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Tumorigenic Transformation of Rat Bone Cells by Benzo(a)pyrene: An *In Vitro* Osteosarcoma Model

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Bone is a specialized connective tissue composed of intercellular calcified material, bone matrix and several types of cells. These mineralized, cellular structures are richly supplied with blood vessels and nerves. To investigate the possibility of tumorigenic conversion of normal bone cells by tobacco-related chemical carcinogens, primary normal rat osteoblast-enriched cells were exposed to benzo(a)pyrene, a tobacco-related chemical carcinogen, alone or in conjunction with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Chemically treated (PTRCC-BaP) and chemically transformed cells (RCC-BaP and RCC-BaP-TPA) were established and their biological properties, *in vitro* and *in vivo* tumorigenicity, and expression of c-*myc*, c-*fos*, c-*jun*, p53, and Rb were studied. The chemically transformed cells demonstrated anchorage-independent growth and developed tumors in nude mice. They showed higher proliferation rate than normal cells and morphological alteration from normal cells. The chemically treated and chemically transformed cells possessed high alkaline phosphatase activities and a low acid phosphatase/alkaline phosphatase ratio, and expressed osteonectin protein. They expressed higher levels of c-*myc* transcripts compared to the parental cells. However, the levels of c-*jun* and c-*fos* transcripts were notably decreased in chemically transformed cells and these cells also contained lower levels of p53 and Rb proteins than normal cells. These results indicate that malignant transformation of rat bone cells can be induced by benzo(a)pyrene and that overexpression of c-*myc* messages, together with the downregulation of p53 and Rb proteins, may be associated with malignant progression of rat bone cells.

Key words : osteoblast-enriched cells, benzo(a)pyrene, malignant transformation, proto-oncogenes, tumor suppressor genes

Introduction

Tobacco is associated with cellular DNA damage and epidemiological studies support the contention that tobacco causes oral cancer in humans (Winn *et al.*, 1981; Talamani *et al.*, 1990; Stich *et al.*, 1992). The constituents of smoked and smokeless tobacco responsible for oral cancer are tobacco-specific *N*-nitrosamines and benzo(a)-pyrene [B(a)P] (Park *et al.*, 1992). Benzo(a)pyrene, a polycyclic aromatic hydrocarbon (PAH), is a potent chemical carcinogen to which humans are routinely exposed (Hutcheon *et al.*, 1983; Harvey, 1991) and a ubiquitous environmental pollutant whose metabolites are mutagenic and carcinogenic in a variety of biological systems.

PAH is composed of fused benzene rings that are essentially water insoluble and non-toxic in biological systems. However, it is widely accepted that the metabolic activation of PAHs to highly electrophilic species and their subsequent covalent interactions with target cell DNA result in mutations in cellular genome(s) (Conney, 1982).

Bone is a specialized connective tissue composed of intercellular calcified material, bone matrix and several types of cells. These mineralized, cellular structures are richly supplied with blood vessels and nerves (Matthews, 1980; Junqueira *et al.*, 1995), and a dynamic tissue of which formation and resorption are maintained throughout life. Generally, serum ions are readily accessible to the fluids surrounding bone mineral. This is evidenced by the rapid isotopic exchange that occurs when a radioisotope tracer such as ^{45}Ca is used, indicating that bone has a rich and extensive blood supply. Osteoblasts are mesenchymal in origin, normally

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reside in bone and are responsible for osteogenesis. This osteogenetic process is executed through the synthesis and secretion of an organic matrix that subsequently undergoes calcification by deposition of crystals of calcium and phosphate (Wong, 1992).

Carcinogenesis results from accumulation of carcinogen-induced genetic and epigenetic damages in susceptible cells. The susceptible cells gain a selective growth advantage and undergo clonal expansion by activation of proto-oncogene or inactivation of tumor suppressor genes during the carcinogenesis (Harris, 1991). In several types of bone tumors, amplification of c-*myc* that plays an important role in regulation of the cell cycle was detected (Barrios *et al.*, 1993; Barrios *et al.*, 1994). Bone tumors, especially osteosarcoma, have high alkaline phosphatase activity and produce osteoid (Cole and Cohen Jr., 1992).

There is evidence that involvement of the retinoblastoma (Rb) gene is important in the development of osteosarcoma and other bone tumors (Friend *et al.*, 1986; Araki *et al.*, 1991; Honoki *et al.*, 1993). Frequently, these tumors have been associated with rearrangements in the Rb gene such that the Rb gene products are lost (Friend *et al.*, 1986), and patients with hereditary retinoblastoma appear to have a particularly high risk of osteosarcoma (Draper *et al.*, 1986). Recently, many studies have indicated that mutations in p53 tumor suppressor gene are the most frequently found genetic disorders in most human cancers, and that mutant p53 exertes a dominant oncogenic function during *in vitro* transformation (Eliyasu *et al.*, 1989; Finlay *et al.*, 1989). The human p53 gene is located on the short arm of chromosome 17, a frequent site of allele loss in osteosarcomas (McBride *et al.*, 1986), and several reports have demonstrated that bone tumors are associated with changes in the p53 gene (Masuda *et al.*, 1987; Ehrhart *et al.*, 1988; Romano *et al.*, 1989; Ueda *et al.*, 1993). Previous reports assume that chemical carcinogens participate in osteosarcoma oncogenesis in humans (Rhim and Dritschilo, 1991). However, despite extensive studies, no apparent cause for this tumor has been identified.

In the present study, to investigate the possibility of tumorigenic conversion of normal bone cells by tobacco-related chemical carcinogens, primary normal rat osteoblast-enriched cells were exposed to benzo(a)pyrene, a tobacco-related chemical carcinogen, alone or in conjunction with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Chemically treated (PTRCC-BaP) and chemically transformed cells (RCC-BaP and RCC-BaP-TPA) were established and their biological properties, *in vitro* and *in vivo* tumorigenicity, and expression of c-*myc*, c-*fos*, c-*jun*, p53 and Rb were studied.

