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Differential Production of Proteolytic Enzymes by Normal (KMS-6), Immortally Transformed (KMST-6), and Tumorigenetically Transformed (Ha-KMST-6) Human Fibroblasts

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ABSTRACT. Production of proteolytic enzymes by human fibroblasts in the process of transformation was investigated in order to learn which step, the immortalizing or tumorigenic step, is more important for malignant transformation of human cells. The cells used were normal human fibroblasts (KMS-6), fibroblasts immortally transformed by treatment with Co-60 gamma rays (KMST-6), and KMST-6 cells further tumorigenetically transformed KMST-6 cells by infection with Harvey murine sarcoma viruses (Ha-KMST-6). Proteolytic enzymes in culture medium or cells were assayed using synthetic substrates, N-a-(p-tosyl)-L-arginine [^{3}H]methyl ester hydrochloride and H-D-val-leu-lys-p-nitroaniline. Our results showed that the immortally transformed KMST-6 cells produced a larger amount of the enzymes than the normal and the tumorigenetically transformed cells. Since elevated production of proteolytic enzymes has been reported to correlate well with malignant transformation of cultured rodent or avian cells by many other investigators, our present results indicate that our immortalized human fibroblasts have already acquired properties characteristic to cancer cells.

Key words : Human cells — Transformation — Proteases

Numerous studies on *in vitro* malignant transformation of rodent cells by treatment with chemicals or physicals have been reported, but there have been only a few successful studies on the transformation of normal human cells. Kakunaga[1] described the transformation of human fibroblasts with 4-nitroquinoline 1-oxide (4NQO) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and Namba *et al.*[2-4] transformed human embryo fibroblasts by treatment with Co-60 gamma rays or 4NQO. Dorman *et al.*[5] reported the malignant transformation of human endothelial cells with MNNG. Recently Yoakum *et al.*[6] described the malignant transformation of human bronchial cells by transfection of the *ras* oncogene.

When we refer to *in vitro* transformation of normal human cells, the determination of transformation is based on the two following criteria[5] : (1) unlimited cell growth, and (2) abnormal karyotypes, since normal cells do not proliferate indefinitely and do not harbor abnormal chromosomes *in vivo*.

As indicated by Roblin *et al.*,[7] Noonan[8] and Danϕ *et al.*[9] in review of studies concerned with enhanced production of proteolytic enzymes and carcinogenesis, positive correlation between enhanced production of proteolytic

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enzymes and malignant transformation of cells has been noted by many investigators, although some exceptions were found.[10-12] The biological roles of the proteolytic enzymes are not yet fully understood, but they may be related to cell growth, anchorage-independent cell growth, invasiveness of cancer cells into surrounding tissues or metastasis.

Since we recently succeeded in obtaining an immortally transformed human fibroblast line by treatment with Co-60 gamma rays (KMST-6) and in further transforming these immortal KMST-6 cells into a tumorigenic cell line (Ha-KMST-6) by infection with Harvey sarcoma viruses,[13] we decided to compare the production for proteolytic enzymes by these two cell lines with their normal counterparts (KMS-6) in order to learn which step, immortalization or tumorigenic transformation of normal human fibroblasts, shows characteristics similar to the malignant transformation of rodent and avian cells.

MATERIALS AND METHODS

Cells and cultures

A normal human diploid fibroblast strain, KMS-6, was established from a 9-week-old embryo in our laboratory. This normal cell strain senescened at the 40th to 50th population doubling level (PDL) under conventional culture conditions. Next, we transformed KMS-6 cells into an immortal cell line, KMST-6, by repeated treatment with Co-60 gamma rays.[3] Then, we further transformed KMST-6 cells by infection with Harvey sarcoma viruses into a tumorigenic cell line, Ha-KMST-6.[14] These immortal and tumorigenic cell lines are now at the 210th PDL. Cellular characteristics of KMS-6, KMST-6 and Ha-KMST-6 are briefly summarized in Table 1. All these cells were maintained in Eagle's minimum essential medium (Nissui Seiyaku, Tokyo) supplemented with 10% fetal calf serum. Culture methods, the irradiation schedule, and the cellular characteristics of these cells have already been described in detail by Namba *et al.*[3]

TABLE 1. Characteristics of normal (KMS-6), immortally transformed (KMST-6), and tumorigenetically transformed (Ha-KMST-6) human fibroblasts

