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Establishment and Characterization of Three Androgen-independent, Metastatic Carcinoma Cell Lines from 3,2′-Dimethyl-4-aminobiphenyl-induced Prostatic Tumors in F344 Rats

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Three stable carcinoma cell lines, designated PLS10, PLS20 and PLS30, have been established from 3,2′-dimethyl-4-aminobiphenyl plus testosterone-induced carcinomas in the dorsolateral prostate of male F344 rats. The cells are keratin-positive and grow as typical epithelial monolayers in culture. When injected into intact male nude mice, PLS10 and PLS30 cells form well-differentiated adenocarcinomas with abundant connective tissue stroma, while PLS20 cells give rise to poorly differentiated adenocarcinomas. Growth of all PLS cell lines in nude mice is not affected by castration and the cells are immunohistochemically negative for androgen receptors. Tumor growth rates in nude mice were found to be PLS20 > PLS10 > PLS30, with significant *in vitro* stimulation by insulin/transferrin, but not epidermal growth factor, dexamethasone or basic fibroblast growth factor. Spontaneous lung metastases were observed in all cases. However, skeletal invasion including bone is essentially observed only with the PLS20 tumors. Gelatin zymography showed predominant secretion of the active form of gelatinase B (Mr 92,000 type IV collagenase) by all the cell lines. Karyotype analysis revealed PLS10, PLS30 and PLS20 to be diploid, hyperdiploid and hypertetraploid, respectively. The results demonstrate that the three PLS cell lines are androgen-independent and metastatic in common, but have different histology, growth potential and invasiveness. They may therefore be useful models for understanding progression and metastasis of human prostatic carcinomas.

Key words: DMAB-induced rat prostatic carcinoma — Androgen-independent tumor — Metastasis — Tumor progression — Gelatinase B

Prostatic cancer is one of the most common malignancies in men in western countries and recently, the incidence has been increasing rapidly in Japan.[1)] Prostatic cancer progression leading from androgen-dependent, nonmetastatic phenotype to a very much more malignant, androgen-independent, metastatic phenotype almost invariably occurs after androgen deprivation by orchiectomy or administration of androgen antagonists.[2)] To gain further insight into human prostate cancer progression and metastasis, experimental *in vivo* models that mimic the natural course of the disease progression are essential. To date, however, only a few human prostatic carcinoma cell lines are available for study and most of them have low rates of spontaneous metastasis.[3–5)] Animal models of human prostate cancer are also rather limited in number,[6–8)] but several rat prostatic carcinomas arising spontaneously in aged rats, such as the Dunning R-3327 and Pollard tumors, do exhibit metastatic potential to varying degrees. The Dunning R-3327 tumor, which is one of the most widely used animal models of prostatic cancer, has given rise to many sublines ranging from androgen-dependent, nonmetastatic to androgen-independent, metastatic cell lines.[9, 10)]

We previously reported induction of invasive prostatic carcinomas in the dorsolateral lobes of male F344 rats by treatment with 3,2′-dimethyl-4-aminobiphenyl (DMAB) plus testosterone.[11)] The tumors differ from those mentioned above in that they are chemically induced rather than spontaneous, and show a uniform androgen-independent and metastatic phenotype.[12, 13)] In the present study, we established novel carcinoma cell lines in culture from DMAB-induced rat prostatic tumors, and observed them to retain their androgen-independent and metastatic phenotype. One cell line was found to have further acquired the potential for bone invasion. Analysis of matrix metalloproteinase activity demonstrated that they all secrete predominantly the active form of gelatinase B.

MATERIALS AND METHODS

Source of cell lines Prostatic carcinomas were induced in the dorsolateral prostates of male F344 rats by administration of DMAB (Matsugaki Pharmaceutical Co., Osaka) and testosterone propionate (Sigma, St. Louis, MO) as detailed earlier.[11)] Three examples of primary tumors were successfully transplanted into the subcutis of 6-week-old male nude mice of the CD-1 strain (Charles River Japan, Inc., Kanagawa) and maintained

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in the same mice. Three independent transplantable tumor lines at passage number 3 were selected for cell culture studies.[14]

Cell culture Tumor tissue was cut into small pieces and minced in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) with sterile scissors after having been washed with DMEM. The tumor pieces were treated with 50 u/ml Dispase (Godo Shusei, Tokyo) for 30–60 min at 37°C and large tumor pieces were allowed to settle. Supernatant fluid containing cell clumps was collected after centrifugation at 100*g*. Cell pellets were washed, resuspended in DMEM containing 10% fetal bovine serum (FBS) (GIBCO), 100 u/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (GIBCO) and incubated for 1–2 h on plastic at 37°C. Medium containing floating cell clusters was collected and centrifuged. The pellets were resuspended in fresh DMEM medium with 10% FBS plus serum extender (MITO) containing various growth factors and hormones and cultured on dishes coated with collagen type I (Iwaki, Tokyo) in a humidified 5% CO_2 incubator at 37°C. After several passages, any remaining fibroblasts were removed by a differential attachment and detachment selection method with trypsin-EDTA, after which cultures of epithelial monolayers were obtained. PLS10, PLS20 and PLS30 cells were established from these monolayers by adaptation to DMEM with 10% FBS and maintained in plastic dishes for several months. The absence of *Mycoplasma pulmonis* contamination of PLS cells was confirmed by a direct agar method. The absence of two viruses (mouse hepatitis virus and Sendai virus) was also confirmed by a polymerase chain reaction method with the assistance of the Experimental Animal Research Center of Japan (Tokyo).