Materials and Methods

Culture of primary normal rat calvarial cells (NRCC)

Sprague-Dawley rats (Animal Laboratory, College of Medicine, Seoul National University), which were 19-days pregnant, were anesthetized with sodium pentobarbital (30 mg/kg) intraperitoneally. The fetuses were removed and the skins were separated from the cranium of the fetuses. The portions of calvaria containing frontal and parietal bone were separated from the brain. Blood residue was removed by repeated washing with calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS, GibcoBRL Life Technologies Inc., Gaithersburg, MD) and the periosteum was removed with an ophthalmic pincette. The calvaria samples were transferred to a Reacti-vial (10 calvariae/vial; Pierce, Rockford, IL) containing 1.5 ml of 0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA. Incubation with continuous and slow stirring was carried out in a 37°C incubator. After 10 min, the supernatant containing the cells was transferred to a 15 ml conical tube and added to an equal volume of ice-cold fetal bovine serum (FBS, GibcoBRL Life Technologies Inc.) to stop the enzyme activity. This was labeled population I.

Fresh enzyme was added to the remaining bone chips and the digestion continued for a further 10 min and population II was obtained. A third 10-min digestion yielded population III. Populations IV and V were harvested after two subsequent 20-min digestion periods. Cell populations isolated in this manner were centrifuged at 300×g for 6 min, resuspended in minimum essential medium (MEM, GibcoBRL Life Technologies Inc.) supplemented with 10% heat-inactivated FBS and antibiotics and plated at 60-mm Petri dish. All cells and cell lines were maintained in MEM supplemented with 10% FBS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were plated at 3×10^5 cells per 60-mm Petri dish and grown for the various assays.

Tumorigenic transformation of cells to B(a)P, alone or in conjunction with TPA

The primary NRCC (population V) were plated at 3×10^5 cells per 60-mm Petri dish. When the cultures reached 70% confluency, they were exposed to B(a)P (0.5 μg/ml; Sigma Chemical Co., St. Louis, MO) for 20 weeks. These chemically treated cells were designated PTRCC-BaP. The primary NRCC (population V) were also exposed to B(a)P (0.5 μg/ml) for 40 weeks. The primary NRCC were exposed to B(a)P (0.5 μg/ml) for 20 weeks, and then further exposed to B(a)P (0.5 μg/ml) and TPA (1.0 μg/ml; Sigma Chemical Co.) for 20 weeks. These cells were named RCC-BaP and RCC-BaP-TPA, respectively. Benzo(a)pyrene and TPA were dissolved in DMSO (Sigma Chemical Co.) before dilution in MEM. The final concentration of DMSO in culture medium was 0.05%.

Light microscopic and ultrastructural examinations of cells

The cultures were washed twice with CMF-HBSS and fixed *in situ* with 2.5% glutaraldehyde solution for 1 h at room temperature. After washing twice with 0.1 M cacodylate buffer, the microscopic features of the cells were photographed.

The cultures were washed twice with CMF-HBSS, scraped out with a policeman and collected in a glass vial. They were immediately fixed in 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, pH 7.4. They were then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated in a graded series of ethanol and embedded in Epon 812 using propylene oxide as a miscible intermediate. Ultrathin sections were stained with uranyl acetate and lead citrate, and were examined with an electron microscope (1200EXII, JEOL, Japan).

Determination of cell proliferation rate

Confluent cells in 100-mm Petri dishes were trypsinized and counted. The cells were suspended in MEM supplemented with 10% FBS and 3×10^5 cells were plated onto 60-mm Petri dishes. The number of viable cells after trypan blue exclusion was counted after 1, 2, 3, 4, 5 and 6 days of incubation at 37°C. There were four cultures in each group at each time.

Determination of the ability for soft agar colony formation

Anchorage-independent growth was assayed for their ability to grow on semisolid agar by the modified method of Macpherson (1970). Two ml of a mixture of 2X medium and 1.0% Noble agar (Difco Laboratories, Detroit, MI) were poured into a 60-mm Petri dish and allowed to gel. The basal layer was overlaid with 1,000 viable cells suspended in an equal volume of 2X medium containing 40% FBS and 0.6% Noble agar. The Petri dishes were then incubated at 37°C for 3 weeks. Colonies being larger than 50 μm in diameter were examined and counted with the aid of an inverted microscope.

Determination of in vivo tumorigenicity of cells

The *in vivo* tumorigenicity of cells was determined in nude mice. Monolayer cultures were trypsinized and resuspended in culture medium and 0.1 ml of phosphate-buffered saline containing 1×10^7 cells were injected subcutaneously into athymic nude mice (*nu/nu*; UCLA Nude Mice Facility, Los Angeles, CA) at 24 h after X-irradiation (300 rads). All mice received injections in the right flank and were monitored twice weekly for the appearance of tumors over a period of 3 months. Tumors that developed were excised for histological examination.

Determination of acid and alkaline phosphatase activities

Monolayer cultures (1×10^6 cells) were trypsinized, harvested, and homogenized by sonication in 0.5 ml of distilled water. By the modified method of Bessy *et al.* (1946), the activity of phosphatase was assayed in a reaction mixture composed of 15 mM *p*-nitrophenyl phosphate, 0.1 M sodium citrate buffer, pH 4.8 (acid phosphatase) or 0.1 M glycine-NaOH buffer, pH 10.4 (alkaline phosphatase), 0.1 ml of 0.1% Triton X-100 in saline, and 0.2 ml of the homogenate, in a final volume of 0.6 ml. The enzymatic reaction was allowed for 30 min in a water bath adjusted to 37°C and stopped by adding 2.5 ml of 0.1 N sodium hydroxide. The content of *p*-nitrophenol in the resulting supernatant was monitored at 410 nm. Protein content was measured by the method of Lowry *et al.* (1951), and bovine serum albumin was used as a reference standard.

