted into the subcutaneous pocket and the wound was closed with clips.

A sterile gelatin sponge (Spongel; Yamanouchi, Tokyo, Japan) was cut into 10 × 5 × 3 mm pieces. A subcutaneous pocket as described above was made, one piece of gelatin sponge was inserted, and the wound was closed with clips. The adenoma cells (5×10^6 cells/0.1 ml) were immediately injected into the pre-inserted gelatin sponge.

A small incision was made 120 days after implantation, the plastic plate was removed with forceps, without injuring the surrounding tissue, and the wound was closed with clips. Then 5×10^6 FPCK-1–1 cells were injected into the tissue surrounding the plastic plate. Sham-operated mice were prepared by making an incision in the skin and a subcutaneous pocket formed, the wound was closed, and the same number of FPCK-1–1 cells was injected.

All the procedures were performed under sterile conditions. Nude mice used in this study had been maintained in the complete barrier condition, lit from 7:00 am to 7:00 pm, at 23 ± 3° C and 50 ± 10% humidity, fed with sterilized mouse diet (Nihon Nosan Kogyo, Yokohama, Japan) and autoclaved distilled water, in the germ-free section of Institute for Animal Experimentation, Hokkaido University School of Medicine.

Establishment of Culture Cell Lines from the In Vivo Arising Tumors and Fibroblast Cell Lines

The subcutaneous tumors in nude mice were aseptically removed about 200 days after implantation. The tumors were subjected to culture by mechanical and enzymatic disaggregation with 0.5% collagenase (Wako Pure Chemicals, Osaka, Japan) and designated as FPCKpP_1-1 ("FPCK" stands for FPCK-1–1-cell-derived, "p" stands for plastic plate, "P_1" stands for first passage, and "-1" represents the individual mouse number).

Reactive stromal tissues around the plastic plate or normal subcutaneous tissues were placed on individual 60-mm dishes (MS-10600; Sumitomo Bakelite, Tokyo, Japan) so that fibroblasts would spread and grow there. The cultured fibroblasts were maintained in the medium containing an equal ratio of Ham's F12 (Nissui) and DMEM supplemented with 10% FBS, and the passages between 4 and 10 were used.

Determination of In Vitro Tumor Cell Growth, Plating Efficiency, and Soft Agar Colony Formation

For in vitro cell growth analyses, cells were seeded into a 6-well plate (Falcon, 3046; 1×10^5 cells per well). The medium was changed every other day. The cells were harvested and counted by trypan blue exclusion test.

For evaluation of plating efficiency, 1×10^3 cells suspended in the medium containing 1% dialyzed serum were plated into 60-mm dishes in quadruplicate. The dishes were incubated for 8 days, and colonies were fixed in Carnoy's fixative, stained with 0.1% crystal violet, and scored.

For determination of the soft agar growth (anchorage independent), 4×10^4 cells were suspended in 1 ml of the medium containing 0.3% agar (GIBCO) and a double volume of dialyzed serum, and applied onto the presolidified 0.6% agar (1 ml) in 6-well plates. Triplicate plates were prepared for each cell line. After 3 weeks of incubation, colonies larger than 0.1 mm in diameter were scored.

Measurement of Human VEGF Protein Levels in Conditioned Medium by ELISA

A commercially available ELISA kit (R and D System, Minneapolis, Minnesota) for human VEGF was used to quantitatively measure the level of the protein in CM according to the manufacturer's instruction. To generate CM, subconfluent cells were harvested, counted, and seeded 5×10^5 in 24-well plates. The next day, medium was replaced by fresh medium and cells were allowed to grow for another 48 hours. The CM was collected and centrifuged at 11,000 rpm at 4° C for 15 minutes. The medium was then stored at −85° C until used for VEGF ELISA analysis.

Preparation of Medium Conditioned with Cultured Fibroblasts

CM from subconfluent fibroblasts (2.0–3.5 $\times$ 10^6 fibroblasts per 100-mm dish) was harvested after 48 hours culture in 10 ml of serum-free Ham's F12/DMEM. The supernatants were collected and centrifuged at 1,200 rpm for 5 minutes at 4° C and then recentrifuged at 20,000 $\times g$ for 60 minutes at 4° C (J2-M1; Beckman Instruments, Palo Alto, California). The supernatants were passed through a 0.22-μm filter to remove cellular debris.

The supernatants were concentrated by membrane ultrafiltration (AmiconYM membrane, cut-off molecular weight: 100,000) in centrifugal concentrators. The resultant CMs were diluted 1:1 with serum-free Ham's F12/DMEM and used for cell growth assay.