	KMS-6	KMST-6	KMST-6
Doubling time (hr)[1]	60	48	36
Saturation density (cells/cm^2, $\times 10^{-3}$)[1]	65	130	260
Colony formation in soft agar (%)[2]	0.001	0.001	0.3
Life span	Definite	Indefinite	Indefinite
Karyotype	Normal	Abnormal	Abnormal
Transplantability[3]	0/5	0/5	5/6

1. These data were obtained using the defined medium described in the text.
2. This experiment was carried out with MEM plus 10% FBS.
3. Number of tumor-bearing mice/Number of mice used.

Assay of proteolytic enzymes in culture medium and in cells

Since serum may contain some proteases and/or protease inhibitors, we used a serum-free defined medium which consisted of a 1 : 1 mixture of

Dulbecco's modified Eagle's medium and Ham's F12 (Nissui) supplemented with 0.1% bovine serum albumin fraction V (Sigma, St. Louis, MO), 10 μg/ml of transferrin (Collaborative Research, Waltham, MA), 1 μg/ml of insulin (Sigma) and 5 μg/ml of oleic acid (Sigma). This defined medium showed no proteolytic activities and contained no inhibitory factors for proteolytic enzymes. In this culture medium, normal KMS-6 cells grew until about the 20th PDL, while the transformed cells proliferated permanently. Therefore normal KMS-6 cells were used for the 8th and 18th PDL. The details of this defined medium were described by Namba *et al.*[13]

We determined proteolyitc activity by a modification of the method of Imanari *et al.*[15] Briefly N-a-(p-tosyl)-L-arginine [^{3}H]methyl ester hydrochloride (^{3}H-TAME, 37.0 GBq/mmole, Amersham, England) was used as the substrate and diluted with distilled water at a concentration of 10^6 dpm/ml. Reaction mixtures containing 10 μl of culture medium (pH 7.4 to 7.8) and 10 μl of ^{3}H-TAME were incubated for 30 min at 37°C. The reaction was terminated by cooling the mixtures in ice and adding 6 ml of scintillator (4 g of PPO and 100 mg of POPOP in toluene per liter) containing 50 μl of stop solution containing 1 volume of acetic acid and 9 volumes of 0.02 M TAME (Nakarai Chemicals, Kyoto). The reaction product [^{3}H-methyl] extracted into the toluene was determined by a liquid scintillation counter. Under these conditions the release of ^{3}H-methyl increased linearly up to 60 min. One unit of the proteolytic activity was defined as the amount of the enzymes that hydrolyzed 1 μM of TAME per minute. The culture medium incubated for 3 days at 37°C without cells showed no proteolytic activity and was used as a blank for each assay. In order to determine types of enzymes produced, bovine pancreas trypsin inhibitor (Sigma), antithrombin III (Boehringer Mannheim, West Germany), esteratin which was generously given to us by Dr. T. Aoyagi, Institute of Microbiol Chemistry, Tokyo, and diisopropylfluorophosphate (Sigma) were added to the reaction mixtures.

The assay for plasminogen activator (PA), one of serine proteases, is based on the conversion of plasminogen by PA to plasmin, which is detected by the chromogenic substrate.[16] We first incubated 100 μl of 10 CU/ml of plasminogen (700 CU/vial, Green Cross, Co., Osaka) for 2 min to 6 min at 37°C. Then we added 50 μl of culture medium to it and incubated the reaction mixture for 10 min at 37°C. Thereafter, 350 μl of 0.3 mM of a substrate solution (H-D-val-leu-lys-p-nitroanilid dihydrochloride, S-2251, Kabi, Stockholm) was added to the reaction mixture. The reaction was incubated for 3 min at 37°C and stopped by adding 50 μl of 50% acetic acid. Absorption was read at 405 nm. Urokinase (1,200 IU/vial, Green Cross) diluted at desired concentrations was used as a reference for PA activity. The results obtained were expressed as the IU of this standard urokinase activity. The plasminogen, S-2251, and the urokinase were dissolved in Tris buffer (0.05 M, pH 7.4) containing 0.7 g/liter of NaCl.

Measurement of cell growth

Cells were detached from dishes with 0.2% trypsin solution, stained with 1% crystal violet in 0.1 M citric acid, and counted by means of a hemocytometer.