Northern analysis

To determine the transcription of c-*myc*, c-*fos*, c-*jun*, p53, Rb and β-actin genes, cytoplasmic poly

(A^+)RNA was extracted from cells using standard procedures. Probes used for northern analysis were as follows: v-*myc* cDNA (ATCC, Rockville, MD), v-*fos* cDNA (ATCC), v-*jun* cDNA (ATCC), p53 cDNA (from Dr. E. Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA), Rb cDNA (from Dr. S. Friend, Massachusetts General Hospital Cancer Center, Charlestown, MA) and human β-actin cDNA (from Dr. L. Kedes, Stanford University, Palo Alto, CA). All were labeled with [^{32}P] dCTP (Amersham Corp., Arlington Heights, IL) by megaprime labeling (Amersham Corp.). Specific radioactivities of labeled probes were always higher than 5×10^8 cpm/μg of DNA. Five μg of poly (A^+)RNAs were denatured and run on a 1.2% formaldehyde agarose gel with marker RNAs (9.5-, 6.2-, 3.9-, 2.8-, 1.9-, 0.9-, 0.6- and 0.4-kb RNA marker, International Biotechnologies, Inc., New Haven, CT). The RNAs were transferred to nylon filters (Amersham Corp.) and crosslinked with ultraviolet light for 5 min. The filters were hybridized to [^{32}P]-labeled probe for 24 h at 42°C in 50% formamide/10% dextran sulfate/5X SSPE (0.15 M NaCl, 0.01 M Na_2HPO_4 and 0.001 M EDTA)/5X Denhardt's solution/denatured salmon sperm DNA (20 μg/ml). Filters were washed twice in 5X SSPE for 15 min at 42°C, then in 1X SSPE/0.1% SDS for 30 min at 42°C and finally in 0.1X SSPE/0.1% SDS for 15 min at room temperature. Filters were then autoradiographed on Hyperfilm-MP (Amersham Corp.) for 12 h at -70°C. After exposure, the probe was stripped off the filter for rehybridization to the next radiolabeled probe.

Western analysis

Cells grown in 100-mm Petri dishes were lysed in a lysis buffer [10 mM Na_2HPO_4 (pH 7.2), 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% sodium azide and 0.004% sodium fluoride] on ice for 15 min. The cell lysate was centrifuged at 15,000×g for 20 min and the supernatant containing 1 mg/ml of protein was denatured by boiling for 3 min in a sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.001% bromophenol blue]. An aliquot of the denatured supernatant containing 100 μg of protein was electrophoresed in a 10% SDS-polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore Corp., Bedford, MA). After incubation in a blocking buffer [0.2% I-block™ (Tropix Inc., Bedford, MA), phosphate buffered saline and 0.05% Tween 20] for 3 h at room temperature, the membrane was exposed to mouse anti-human osteonectin monoclonal antibody (Biodesign International, Kennebunk, ME) and mouse anti-mouse monoclonal antibody for p53 (PAb240) or Rb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at room temperature for 1 h. After washing with the blocking buffer, the membrane was treated with anti-mouse IgG-alkaline phosphatase conjugate antibody (Tropix Inc.) and again washed with the blocking buffer and an assay buffer (0.1 M diethanolamine and 1 mM $MgCl_2$). The membrane was then incubated in Nitroblock Reagent (Tropix Inc.), washed again with the assay buffer and incubated in chemiluminescent substrate solution using the Western-Light kit (Tropix Inc.). The membrane was exposed to Hyperfilm-MP for 4 min at room temperature.

Results

Morphology, ultrastructural finding, and proliferation rate

The PTRCC-BaP cells were morphologically similar to its normal counterpart, but the RCC-BaP and RCC-BaP-TPA cells showed morphological alterations from its normal counterpart (Fig. 1). Primary NRCC (population V) were pleomorphic in shape, and the rather invaginated, euchromatin-rich nuclei with marginated nucleoli were eccentrically located. The cytoplasm had well-developed rough endoplasmic reticulum with some dilation. Round or oval mitochondria were scattered throughout the cytoplasm. The Golgi apparatus was less prominent and was situated near the cytocenter of the cells. The cytoplasm contained a few vacuoles that varied in size. Many finger-like microvilli on the cell surface were observed (Fig. 2A).

The PTRCC-BaP cells were pleomorphic, and had a euchromatin-rich nucleus situated eccentrically. The cytoplasm showed a moderate amount of mitochondria and Golgi apparatus. These cells had well-developed rough endoplasmic reticulum with microcystic dilation and were characterized by many bulbous microvilli on the cell surface (Fig. 2B).

The RCC-BaP cells had a eccentric nucleus with large nucleoli. In the cytoplasm, well-developed free polyribosomes and rough endoplasmic reticulum were visible. Some cells showed numerous