Immunofluorescence microscopy and immunoperoxidase staining Immunofluorescence microscopy for keratin was performed on the cultured cells grown on Lab-Tek chamber slides (Nunc, Naperville, IL) as described previously.[15] For immunostaining of androgen receptor, tissue slices were fixed in ice-cold acetone and routinely processed for paraffin embedding. The sections were deparaffinized, rehydrated in water, and heated in distilled water in a microwave oven for 5 min. They were then cooled to room temperature, rinsed in PBS (pH7.3) with polyvinyl pyrolidone (PVP), washed several times at 4°C in 85% ethanol-PVP, PBS/PVP and sodium borohydride/PBS-PVP, and treated with 2% goat serum in PBS-gelatin at 4°C to block nonspecific binding. Incubation was carried out overnight at 4°C with the polyclonal rabbit antibody PG21 (Signet Lab., Dedham, MA). Binding was visualized by the avidin-biotin-peroxidase complex method, and the sections were counterstained weakly with hematoxylin.[16]

Cell-growth analysis In order to study the growth factor responsiveness of the PLS cell lines, DMEM medium containing 2.5% FBS without supplements was used as the control medium. The cells were plated at 1×10^5 cells/35-mm dish in control medium alone or supplemented with various growth factors and hormones, singly or in combination, and representative samples were counted in triplicate 3 days after seeding with a hemocytometer. Concentrations of growth factors and hormones used were; insulin/transferrin (each, 5 μg/ml), epidermal growth factor (EGF, 10 ng/ml), basic fibroblast growth factor (bFGF, 10 ng/ml), hepatocyte growth factor (50 ng/ml), dexamethasone (1 μM), testosterone propionate (0.1 μM), dihydrotestosterone (0.1 μM), 17β-estradiol (1 μM). All the reagents were obtained from Sigma. MITO containing insulin, transferrin, EGF, endothelial growth supplement, triiodothyronine, hydrocortisone, progesterone, testosterone, and 17β-estradiol was obtained from Collaborative Biomedical Products (Bedford, MA).

Hormone-dependency of tumor cells Exponentially growing cells were harvested with trypsin-EDTA, washed with PBS and resuspended in Hank's balanced salt solution (HBSS). The cells (5×10^6) were then injected subcutaneously (s.c.) into the left abdominal flanks of 8-week-old athymic nude mice of the CD-1 strain with or without hormonal treatments. Tumor size, measured in 2 dimensions with a slide caliper every week, was estimated from the mean diameter.

Spontaneous invasion and metastasis in nude mice Mice bearing tumors were autopsied 10 weeks after s.c.-injection of PLS cells into the left abdominal flank. Lungs were removed and fixed in Bouin's solution. The numbers of macroscopic lung metastases were determined by counting visible parietal nodules. To determine the extent of skeletal invasion from primary subcutaneous tumors, the femoral muscle, sciatic nerve and femur of each mouse were examined. Skeletal invasion was assessed on the basis of swelling of the hind thigh, with or without hemiplegia. Lung metastasis and local invasion were confirmed by histological examination.

Gelatinase analysis Cultured tumor cells were washed twice with HBSS and then incubated in serum-free RPMI 1640 containing 0.1 mg/ml BSA for 30 h at 37°C. The resultant conditioned medium was concentrated and used for the measurement of gelatinase activity by zymography with gelatin as a substrate as described previously.[17]

Karyotype analysis Chromosome analysis was performed on metaphase cells. Cells were treated with 0.01–0.02 μg/ml colcemid for 2 h and subjected to hypotonic conditions for 30 min, then fixed in methanol : acetic acid (3 : 1). Air-dried slides were G-banded for analysis. At least twenty metaphases were examined for each cell line.