RESULTS

The cell growth and production of proteolytic enzymes of normal KMS-6, immortally transformed KMST-6 cells, and tumorigenetically transformed Ha-KMST-6 cells are shown in Figure 1. The immortally transformed KMST-6 cells revealed about a 3 to 4-fold increase in the production of proteolytic enzymes over those of the normal and the tumorigenetically transformed human fibroblasts. Interestingly, tumorigenetically transformed Ha-KMST-6 cells showed no significant increase in the amount of the enzymes. These results led us to investigate the intracellular proteolytic enzymes, because the decreased amount of the enzymes in the culture media of KMS-6 and Ha-KMST-6 might be due to retarded secretion of the enzymes. As shown in Table 2, KMS-6 and Ha-KMST-6 cells had a smaller amount of intracellular enzymes than KMST-6 cells.

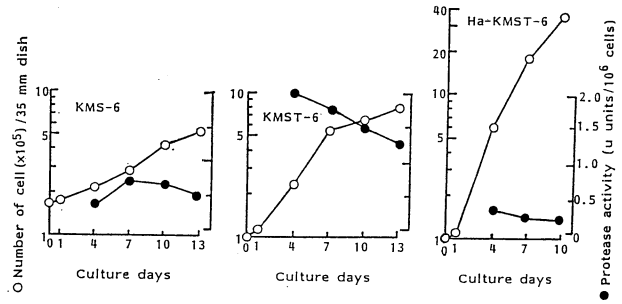

Fig. 1 Relation of cell growth and production of proteases of normal (KMS-6), immortally transformed (KMS-6), and tumorigenetically transformed (Ha-KMST-6) human fibroblasts. Each point represents a mean of triplicate cultures.

TABLE 2. Proteolytic activity of culture medium and cell lysate of normal (KMS-6), immortally transformed (KMST-6), and tumorigenetically transformed (Ha-KMST-6) human fibroblasts

	Proteolytic activity (μ Unit/10^6 cells)[1]		
	KMS-6	KMST-6	Ha-KMST-6
Medium[2]	0.30	2.37	0.21
Cell lysate[3]	0.30	2.39	0.11

1. Cells in the logarithmic growth stage were used. Each value of the activity represents a mean of triplicate cultures.
2. Cells were cultured in 2 ml of culture medium.
3. Cells were dissolved in 2 ml of cold 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100.

The enzyme production of KMS-6 and KMST-6 cells was high in the exponential growth stage and then decreased as the cells reached confluency. This decreased proteolytic activity in the stationary stage was not due to the production of inhibitors by confluent cells. Mixing of the culture medium in

the logarithmic growth stage with the one in the stationary stage resulted in a decrease in proteolytic activity due only to dilution (data not shown).

Using various protease inhibitors, we then examined what kinds of proteolytic enzymes were produced by the cells. Table 3 shows that protease activity of the normal KMS-6 cells was inhibited either by a bovine pancreatic trypsin inhibitor (BPTI) or by diisopropylfluorophosphate, while the activity of the enzymes of the immortal KMST-6 cells was reduced about 35% by all of the inhibitors, BPTI, antithrombin III (AT) and esteratin (ES). These results indicated that the normal human cells produced mainly trypsin-like enzymes and that the immortal cells yielded at least three types of enzymes, including trypsin-like enzyme, thrombin and esterase, in substantial amount. On incubation of the culture medium at 100°C for 10 min, no proteolytic activity was detected (Table 3). After the KMST-6 cells were treated with 0.4 μg/ml of cycloheximide for 24 hr, they produced only 17.5% of the enzymes compared with the untreated KMST-6 cells (data not shown).

We investigated the production of plasminogen activator (PA) by KMS-6, KMST-6, and Ha-KMST-6 cells. The PA activity of the immortally transformed KMST-6 cells was the highest (25.9 IU/ml/10^6 cells), followed in order by that of Ha-KMST-6 (13.9 IU/ml/10^6 cells) and that of KMS-6 (1.7 IU/ml/10^6 cells). These results were similar to those of the proteolytic enzymes.