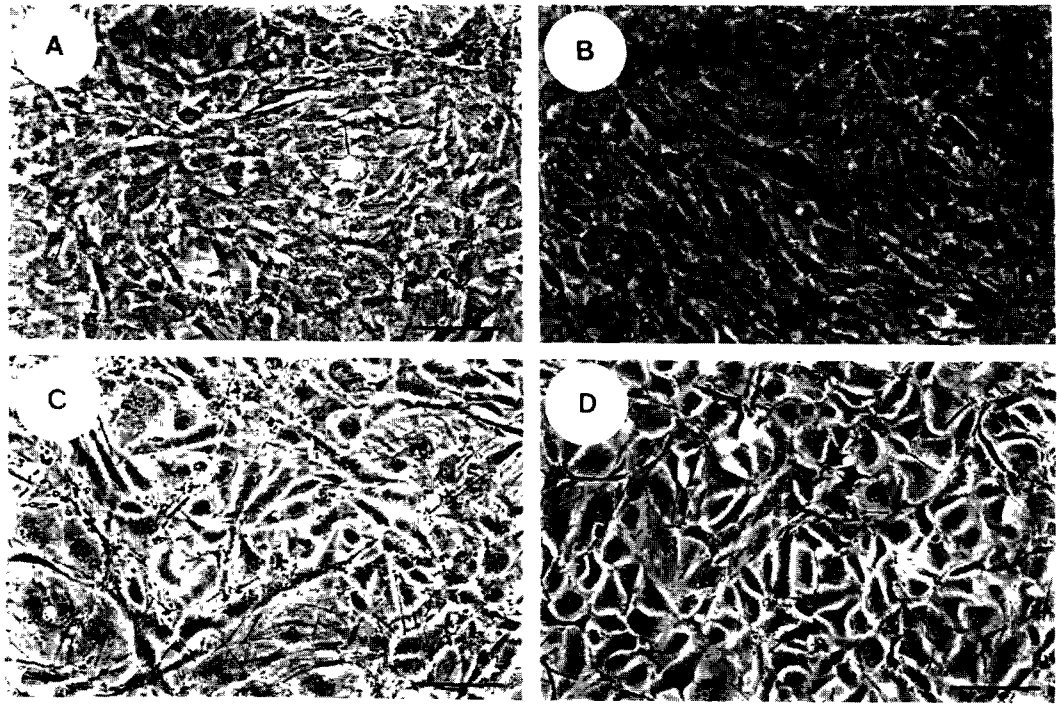

Fig. 1. Microscopic features of NRCC (**A**), PTRCC-BaP (**B**), RCC-BaP (**C**), and RCC-BaP-TPA cells (**D**). The cells were plated and grown to confluence. The chemically transformed cell lines RCC-BaP and RCC-BaP-TPA showed morphological alterations from their parental counterpart, NRCC. Bar length=100 μm.

vacuoles and dilation of rough endoplasmic reticulum (Fig. 2C).

The RCC-BaP-TPA cells contained a rather invaginated, euchromatin-rich nucleus with large nucleoli situated eccentrically. The cytoplasm had well-developed rough endoplasmic reticulum. The Golgi apparatus was not as well-developed (Fig. 2D). However, in spite of careful examination there was much difficulty in drawing meaningful

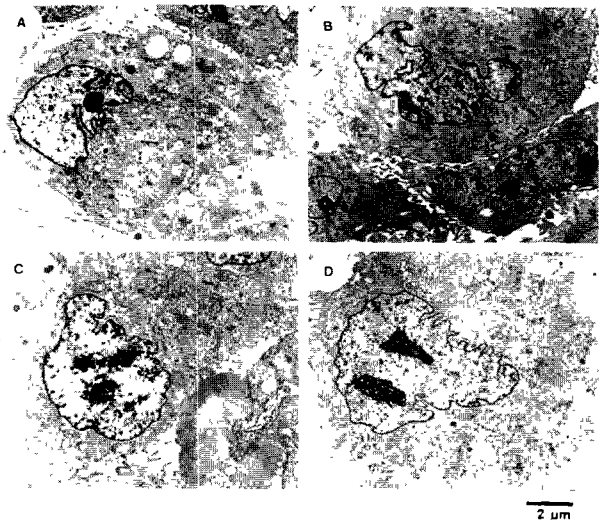

Fig. 2. Ultrastructural findings of NRCC (**A**), PTRCC-BaP (**B**), RCC-BaP (**C**), and RCC-BaP-TPA cells (**D**) as shown by transmission electron microscopy.

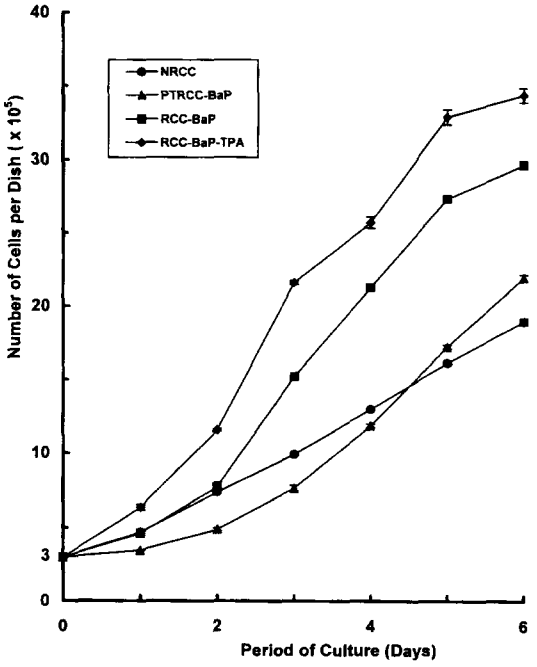

Fig. 3. Growth curves of NRCC, PTRCC-BaP, RCC-BaP and RCC-BaP-TPA cells grown in MEM supplemented with 10% FBS.

positive correlations between the ultrastructural characteristics of the cells and the degree of their tumorigenicities.

The chemically transformed cells proliferated well in MEM supplemented with 10% FBS. Population doubling times of the NRCC, PTRCC-BaP, RCC-BaP, and RCC-BaP-TPA cells in MEM were approximately 44, 39, 32 and 27 h, respectively (Fig. 3).

