Tumorigenic Conversion and Characterization of Rat Osteoblast-like Cells Transformed by 7,12-Dimethylbenz(a)anthracene

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국문초록

7,12-Dimethylbenz(a)anthracene에 의한 흰쥐 골모세포유사세포의 악성형질전환과 특성에 관한 연구

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이 진

본 연구는 태령 19일된 백서 태자 두개관에서 분리한 골모세포유사세포에 화학발암물질인 7,12-Dimethylbenz(a)anthracene (DMBA: 0.5 ㎍/ml) 및 tumor promotor인 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA: 1.0 ㎍/ml)를 단독 혹은 복합처리하여 PTRCC-DMBA, RCC-DMBA 및 RCC-DMBA-TPA 세포주를 확립시키고, 각 세포의 세포형태, 세포성장곡선, alkaline phosphatase와 acid phosphatase 활성 및 *in vitro* tumorigenicity를 연구하였다. 또한 c-*myc*, c-*fos*, c-*jun*, p53 및 Rb 유전자의 발현변화와 항암단백질인 p53 및 pRb 단백질의 발현변화를 관찰하여 골모세포유사세포가 악성형질전환되는 분자기전의 일단을 연구하고자 시행하였다. 본 실험에 사용한 모든 세포군에서 높은 alkaline phosphatase 활성과 낮은 acid phosphatase/alkaline phosphatase ratio를 보여 골모세포의 특성을 나타내었다. RCC-DMBA와 RCC-DMBA-TPA 세포는 정상세포나 PTRCC-DMBA에 비해 빠른 성장속도를 보였으며, 또한 soft agar상에서 colony를 형성하여 anchorage-independent growth를 나타내었다. 화학발암물질로 악성변형된 세포들은 정상세포나 PTRCC-DMBA 세포에 비해 c-*myc* 유전자의 과발현이 관찰되었다. 정상세포에서 p53 유전자의 발현은 1.9 kb의 message만이 발현되었다. 그러나 화학발암물질로 형질전환된 세포에서는 1.9 kb message외에도 1.6 kb의 message가 더 발현되었으며, message의 양도 현저히 증가되었다. p53 단백질의 발현은 RCC-DMBA-TPA 세포에서 정상세포에 비해 현저히 감소하였으나, RCC-DMBA 세포에서는 유사한 경향을 보였다. Rb 유전자의 발현은 RCC-DMBA-TPA 세포에서만 현저히 감소하였으나, Rb 단백질의 발현은 정상세포에 비해 형질전환된 세포들에서 모두 현저히 감소되었고, 특히 RCC-DMBA-TPA 세포에서는 거의 발현되지 않았다. 이상의 결과에서 백서 태자 두개관에서 분리한 골모세포유사세포는 화학발암물질인 DMBA에 의해 악성형질전환이 유도되었으며, c-*myc*의 과발현 및 p53과 Rb 단백질의 발현감소가 정상 골모세포유사세포의 악성변형과정에 밀접히 연관되어 있음을 시사한다.

주요어 : 골모세포유사세포, DMBA, 악성형질전환, c-*myc*, p53, Rb

Introduction

Cancer is characterized by uncontrolled cell growth leading to invasion of surrounding tissues and spread to other parts of body. Humans are exposed to a variety of known carcinogens in tobacco, the environment, diet, and alcoholic drinks. Many genotoxic carcinogens including polycyclic aromatic hydrocarbons(PAHs), aromatic amines, nitrosamines, and alkylating agents have been identified. And these environmental carcino-gens can be taken up by human body[1] and may make contact with target tissues directly or through blood stream. PAHs are probably the most widely distributed class of potent carcinogens present in the human environ-ment[2]. Although some carcinogens are responsible for direct DNA damage, most of the carcinogens such as PAH are converted to their electrophilic derivatives as an ultimate carcinogen through normal cellular metabolism. This process is known as metabolic activation and sometimes occurs in several steps. Activation is a normal enzymatic process such as cytochrome P450 system that converts a particular chemical into a more water-soluble, but reactive form that can then bind to cell macromolecules such as DNA, RNA, proteins and lipids[1,3]. 7,12-Dimethylbenz (a)anthracene (DMBA) is one of the most potent carcinogenic PAHs known. In the mouse skin tumorigenesis system, multiple papillomas and carcinomas can be produced on the back of mice following a single application of DMBA[4]. And administration of DMBA by oral stomach tube elicited mammary cancer or leukemia in rats[5,6].