RESULTS

Morphological characteristics The three cell lines showed similar morphological features on plastic, forming typical epithelial monolayers (Fig. 1). Acinar-like structures were seen with the PLS10 and PLS30 cells. Indirect immunofluorescence microscopy using antiserum to keratin demonstrated that all the PLS cells were

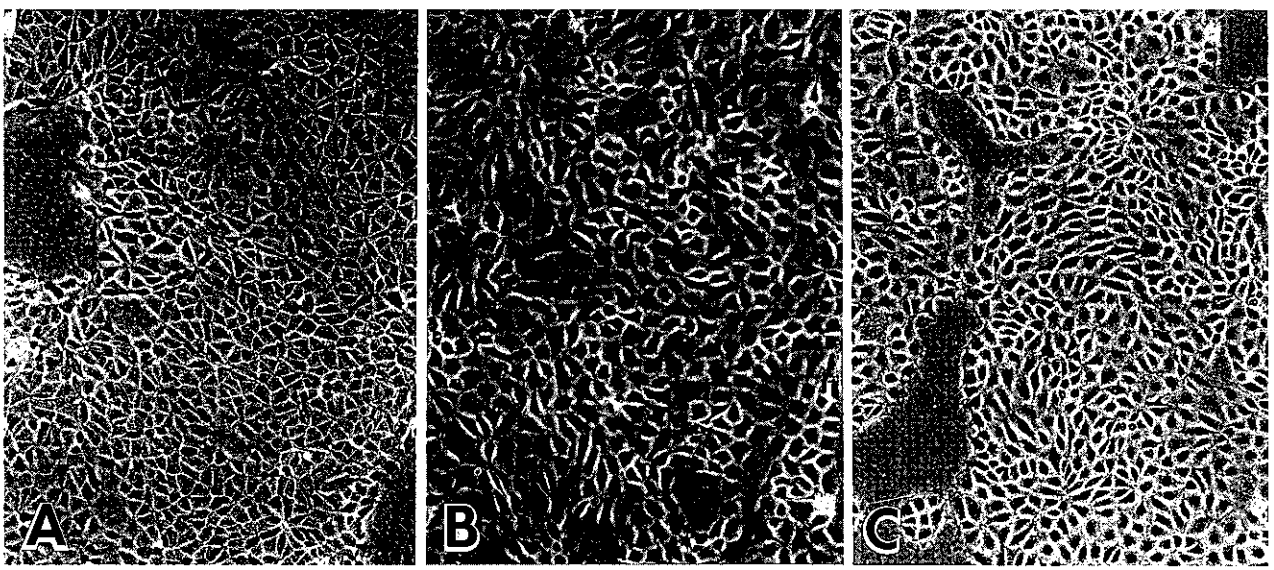

Fig. 1. Morphology of the established PLS10 (A), PLS20 (B) and PLS30 (C) rat prostatic tumor cell lines grown on plastic. All cell lines form typical epithelial monolayers. ×80.

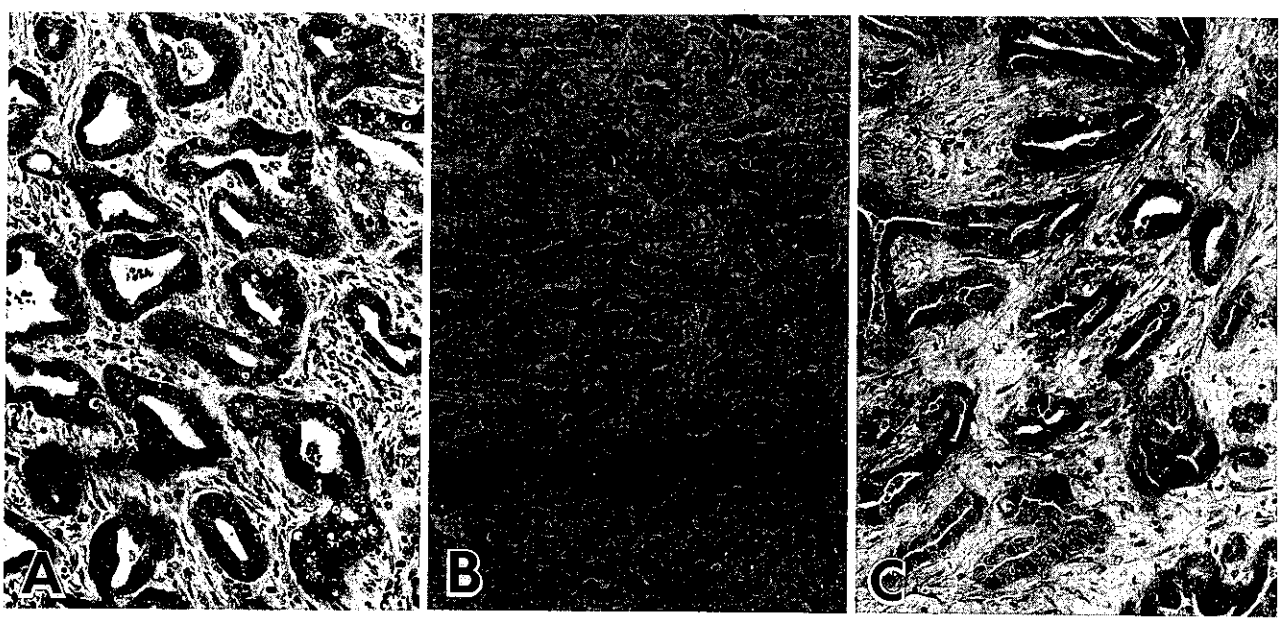

Fig. 2. Histology of primary tumors grown in nude mice after s.c.-injection of PLS10 (A), PLS20 (B) and PLS30 (C) cells. The PLS10 and PLS30 tumors are well-differentiated adenocarcinomas and the PLS20 tumor shows a poorly differentiated carcinoma appearance. H-E ×200.