Acid and alkaline phosphatase activities and its ratio

To characterize the nature of the cell populations isolated from fetal rat calvaria, acid and alkaline phosphatase activities and its ratio were studied. Cells released early during the digestion period (populations I and II) have been shown to possess the high acid phosphatase activity and low alkaline phosphatase activity usually attributed to osteoclasts, whereas those released in the later stages of digestion (populations IV and V) have been shown to have low acid phosphatase activity and high alkaline phosphatase activity (Fig. 4A). All cells derived from population V by treatment of chemicals have been shown to possess low acid phosphatase activity and high alkaline phosphatase activity (Fig. 4B) and the very low acid

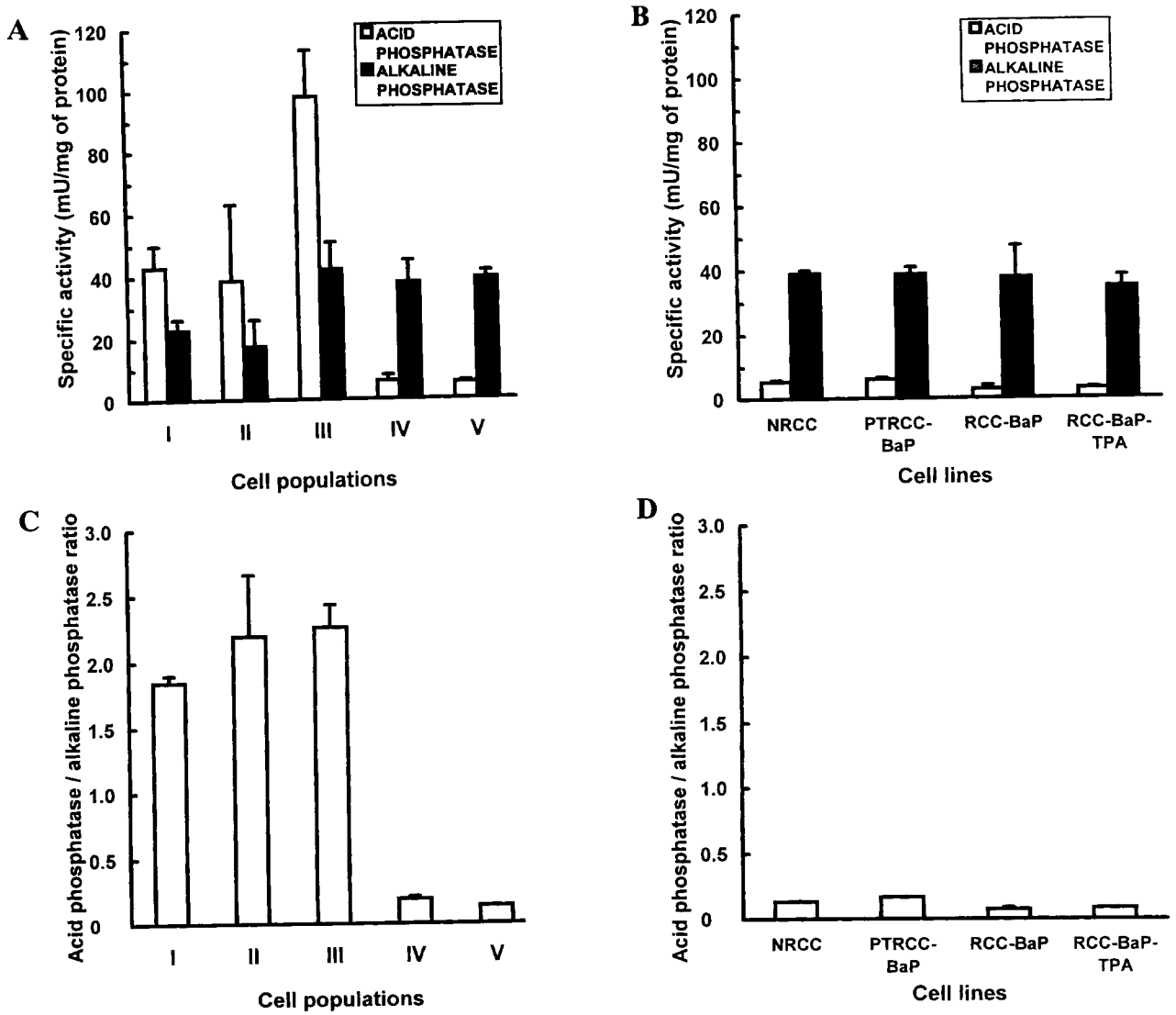

Fig. 4. Activities of acid phosphatase and alkaline phosphatase in rat calvarial cell populations (**A**) and cell lines (**B**). Acid phosphatase/alkaline phosphatase ratio in rat calvarial cell populations (**C**) and cell lines (**D**). Enzyme activity was assayed with 15 mM *p*-nitrophenyl phosphate in 0.1 M sodium citrate buffer, pH 4.8 (acid phosphatase) and 0.1 M glycine-NaOH buffer, pH 10.4 (alkaline phosphatase) at 37°C. The bars represent the average±standard deviation of the values obtained from 6 experiments. One unit of enzyme activity was defined as that amount of enzyme which catalyzes the transformation of 1 mol of substrate per min at 37°C.

phosphatase/alkaline phosphatase ratio (Fig. 4D) usually attributed to osteoblastic cells. However, cells released early during the digestion (populations I-III) showed a markedly higher ratio of acid phosphatase/alkaline phosphatase than those in the cell populations IV and V (Fig. 4C).

Expression of osteonectin protein

To characterize the nature of the PTRCC-BaP, RCC-BaP and RCC-BaP-TPA cells, expressions of osteonectin, a bone-specific protein, were studied. The level of osteonectin protein in NRCC cells were higher than those of cells exposed to chemicals. The other cells also expressed osteonectin proteins (Fig. 5), suggesting that the PTRCC-BaP, RCC-BaP and RCC-BaP-TPA cells may originate from osteoblasts.

In vitro and in vivo tumorigenicity

NRCC and PTRCC-BaP cells did not proliferate in the semi-solid soft agar, indicating the inability of anchorage independency of the cells. The RCC-BaP and RCC-BaP-TPA cells, however, grew in the soft agar with colony-forming efficiencies of 1.0 to 2.6%, indicating the ability of anchorage independency of the cells (Table 1). Further, 100% of mice receiving the RCC-BaP and RCC-BaP-TPA cells developed tumors (Table 1). Tumors began to appear at 3 weeks after injection, followed by a rapid growth. Microscopically, the tumor consisted of spindle-shaped cells arranged in a sheet without any polarity. Tumor cells were characterized by bizarre and pleomorphic cells with hyperchromatic nuclei. There were many intracytoplasmic fat vacuoles and numerous mitotic figures, often abnormal. They did not have fibrotic tissue capsules and showed infiltration to surrounding tissues. They also exhibited a storiform pattern (Fig. 6). However, no tumors developed in animals receiving NRCC or PTRCC-BaP cells.