Bone is a complex living tissue in which the extracellular matrix is mineralized, conferring marked rigidity and strength to the skeleton. In addition to its supportive and protective functions, it actively partici-pates in maintaining calcium homeostasis in the body. Bone is composed of several types of cells such as osteoblasts, osteocytes, and osteoclasts. Also bone is composed of an organic matrix of which Type I collagen constitutes appro-ximately 95%, and that is strengthened by deposits of calcium salts[7]. Mature bones have an elaborate vascular system that supplies the cells of the marrow, bone tissue, and periosteum. Even within

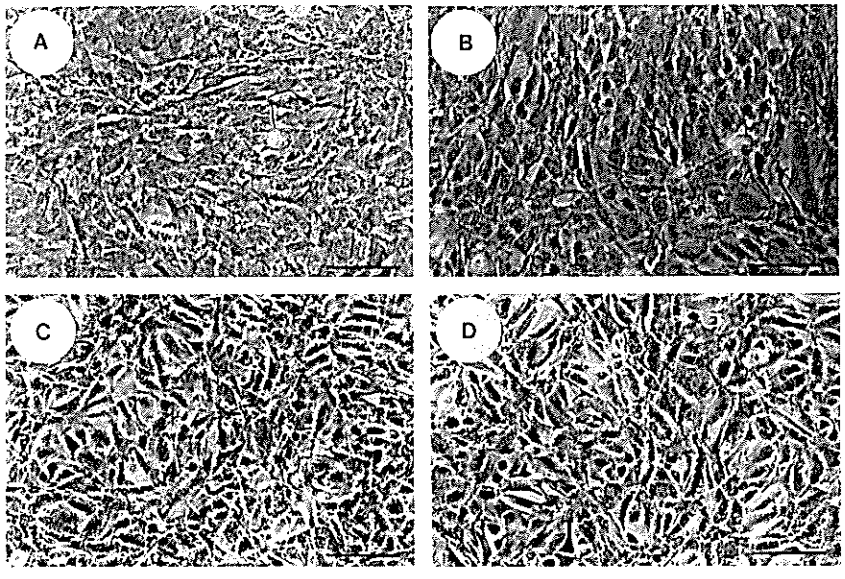

Fig. 1. Microscopic features of NRCC (A), PTRCC-DMBA (B), RCC-DMBA (C), and RCC-DMBA-TPA (D). The cells were plated and grown to confluence. The chemically transformed cell lines RCC-DMBA and RCC-DMBA-TPA shown morphological alterations from their parental counterpart, NRCC. Bar length = 100 μm.

dense cortical bone tissue, the organization of vascular canals ensures that no cell lies more than 300 micrometers from a blood vessel[18]. Hence, serum ions are readily accessible to the bone cells and minerals.

It is now firmly established that oncogenes and tumor suppressor genes are key molecules that have diverse roles in signal transduction, cell proliferation, differentiation, and embryonic development, as well as in cellular transformation and oncogenesis[9-12]. Among the more than 100 transforming and tumor suppressor genes that have been identified, only a few have been shown to play a significant role in the biology of human neoplasia, including that of bone cancers[13,14]. Recent advances have indicated that oncogenes also have important roles in the control of bone cell activity.

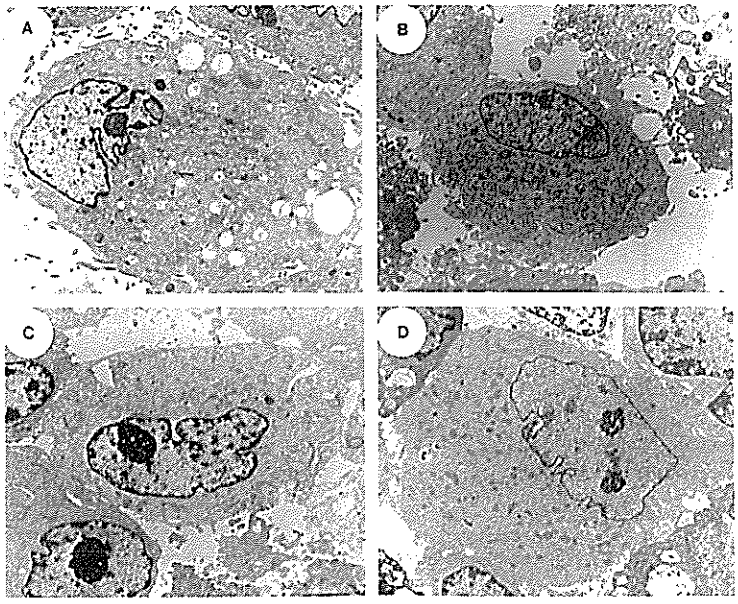

Fig. 2. Ultrastructural findings of NRCC (A), PTRCC-DMBA (B), RCC-DMBA (C), and RCC-DMBA-TPA cells (D) as shown by transmission electron microscopy.