positive (data not shown). Histologically, the tumors that developed in nude mice after s.c.-injection of cultured PLS10 and PLS30 cells were well-differentiated adenocarcinomas. With PLS20 cells, poorly differentiated adenocarcinoma developed (Fig. 2). All tumors contained abundant fibrous connective tissue, characteristic of prostatic carcinomas. Their histological features were essentially the same as those of the transplantable tumors in nude mice.

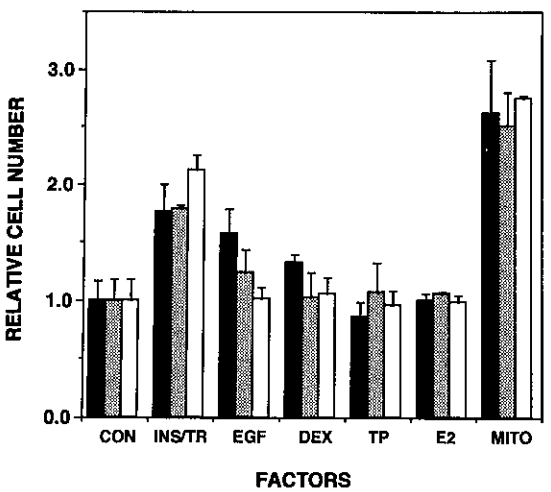

Fig. 3. Growth response of PLS cell lines to growth factors and hormones in culture. PLS10 (■), PLS20 (▨) and PLS 30 (□) cells (1×10^5/35 mm well) were cultured in control medium (CON) or medium supplemented with the following factors: insulin/transferrin (INS/TR), epidermal growth factor (EGF), dexamethasone (DEX), testosterone propionate (TP), 17β-estradiol (E2) and MITO. Cells were counted after 4 days of growth. Values are means ($n=3$)$\pm$SD.

***In vitro* growth characteristics** Growth responsiveness of the cells to hormones and growth factors was examined during early passages. Insulin/transferrin ($P<0.05$), EGF ($P<0.05$), dexamethasone ($P<0.05$) and MITO ($P<0.01$) significantly stimulated the growth of PLS10 cells. In the cases of PLS20 and PLS30, only insulin/transferrin ($P<0.05$) stimulated the cell growth significantly (Fig. 3). After an additional 10–20 passages, the PLS 10 cell line was still stimulated by insulin/transferrin, but had become refractory to EGF and dexamethasone (data not shown). A similar result was obtained when DMEM medium containing 0.5% FBS was used as the control medium. bFGF, testosterone propionate, dihydrotestosterone and 17β-estradiol alone had no effect on the growth of these cell lines, irrespective of the passage number.

Hormone-dependency Growth of PLS10, PLS20 and PLS30 cells in castrated adult male nude mice, castrated male mice given daily testosterone injections, intact female mice and ovariectomized female mice was not significantly different from that in intact male mice (data not shown). Androgen receptors were detected in ventral and dorsolateral lobes of normal rat prostate, but not in any PLS tumor tissues by immunohistochemistry using polyclonal antibody PG21 (Fig. 4). The results thus indicated the PLS cell lines to be androgen-independent.

Tumorigenicity, invasion and metastasis in nude mice As shown in Table I, all the cell lines produced tumors in male nude mice with 100% incidence. Tumor growth rate was significantly higher with PLS20 than with