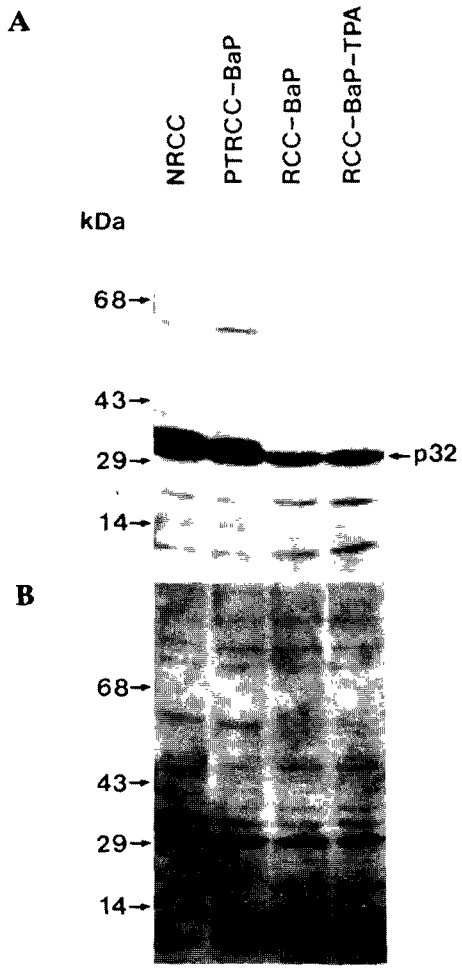

Fig. 5. Western blot analysis of osteonectin protein in NRCC, PTRCC-BaP, RCC-BaP and RCC-BaP-TPA cells (**A**) and total protein levels of the blot with Coomassie Brilliant Blue staining (**B**).

Expression of c-myc, c-jun, and c-fos

Expression of 2.4 kb *c-myc* messages in the PTRCC-BaP cells was higher than that of NRCC, but further increased in the RCC-BaP cell line (Fig.

Table 1. Characteristics of rat calvarial cells transformed by benzo(a)pyrene

Cells or cell lines	Primary	Transformation step	Soft agar colony formation (%)*	Tumorigenicity in nude mice (no. of mice with tumor/no. of mice tested)
NRCC	+		None	0/5
PTRCC-BaP	–	B(a)P	None	0/5
RCC-BaP**	–	B(a)P	1.0	5/5
RCC-BaP-TPA**	–	B(a)P + TPA	2.6	5/5

*Cell suspensions were plated on 0.3% soft agar medium containing 20% fetal bovine serum, and colonies larger than 50 μm in diameter were counted after 21 days incubation at 37°C from 60-mm Petri dishes receiving 1,000 cells each. Three separate experiments were averaged.

**RCC-BaP and RCC-BaP-TPA are chemically transformed tumorigenic cells derived from the NRCC as described in the text.

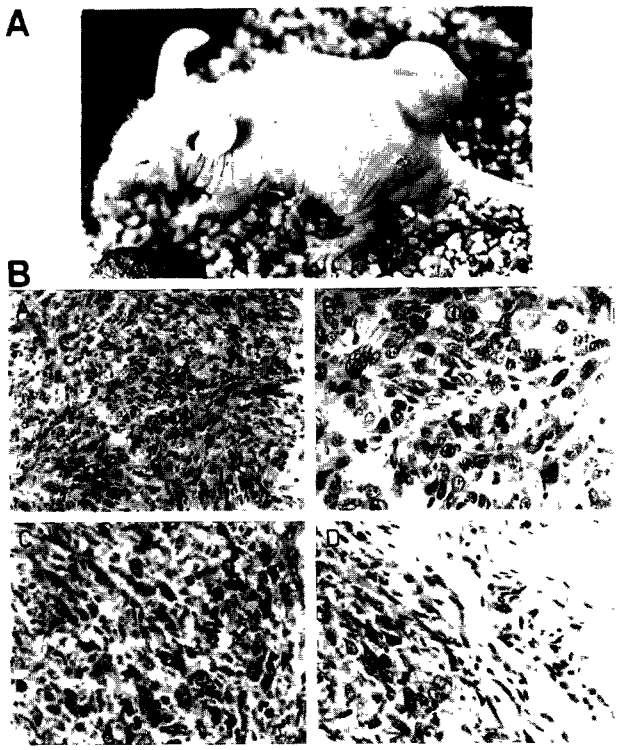

Fig. 6. High-grade osteosarcoma developed subcutaneously in nude mice by RCC-BaP cells. **(A)** All 5 mice injected with 1×10^7 cells developed tumor within 6 weeks. **(B)** Tumor cells were characterized by bizarre and pleomorphic cells (A, X100 magnification; B, X400). Microscopic features of tumor cells with capillaries (C, X 400). Infiltration of tumor cells into mouse skeletal muscle cells (D, X400).