In order to learn the biological roles of these proteolytic enzymes produced by cells, KMS-6 and KMST-6 cells were cultured for 7 days in the presence of a mixture of three inhibitors, BPTI, ES and AT. Concentrations of these inhibitors were the same as those in Table 3. Neither cell growth nor DNA synthesis was inhibited by these inhibitors (data not shown).

TABLE 3. Proteolytic activity in culture medium of normal (KMS-6) and immortally transformed (KMST-6) human fibroblasts after treatment with protease inhibitors or heat

Inhibitors[1]	Proteolytic activity (% of control)[2]	
	KMS-6	KMST-6
None	100	100
BPTI	41.1	63.0
AT	85.7	62.7
ES	92.8	68.0
DFP	40.8	32.0
BPTI + ES	ND	30.0
BPTI + ES + AT	ND	15.3
100°C, 10 min	0	0

1. Abbreviation (concentrations used) ; BPTI : bovine pancreas trypsin inhibitor (10 μg/ml), AT : antithrombin III inhibitor (0.5 IU/ml), ES : esteratin (1 μg/ml), DFP : diisopropylfluorophosphate (0.5 mM), ND : Not done
2. Each value of the activity represents a mean of triplicate cultures.

DISCUSSION

Our current work showed that the immortally transformed human fibroblasts (KMST-6) produced a larger amount of proteolytic enzymes and plas-

minogen activator than their normal counterparts and the tumorigenetically transformed Ha-KMST-6 cells. Interestingly, we observed a lack of correlation between high proteolytic activity and tumorigenicity. The similar results were reported by Jones *et al.*,[10] who studied on several clones of a human osteosarcoma cell line.

The positive relation between enhanced production of proteolytic enzymes and malignant transformation has generally been recognized, as reviewed by other investigators.[7-9] If it is so, it is probable that our human cells immortally transformed by treatment with Co-60 gamma rays have already been changed into malignant cells. The two criteria for *in vitro* malignant transformation of human cells, immortality and karyological aberration, which were previously proposed by Namba *et al.*,[2] also appear to indicate that these immortally transformed human cells have already been changed to cancer cells.

Interestingly, KMST-6 cells produced various types of proteolytic enzymes in considerable amounts, while the normal cells yielded mainly the trypsin-like enzyme. Chen and Buchanan[17] also reported that transformed chick embryo fibroblasts in contrast to untransformed cells secreted proteases other than plasminogen activator. Although the roles of these enzymes have not been fully clarified yet, they may in part contribute to cell growth, invasiveness or metastasis of cancer cells. Noonan[8] reported that the addition of trypsin or thrombin to cell cultures could stimulate glucose transport, alter cellular morphology, induce the rearrangement of filamentous structures, alter cyclic nucleotide levels, and induce DNA synthesis and cell division. Similar results were obtained by Quingley *et al.*[18] who demonstrated that exogenous addition of thrombin to normal chick embryo fibroblast cultures induced cell growth. However, they reported that various inhibitors of proteases did not have any significant effect on cell growth, RNA, and protein synthesis of RSV transformed chick embryo fibroblasts. These results were consistent with our present results. We added inhibitors to the normal and immortally transformed cultures, but no significant growth inhibition, no morphological alteration, or no decrease in cell saturation density was detected. However, there is a possibility that endogenously produced proteases work intracellularly to regulate the growth of transformed cells. Endogenous proteolytic enzymes may intracellularly destroy some proteins inhibitory to cell growth or activate some proteins which contribute to the abnormal growth behaviour of the transformed cells. The inhibitors added exogenously to the cultures may have no access to intracellular proteases. Otherwise, the biological function of these enzymes is expressed at the plasma membrane.[19]

Which is more crucial for malignant transformation of human cells, immortalization or tumorigenicity? As mentioned above, we suppose that immortalization is more crucial than tumorigenicity for malignant transformation of human cells. When normal human fibroblasts which were mortal were infected with Ha-MSV, they did not produce tumors or become immortal cells.[14] Similar results were obtained by Rhim *et al.*, who first immortalized normal human keratinocytes with Ad-12-SV40 and then made these immortal cells tumorigenic by infection with Kirsten MSV.[20] These results, together with ours, indicate that factors involved in the immortalization of cells work as initiators of carcinogenesis and that viruses containing *ras* oncogene act as promotors. For

this reason, studies on the cellular aging of normal human cells should shed light on the mechanisms of malignant transformation of normal human cells.

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