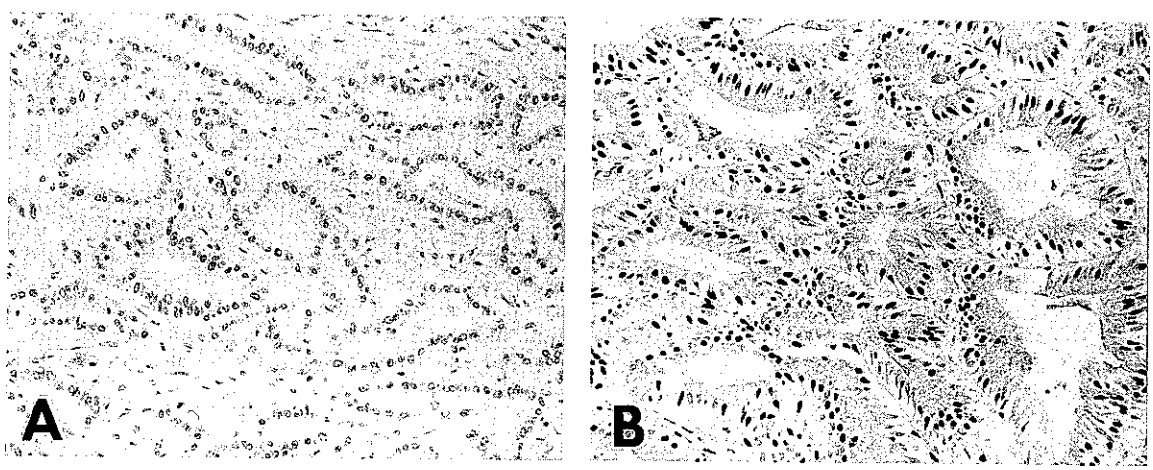

Fig. 4. Androgen receptor immunohistochemistry of subcutaneous tumor in a nude mouse given an s.c.-injection of PLS10 cells. A, PLS10 tumor, showing no positive reaction to androgen receptor in tumor cells. B, Normal rat prostate (ventral lobe). The nuclei stain positive for androgen receptor. ×200.

Table 1. Tumorigenicity and Potentials for Spotaneous Invasion and Metastasis of PLS10, PLS20 and PLS30 Cells in Male Nude Mice of the CD-1 Strain

Cell line	Tumorigenicity	Spontaneous regression	Lung metastasis		Local invasion[b]
			incidence	mean (range)[a]	
PLS10	11/11	1/11	9/10	15.3 (0–43)	1/10
PLS20	9/9	1/9	8/8	10.8 (1–29)	8/8
PLS30	7/7	1/7	6/6	23.0 (11–35)	0/6

Cells (5×10^6) were injected subcutaneously into the abdominal flanks of male nude mice of the CD-1 strain. Autopsy of mice bearing tumors was performed 10 weeks after injection.
a) Number of nodules per lung.
b) Including invasion of femoral muscle, the sciatic nerve and occasionally the femur.