Trypsin and neuraminidase sensitivities of the active entities in the CM were detected by incubating the CM with 0.13 U/ml of trypsin (Sigma, St. Louis, Missouri) or 0.05 U/ml of neuraminidase (Sigma), respectively, at 37° C for 24 hours. Both enzymes were attached to beaded agarose; the supernatants obtained after centrifugation of the mixture of CM with the respective enzymes were used as enzyme-treated CM.

Measurement of Tumor Cell-Growth Stimulated with Conditioned Medium

The cell number in the cultures was quantitatively measured by the crystal violet staining technique (Gillies et al, 1976). Five thousand cells were seeded in each well of 96-well plates (353072; Beckton Dickinson) containing 100 μl of serum-free 6052/DM-160 medium, and the CM with or without treatment was added into each well. Four days later the attached cells were fixed with 5% glutaraldehyde solution for 30 minutes and then stained with the mixture of 0.1% crystal violet (Kanto Chemicals, Tokyo, Japan) and CAPS buffer (50 mM, pH 9.4) for 30 minutes. Subsequently the solution was removed and the plates were rinsed 3 times with distilled water. Crystal violet in the stained cells was extracted for 30 minutes with 50 μl of 10% acetic acid solution, and the absorbance in each well was determined by wave length at 540 nm on a microplate reader (Model 550; BioRad, Tokyo, Japan). The results were expressed in percentages relative to control without CM.

There was a linear correlation between the concentration of crystal violet extracted from nuclei of the cells and the number of cells plated. This correlation was statistically significant in FPCK-1–1 cells (r = 0.88, $p < 0.02$). Significance in other cells were: FPCKpP_1-1 cells (r = 0.91, $p < 0.02$), FPCKpP_1-2 cells (r = 0.93, $p < 0.01$), FPCKpP_1-3 cells (r = 0.90, $p < 0.02$), and FPCKpP_1-4 cells (r = 0.90, $p < 0.02$).

mRNA Extraction and RT-PCR Analysis

Total RNA was isolated from exponentially growing cells cultured in vitro with a TRIZOL reagent (GIBCO). For synthesis of the first-strand cDNA, 300 ng of total RNA was used in a 20 μl reaction mixture containing 1$\times$ first-strand buffer (GIBCO), 7.5 mM DTT, 0.5 mM $MgCl_2$, 0.5 mM dNTP, 100 pg random primer (GIBCO), and MML-V reverse transcriptase (GIBCO). The reverse transcription was performed in a block incubator (BI-525; Astec, Tokyo, Japan) for 50 minutes at 37° C after annealing at 25° C for 10 minutes. PCR was performed 95° C for 5 minutes and on ice for 5 minutes in a block incubator. PCR was performed in a 50 μl reaction mixture containing 1$\times$ universal buffer (Nippon Gene, Tokyo, Japan), 200 nM of each primer, 0.2 mM dNTPs, and 2.5 units of *Taq* polymerase (Nippon Gene). Gene-specific primers were designated to span the coding region of human and mouse thymosin β4 (5' to 3'); human thymosin β4 upstream, GACTTCGCTCGTACTCGTGC; human thymosin β4 downstream, AATGTACAGTGCATATTGGCG; mouse thymosin β4 upstream, CCTCATCCTCCTCGTCCTTA; mouse thymosin β4 downstream, TGATCCAACCTCTTTGCATC. RT-PCR of mRNA encoding the human and mouse thymosin β4 resulted in PCR products of 217 and 295 bp long, respectively. The PCR cycles consisted of 5 minutes initial denaturation at 95° C, followed by 25 cycles of 94° C for 1 minute, 57° C for 1 minute, and 72° C for 2 minutes in a thermal cycler (2400R; Perkin Elmer Cetus, Norwalk, Connecticut). Each PCR amplification included a negative control containing all of the reaction products except cDNA. Of each PCR product, 5 μl was separated in 2% agarose (Iwai Kagaku, Tokyo, Japan), stained with ethidium bromide, and photographed under ultraviolet light.

Histopathologic Examination

Tumor tissues were fixed in phosphate-buffered 10% formaldehyde and embedded in paraffin. Sections 4-μm thick were prepared and stained with hematoxylin and eosin. Staining with Azan technique was also used for positive identification of collagen fiber. Culture cell lines attached to microcover glass (22 $\times$ 22 mm; Matsunami Glass, Tokyo, Japan) were stained with May-Gruenwald's and Giemsa solutions (Merck, Tokyo, Japan).

Statistical Analysis

The differences in the tumor incidences were calculated by χ^2 test. The statistical significance of the correlation between the number of cells plated and the concentration of crystal violet extracted from nuclei of the cells was evaluated by regression analysis.

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