Osteosarcoma is the most common primary malignant tumor of bone[15]. Amplification and overexpression of several proto-oncogenes have been demonstrated in some spontaneous and radiation-induced osteosarcomas[16-18]. The amplification of the c-*myc* gene or overexpression of its protein[19-21], and deregulation of c-*fos*[21-23] are frequently found in human osteosarcomas. The tumor suppressor genes such as pRb and p53, have been shown to be important for the pathogenesis of osteosarcomas, as evidenced by the fact that mutations in, or inactivation of, these genes are associated with deregulated cell growth and human neoplasias, including osteosarcomas[12,24-26]. Several key molecular mechanisms have been implicated in the development of osteosarcoma, but they seem to play a major role only in distinct clinical subsets of tumors. The mechanisms involved in the development of common *de novo* high-grade osteosarcomas are still obscure.

We previously established tumorigenic transformed rat bone cell lines by benzo(a)pyrene (BaP), a tobacco-related chemical carcinogens[27]. In the present study, we investigated the possibility of tumorigenic conversion of normal bone cells by DMBA. Its activity as tumor initiator is more than ten-fold greater than that of BaP in mouse skin[28]. Primary normal rat osteoblast-enriched cells were exposed to DMBA, alone or in conjunction with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Chemically treated (PTRCC-DMBA) and chemically transformed cells (RCC-DMBA and RCC-DMBA-TPA) were established and their biological properties, *in vitro* tumorigenicity, and

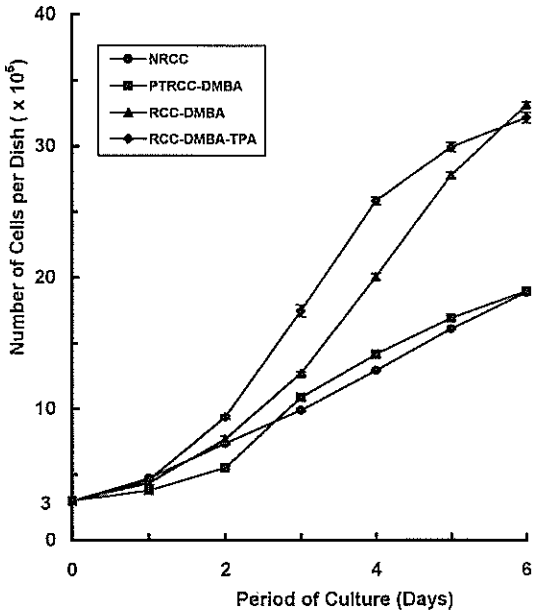

Fig. 3. Growth curves of NRCC, PTRCC-DMBA, RCC-DMBA, and RCC-DMBA-TPA cells grown in MEM supplemented with 10% FBS.

expression of c-*myc*, c-*fos*, c-*jun*, p53, and Rb were studied.

Materials and Methods

Culture of primary normal rat calvarial cells (NRCC)

19-days pregnant Sprague-Dawley rats (Animal laboratory, College of Medicine, Seoul National University) were anesthetized with sodium pentobarbital (30mg/kg) intraperitoneally. The fetuses were removed and the portions of calvaria containing frontal and parietal bone were separated from the brain. Blood residues and the periosteum of the calvaria were removed by an ophthalmic pincette and washing with Hanks balanced salt solution (HBSS, GibcoBRL Life Technologies Inc., Gaithersburg, MD, USA). The calvaria samples were transferred to a Reacti-vial (10 calvariae/vial; Pierce, Rockford, IL, USA) with 1.5 ml of enzyme solution containing of 0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA. Bone cell population I to V were obtained by serial enzyme digestion as previously[27]. All cells and cell lines were maintained in MEM supplemented with 10% fetal bovine serum (FBS, GibcoBRL Life Technology Inc.) and antibiotics at 37°C in humidified atmosphere of 5% CO_2 in air. The cells were plated at 3 x 10^5 cells per 60-mm Petri dish and grown for the various assays.