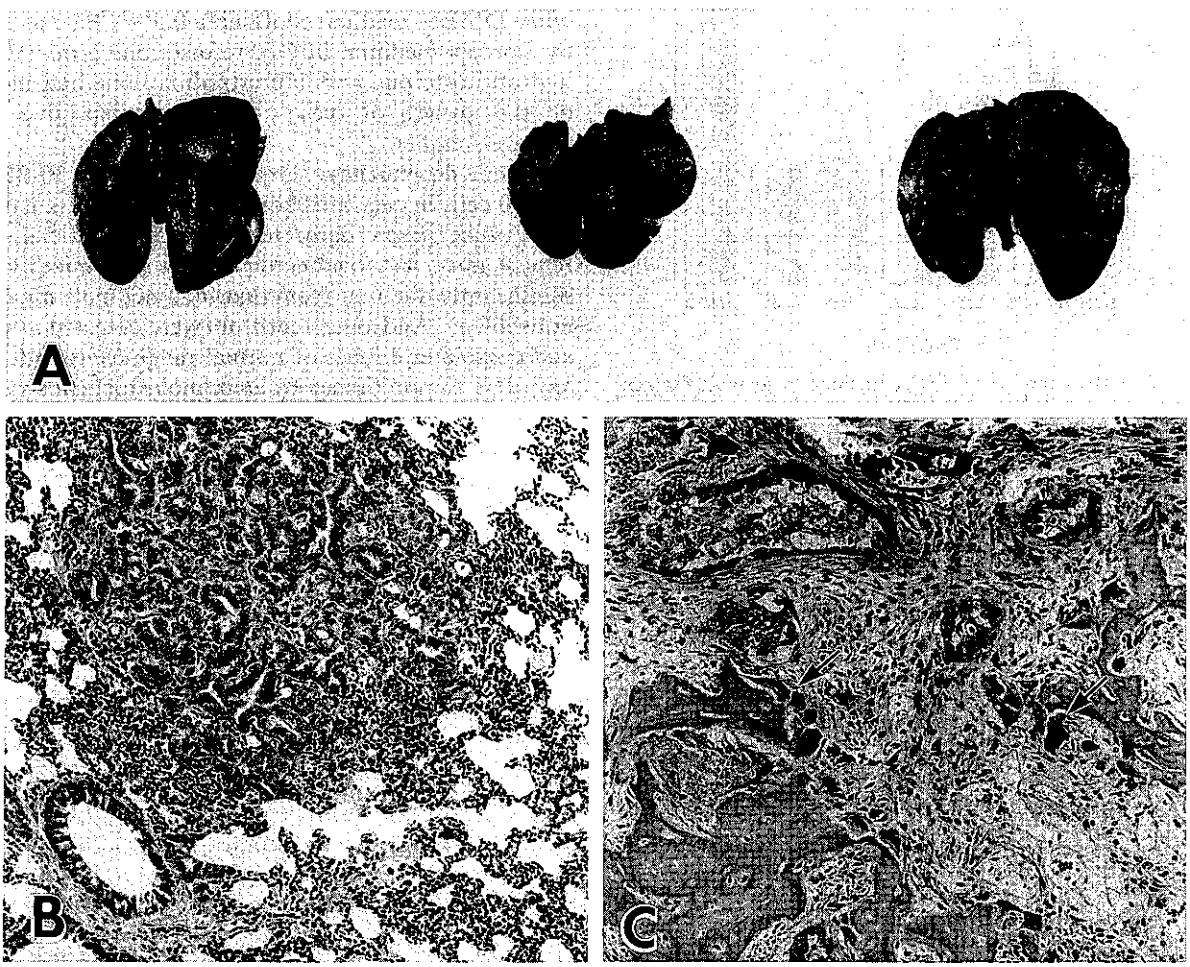

Fig. 5. Lung metastasis and bone invasion in nude mice after s.c.-injection of tumor cells into the abdominal flank. A, Metastatic PLS10 (left), PLS20 (center) and PLS30 (right) tumors in the lungs fixed in Bouin's solution. B, Histology of a metastatic PLS10 tumor in the lung. H-E ×40. C, Histology of a PLS20 tumor infiltrating femoral bone. An osteolytic reaction by osteoclast-like cells is apparent (arrows). H-E ×120.

PLS10 or PLS30 tumors ($P<0.02$, 10 weeks after injection). Spontaneous regression after initial tumor growth was observed for all three lines, ranging from 9–14% in incidence. The PLS tumors produced spontaneous lung metastases in intact male mice at incidences of nearly 100%, with the relative metastatic potentials of the lines

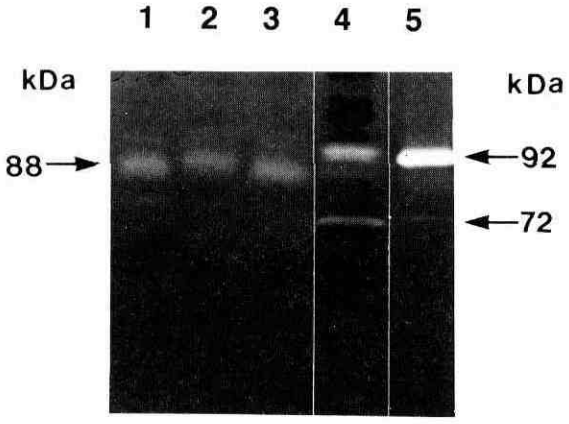

Fig. 6. Zymography of gelatinase activities in serum-free conditioned medium derived from cultured PLS cell lines. Conditioned medium was concentrated 30- to 40-fold and equal amounts, based on cell number equivalents, were electrophoresed in SDS PAGE gels containing gelatin. Gelatinolytic activity is indicated by the white bands where the gelatin has been hydrolyzed. Molecular weights are indicated on the left and right. Lane 1, PLS10; lane 2, PLS20; lane 3, PLS30; lane 4, normal rat serum; lane 5, rat mammary carcinoma cell line (RM22-F5).

being PLS30>PLS10>PLS20 (Fig. 5A). Spontaneous lung metastases were also seen at similar incidences after s.c.-injection into castrated male mice (data not shown). PLS20 tumors demonstrated skeletal invasion including thigh muscle, sciatic nerve at nearly 100% incidence and femoral bone at 38% incidence. The bone lesions resulted from direct and local invasion by subcutaneous tumor and were not distant metastases. Histological features of the lung metastatic nodules and skeletal lesions were essentially the same as those of the respective primary s.c.-tumors (Fig. 5, B and C). In bone lesions infiltrated by PLS20 cells, an osteolytic rather than osteoblastic reaction was observed with activation of osteoclast-like cells being apparent histologically. Distant metastases to other organs such as liver and kidney, as well as lymph node metastasis, were not observed histologically.

Secretion of gelatinase Gelatinase secreted into the conditioned medium by tumor cells was detected using gelatin zymography. Two major gelatin-degrading activities were observed, corresponding to molecular weights of 92 kDa and 88 kDa. These bands were abolished by addition of EDTA to the reaction buffer (data not shown), confirming that the activity was due to metalloproteinase. These species were respectively identical to the inactive and active forms of gelatinase B (MMP-9) present in normal rat serum. The gelatin-degrading activity at 72 kDa was almost negligible. A low-metastatic rat mammary carcinoma cell line (RM22-F5) contained gelatinase B proenzyme. The active form of gelatinase B, however, was almost undetectable in this cell line, unlike the PLS cases (Fig. 6).