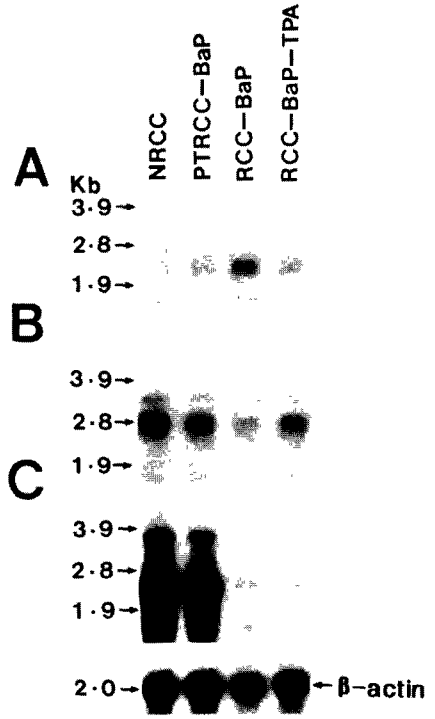

Fig. 7. Transcription of c-*myc*, c-*jun*, and c-*fos* genes from cells grown in MEM supplemented with 10% FBS. **(A)** Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled v-*myc* cDNA; **(B)** Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled v-*jun* cDNA; **(C)** Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled v-*fos* cDNA. The polyadenylated RNAs were electrophoresed, transferred to a nylon filter and hybridized to radiolabeled v-*myc* cDNA. After autoradiography, the probe was stripped off the filter for rehybridization to the next radiolabeled probe.

7A). Since the alteration in c-*myc* mRNA transcripts was observed, we further investigated on the c-*jun* and c-*fos* mRNA levels. All cells expressed two c-*jun* transcripts with sizes of 3.2 kb and 2.7 kb, and c-*fos* with 2.2 kb mRNA transcripts. Interestingly, the amount of the c-*jun* and c-*fos* messages was high in normal and PTRCC-BaP cells, but markedly reduced when the normal cells were transformed to tumorigenic cells (Fig. 7B and C).

Analyses of p53 and Rb

Fig. 8A showed that two p53 transcripts with sizes of 1.9 kb and 1.6 kb were expressed from the chemically treated (PTRCC-BaP) and chemically transformed cells (RCC-BaP and RCC-BaP-TPA), whereas only 1.9 kb p53 transcripts were expressed in NRCC. The chemically treated and chemically transformed cells transcribed notably higher amounts of p53 mRNAs than did NRCC. Western blot analysis showed that p53 protein levels in NRCC were similar to RCC-BaP-TPA cells, but slightly higher than those of the PTRCC-BaP cells (Fig. 8B). Further, the p53 protein expression was almost negligible in the RCC-BaP cells.

The levels of Rb transcripts in the NRCC and PTRCC-BaP cells were similar to each other and significantly higher than those in the RCC-BaP cells. However, RCC-BaP-TPA cells contained similar level of Rb messages when compared to NRCC (Fig. 9A). The levels of $p110^{Rb}$ in the PTRCC-BaP and RCC-BaP cells were similar, but they were markedly lower than that of NRCC. Further, the $p110^{Rb}$ expression was almost negligible in the RCC-BaP-TPA cells (Fig. 9B).

Discussion

These data show that exposure of primary NRCC (population V) to B(a)P, alone or in conjunction

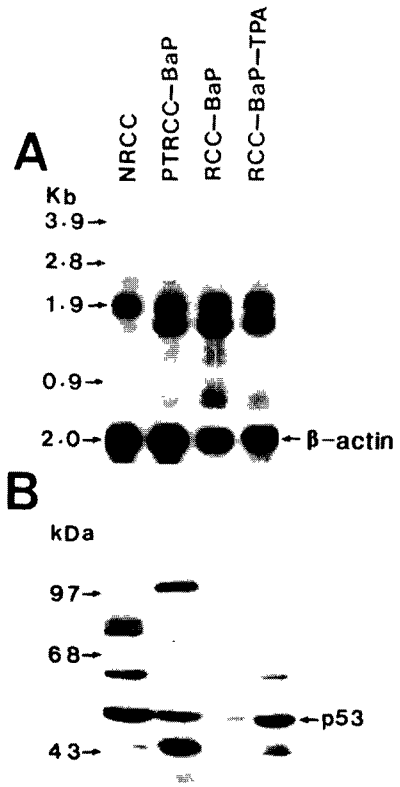

Fig. 8. Expression of p53 in cells or cell lines. **(A)** Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled p53 cDNA; **(B)** Western blot analysis of p53 protein expression from the cells or cell lines.

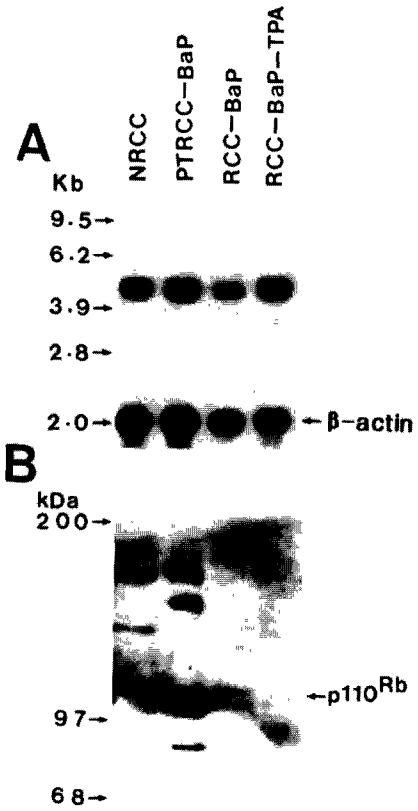

Fig. 9. Expression of Rb in cells or cell lines. **(A)** Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled Rb cDNA; **(B)** Western blot analysis of Rb protein expression from the cells or cell lines.

with TPA, resulted in malignant transformation of cells, evidenced by the ability to grow in soft agar and tumor formation in nude mice. These results indicate that bone cells can be malignantly transformed by exposure of benzo(a)pyrene, a tobacco-related chemical carcinogen. A previous study reported that chemical carcinogens participated in osteosarcoma oncogenesis in humans (Rhim and Dritschilo, 1991).