Tumorigenic transformation of cells to DMBA, alone or in conjunction with TPA

The primary NRCC (population V) were plated at 3 x 10^5 cells per 60-mm Petri dish. When the cultures reached 70% confluency, they were exposed to DMBA (0.5 μg/ml; Sigma Chemical Co., St Louis, MO, USA) for 20 weeks. These chemically treated cells were designated PTRCC-DMBA. The primary NRCC (population V) were also exposed to DMBA (0.5 μg/ml) for 40 weeks. The primary NRCC were exposed to DMBA (0.5 μg/ml) for 20 weeks, and then further exposed to DMBA (0.5 μg/ml) and TPA (1.0 μg/ml; Sigma Chemical Co.) for 20 weeks. These cells were named RCC-DMBA and RCC-DMBA-TPA, respectively. DMBA 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 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 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. Ultra-thin 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 x 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℃. 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[29]. Two ml of a mixture of 2X medium and 1.0% Noble agar (Difco Laboratories, Detroit, MI, USA) 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℃ for 3 weeks. Colonies being larger than 50 ㎛ in diameter were examined and counted with the aid of an inverted microscope.

Determination of acid and alkaline phosphatase activities

Monolayer cultures (1 x 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.*[30], 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℃ 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.*[31], 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.

The obtained poly(A^+)RNAs were resolved in a 1.2% agarose-formaldehyde gel and transferred onto nylon filters (Amersham Corp. Arlington Heights, IL, USA). Probes used for Northern

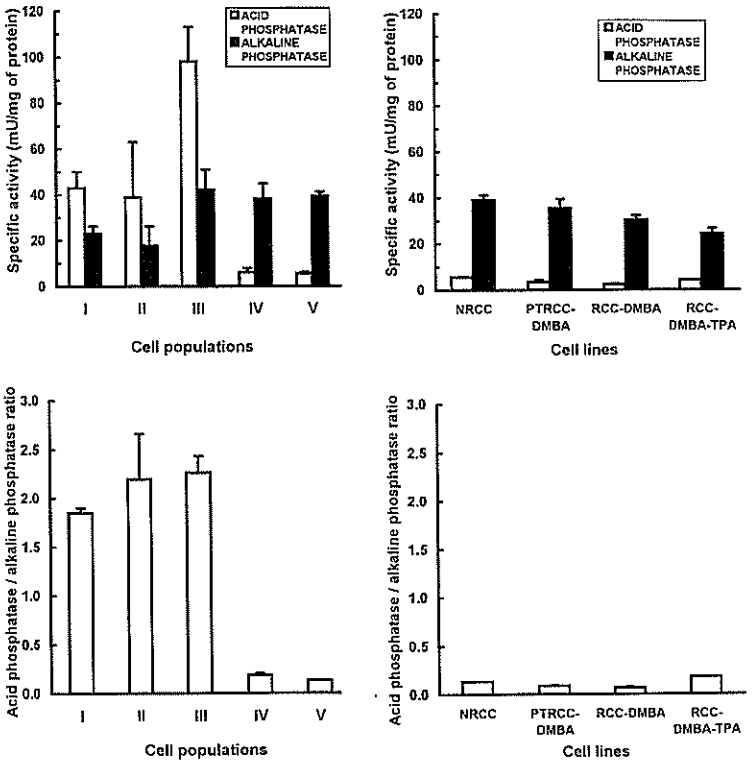

Fig. 4. Activities of acid phosphatase and alkaline phosphatase in rat calvarial cell populations (A), and cell lines (B). Acid phosphatase / alkaline phosphatase ratio in rat calvarial cell populations (C) and cell lines (D). Enzyme activity was assayed with 15 mM *p*-nitrophenyl phosphate in 0.1 M sodium citrate buffer, pH 4.8 (acid phosphatase) and 0.1 M glycine-NaOH buffer, pH 10.4 (alkaline phosphatase) at 37℃. 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℃.

analysis were as follows: v-*myc* cDNA (ATCC, Rockville, MD, USA), v-*fos* cDNA (ATCC), v-*jun* cDNA (ATCC), p53 cDNA (from Dr. E. Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA, USA), Rb cDNA (from Dr. S. Friend, Massachusetts General Hospital Cancer Center, Charlestown, MA, USA) and human β-actin cDNA (from Dr. L. Kedes, Stanford University, Palo Alto, CA, USA). The probes were labeled with [^{32}P]dCTP (Amersham Corp.) by megaprime labeling kit (Amersham Corp.). Hybridization was done as recommended by the membrane manufacturer. Filters were then autoradiographed on Hyperfilm-MP (Amersham Corp.) for 12 h at -70 ℃. After exposure, the probe was stripped off the filter for rehybridization to the next radiolabeled probe.