Karyotype analysis PLS10 cells were diploid and 42 XY, without any nonrandom structural abnormalities (Fig. 7A). PLS20 cells had a hypertetraploid chromosome number with a modal number of 94 chromosomes. Chromosomes were generally present in 4 copies but many karyotyped cells lacked 4 copies of chromosome 5 and 2 of chromosomes 11, 12, 19 and 20, and 1 Y chromosome. Most cells contained many derivative chromosomes whose origin could not be identified (Fig. 7B). In PLS30 cells, the chromosome count was hyperdiploid with a modal chromosome number of 46. Three extra chromosomes 1, 19 and X and structural rearrangements of chromosomes 1, 3 and 11 were observed (Fig. 7C).

DISCUSSION

The reported human prostatic cancer cell lines are limited in number and they only metastasize spontaneously with a low incidence.[3-5] One variant cell line isolated from a secondary tumor deposit of an androgen-independent PC-3 prostatic cancer cell line, metastasizes to the lung after s.c.-injection at incidences of 30–80%.[18] Androgen-independent sublines derived from LNCaP tumors maintained in castrated mice also metastasize to lymph nodes and bone in 11–50% of cases.[19] In contrast, rat Dunning R3327-AT 3 or 6 sublines exhibit spontaneous metastasis at a 100% incidence with plural lung metastatic nodules per lung, but they are histologically anaplastic.[20] In the present study, we established metastatic carcinoma cell lines from prostatic tumors induced in F344 rat with DMAB and testosterone. They are unique for the following reasons. 1) They are cultured cell lines derived from chemically induced rat prostatic tumors, in contrast to the spontaneous origin of all other rat prostatic cancer cell lines except that established from an MNU-induced prostatic tumor in Lobund/Wistar rat,[21] which was not characterized with respect to metastasis. 2) They are androgen-independent but give rise to differentiated adenocarcinomas, unlike the Dunning R3327 model. 3) They are highly metastatic with a 100% incidence of lung metastasis and an average metastatic nodule count of 10–25 per lung after s.c.-injection, making them suitable for quantitative studies. 4) At least one (PLS20) exhibits a more aggressive phenotype, with rapid growth and skeletal invasion, involving muscle, nerve and bone, leading to hemiplegia in mice.

The androgen independence of the PLS cell lines and their transplantable tumors was evidenced by the fact that their growth rates in mice were not affected by castration. The lack of detectable androgen receptors in the cell lines and transplantable tumors is in line with