A mixture of proteolytic enzymes containing collagenase and trypsin is routinely used to release bone cells from rat calvaria (Wong and Cone, 1975; Rao *et al.*, 1977). There is much evidence to support the osteoblastic characteristics of populations IV and V cells. This evidence includes responsiveness to hormones such as parathyroid hormone, calcitonin, and 1,25-dihydroxyvitamin D_3; high alkaline phosphatase activity, low acid phosphatase/alkaline phosphatase ratio (Wong, 1982); synthesis of a type I collagen matrix that has the capacity to mineralize; and synthesis of the bone-associated proteins such as osteocalcin (Hauschka *et al.*, 1975; Price *et al.*, 1976) and osteonectin (Termine *et al.*, 1981). In the present study, primary NRCC (population V) were used in the transformation assay. Primary NRCC, chemically treated and chemically transformed cells have been shown to possess high alkaline phosphatase activity and very low acid phosphatase/alkaline phosphatase ratio. All cells and cell lines also expressed osteonectin protein (Fig. 5), used as a typical marker for osteoblastic phenotypes (Wong, 1982). These results indicate that the PTRCC-BaP, RCC-BaP and RCC-BaP-TPA cells may originate from osteoblasts.

In vivo tumorigenicity study showed that 100% of mice receiving the RCC-BaP and RCC-BaP-TPA cell lines developed tumors. No osteoids or bone-like structures were found in tumors; however, the tumor cells consisted of spindle-shaped cells and were characterized by bizarre and pleomorphic cells with hyperchromatic nuclei. There were many intracytoplasmic fat vacuoles and numerous mitotic figures, often abnormal. They showed

infiltration to surrounding tissues and exhibited a storiform pattern. Most spindle cell sarcomas of soft tissue and bone can be associated with a storiform pattern. Therefore, considering the high alkaline phosphatase activities in the cells, which can be associated with bone cell tumor and expression of osteonectin, the tumors seemed to exhibit a high-grade osteosarcoma histology.

Since the expression of c-*myc* is closely associated with proliferation status of cells (Hirning *et al.*, 1991) and frequently deregulated in cancer cells (Field and Spandidos, 1990; Spencer and Groudine, 1991), the level of c-*myc* transcripts were determined. As might be expected, chemically transformed cells expressed a notably higher level of c-*myc* messages compared to their normal counterpart. This result indicates that overexpression of c-*myc* may be involved in the neoplastic transformation of normal bone cells. The c-*jun* and c-*fos* genes are normal cellular genes that control cell growth, proliferation and differentiation. Furthermore, the c-*fos* gene, when expressed at high levels, transforms fibroblasts and induces tumors in transgenic mice (Miller *et al.*, 1984; Grigoriadis *et al.*, 1993). Contrary to prediction, the levels of c-*jun* and c-*fos* transcripts were notably decreased in chemically transformed cells. The reason for the underexpression of c-*jun* and c-*fos* in these cells is presently unknown, but it is assumed that lower levels of c-*jun* and c-*fos* expression may be associated with diminished differentiation potential of these transformed cells. In fact, downregulation of the c-*fos* expression has been reported in our previous study (Min *et al.*, 1995). Furthermore, an osteosarcoma cell line which was established by treatment of a chemical carcinogen, 4-hydroxyaminoquinoline 1-oxide, in F344 rat *in vivo* showed low levels of expression of c-*fos* and c-*jun* messages (Honoki *et al.*, 1993).

Though molecular biological events underlying cell transformation by chemical carcinogens are largely unknown, it appears that the inactivation of tumor suppressor genes, along with the dominant activation of proto-oncogenes, is involved in tumorigenesis. Among the tumor suppressor genes, the p53 and Rb genes have received the most attention because of their relation to most types of tumor (Kim *et al.*, 1993). Our results showed that the p53 transcripts expressed from NRCC were 1.9 kb, whereas p53 mRNAs expressed from chemically treated and chemically transformed cell lines were 1.9 kb and 1.6 kb. The expression of 1.6 kb mRNA from the cells might be due to failure of the RNA splicing, but the mechanism of generation of 1.6 kb p53 mRNA and its function in these cells are presently unknown. Interestingly, however, p53 protein level in the RCC-BaP-TPA cells were similar to NRCC. Furthermore, p53 protein level in the RCC-BaP cells was almost negligible. Because monoclonal antibody PAb240 detected both wild-type and mutant p53 protein under denaturing conditions, it is presently unknown whether the p53 proteins in chemically treated and chemically transformed cells were wild-type or mutant p53, which might be confirmed by determining the DNA sequence of the p53 gene.

Although the etiology of most common malignant bone tumors remains unknown, previous studies have revealed the relationship between these tumors and the mutation of the Rb gene (Hansen *et al.*, 1985; Toguchida *et al.*, 1988; Araki *et al.*, 1991). Mutations of the Rb gene result in expression of mutant Rb proteins that lack tumor suppressor activity. These mutations are frequently found in retinoblastoma, osteosarcoma, lymphoma, small-cell lung carcinoma, adenocarcinoma of the breast and squamous carcinoma cells (DeCaprio, *et al.*, 1988; T'Ang *et al.*, 1988; Whyte *et al.*, 1988; Horowitz *et al.*, 1990). Approximately 40% of the osteosarcoma showed apparent structural changes in the Rb gene (Toguchida *et al.*, 1988). The reason that chemically treated and chemically transformed cells contain a notably lower amount of Rb protein compared with normal cells is presently unknown. It is possible that these processes could allow cells to escape from senescence, resulting in an increased incidence of cell transformation. Moreover, it was reported that structural alteration of the Rb gene and amplification of c-*myc* might play an important role in the clinical course and pathogenesis of osteosarcoma (Ozaki *et al.*, 1993). In fact, inactivation of tumor suppressor genes or rapid destruction of tumor suppressor protein by viral infections can result in an increased incidence of cancer and immortalization *in vitro* (Mullaart *et al.*, 1990). In the present study, the results indicate that malignant transformation of rat bone cells can be caused by benzo(a)pyrene, a tobacco-related chemical carcinogen. It also demonstrates that overexpression of c-*myc* messages, together with the downregulation of p53 and Rb proteins, may be

associated with malignant progression of rat bone cells.

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