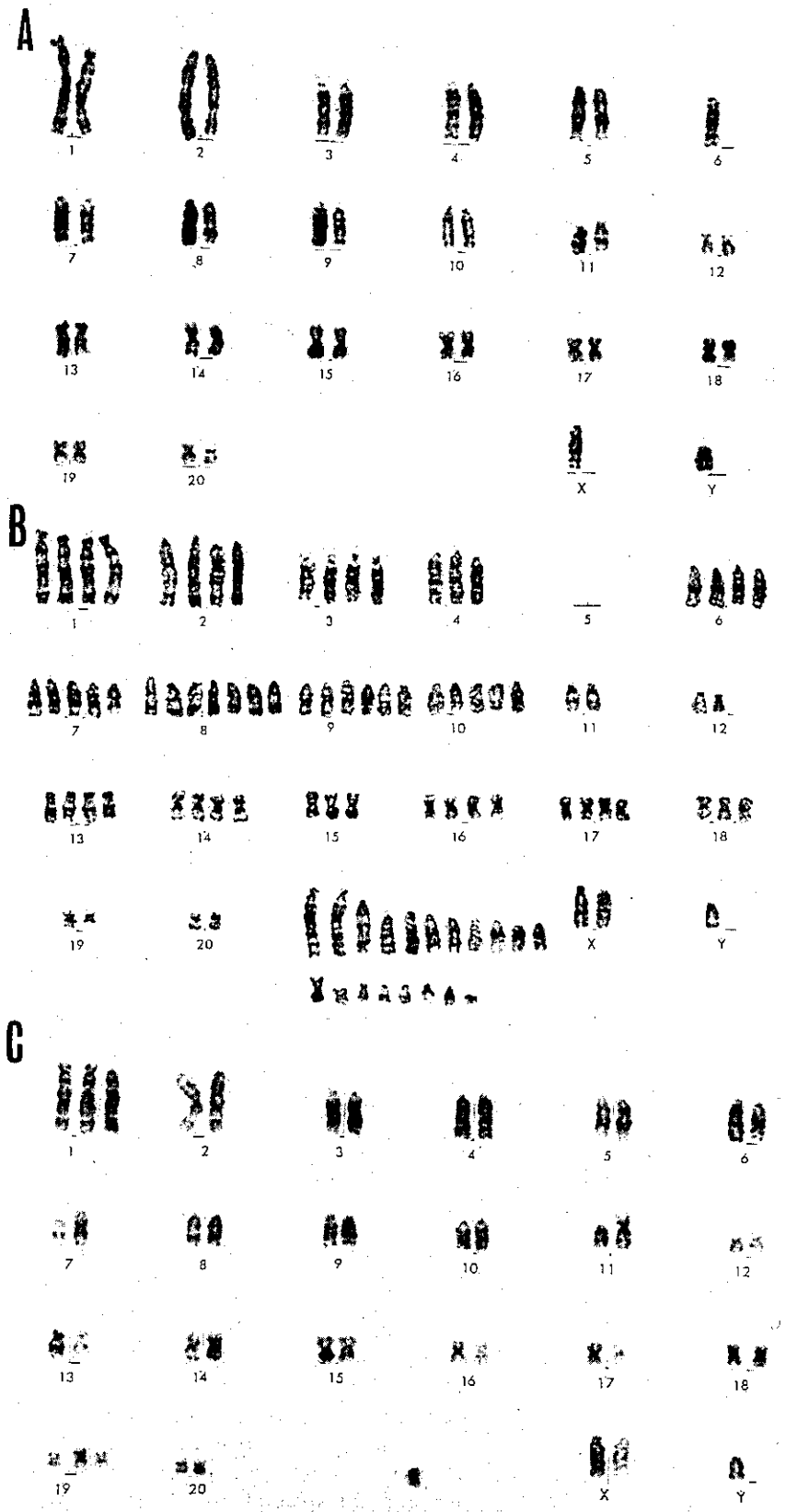

Fig. 7. Karyotypes of PLS10, PLS20 and PLS30 cells. A, PLS 10 cells, 42 XY. B, PLS20 cells, 94 XXY with multiple numerical and structural abnormalities. C, PLS30 cells, 46 XXY with three extra chromosomes and structural rearrangements.

these findings. Although testosterone is essential for induction of primary dorsolateral prostate tumors by DMAB, androgen receptors are rarely detectable in these carcinomas developing in rats.[16] Thus, progression from androgen-dependent to an androgen-independent form may not occur during serial transplantation or culture but rather at an early stage of prostate carcinogenesis in the rats. Rat primary prostatic epithelial cells or non-tumorigenic rat prostatic cell lines require EGF, insulin, dexamethasone and cholera toxin for optimal growth.[21] PLS10 cell lines were also found to be stimulated by insulin/transferrin, EGF and dexamethasone during early passages of culture, but the cells became refractory to EGF and dexamethasone after an additional 10–20 passages. These results indicate that while the PLS cell lines were androgen-independent from the start, they became more growth factor-insensitive to acquire growth autonomy with time.

The well-differentiated, low-invasive PLS10 and 30 cell lines were diploid and hyperdiploid, while the poorly differentiated and highly invasive PLS20 cell line was found to be hypertetraploid and to demonstrate a number of structural abnormalities. The results suggest that rat prostatic adenocarcinomas start development as diploid lesions with cytogenetically normal karyotypes, and subsequent development of hyperdiploidy and hypertetraploidy occur during *in vivo* passage of tumors, as reported for MNU-induced rat mammary carcinomas.[22] The results are also suggestive of an association between tetraploidization of karyotypes and acquisition of a more malignant phenotype in the PLS20 cell line. Thus, the present cell lines might be considered as representing different stages in tumor progression. Dunning R3327 tumors show remarkable instability in their biological phenotypes during serial transplantation.[23, 24] DMAB plus testosterone-induced prostatic tumors in F344 rats are also relatively unstable in terms of their biological phenotype and tend to progress to more aggresive forms under restrained growth conditions both *in vitro* and *in vivo*.

To cast light on the mechanisms responsible for metastasis to the lung, we examined the secretion of gelatinases A and B, which are considered to play important roles in tumor invasion and metastasis.[25, 26] We found that PLS cell lines constitutively secrete the active form of gelatinase B rather than the proenzyme form. This unique feature appears to be specific to highly metastatic PLS cell lines, among the rat cell lines studied. Low-metastatic rat mammary carcinoma cell lines exclusively secrete gelatinase B proenzyme, and non-metastatic mammary carcinoma and normal rat kidney cell lines predominantly secrete gelatinase A proenzyme (data not shown). Thus, secretion of the active form of gelatinase B may be correlated with the metastatic ability of the cells. It is possible that unusual activation of gelatinase B secreted by PLS cell lines *in vivo* may be mediated by a serine protease pathway such as that involving plasmin or by metalloproteinases including stromelysin-1 and gelatinase A produced by tumor cells themselves. The role of the latter two metalloproteinases, however, would appear to be limited because their activities in the PLS cell lines were found to be very weak or absent, as demonstrated by casein and gelatin zymography, respectively. It has been reported that gelatinase B activity is increased in human prostatic carcinomas as compared with benign prostatic hypertrophy.[27] Gelatinase B activity has also been demonstrated in Dunning rat prostatic tumors.[28] The precise role of gelatinase B in metastasis of human and rat prostatic carcinomas, however, remains unclear. The presently described PLS cell lines may therefore, be useful tools for understanding the role of gelatinase B in metastasis of prostatic carcinoma.

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