Western analysis

80% confluent cells were lysed, and the cell extracts were processed for Western analysis to determined the intracellular levels of p53 and Rb proteins using Western-Light kit (Tropix Inc., Bedford, MA, USA) as previously[27]. Monoclonal antibody for p53 (PAb240) or Rb were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The membrane was exposed to Hyperfilm-MP for 4 min at room temperature.

Results

Morphology, ultrastructural finding, proliferation rate, and in vitro tumorigenicity

The PTRCC-DMBA cells were morphologically similar to its normal counterpart, but RCC-DMBA and RCC-DMBA-TPA cells showed morphological alterations from its normal counterpart (Fig. 1). Primary NRCC 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. Golgi apparatus were less prominent and were situated near the cytocenter of the cells. The cytoplsm contained a few vacuoles variable in size.

The many finger-like microvilli on the cell surface were observed. Also ultrastructural characteristics of PTRCC-DMBA, RCC-DMBA, and RCC-DMBA-TPA cells were similar to NRCC (Fig. 2). Therefore, in spite of careful examinations, there was much difficulty in drawing meaningful 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-DMBA, RCC-DMBA, and RCC-DMBA-TPA cells were approximately 44, 37, 32, and 29 h, respectively (Fig. 3).

NRCC and PTRCC-DMBA cells did not proliferate in the semi-solid soft agar, indicating the lack of ability of anchorage independency of these cells. The chemically transformed cell lines (RCC-DMBA and RCC-DMBA-TPA), however, grew in the soft agar with colony-forming efficiencies of 7.6 to 4.2%, indicating the ability of anchorage independency of these cells (Table 1).

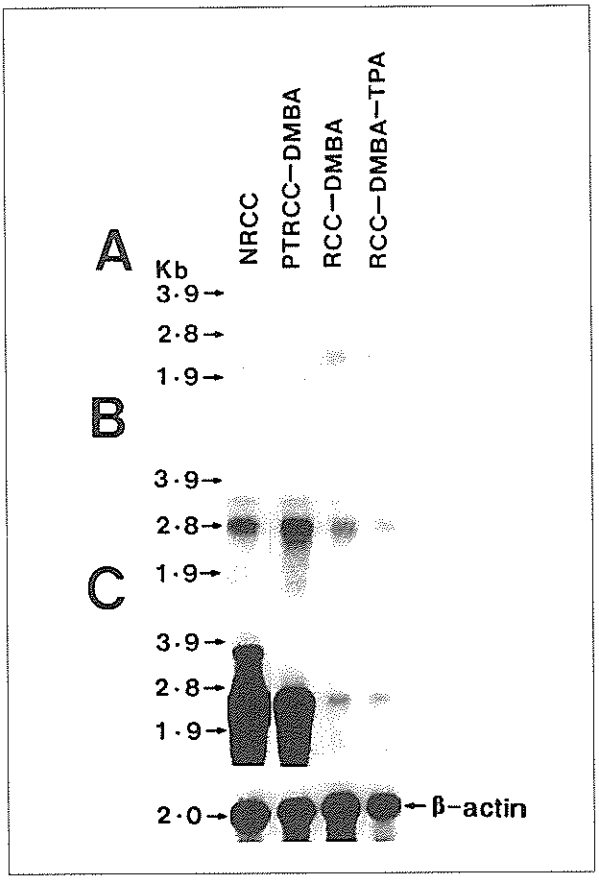

Fig. 5. Transcription of c-*myc*, c-*jun*, and c-*fos* genes from cells grown in MEM supplemented with 10% FBS. (A) Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled v-*myc* cDNA; (B) Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled v-*jun* cDNA; (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 rediolabeled probe.

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 high acid phosphatase (ACP) activity and low alkaline phosphatase (ALP) activity usually attributed to osteoclast, whereas those released in the late stages of digestion (population IV and V) have been shown low ACP activity and high ALP activity (Fig. 4A). All cells derived from population V by treatment of chemicals have been shown to possess low

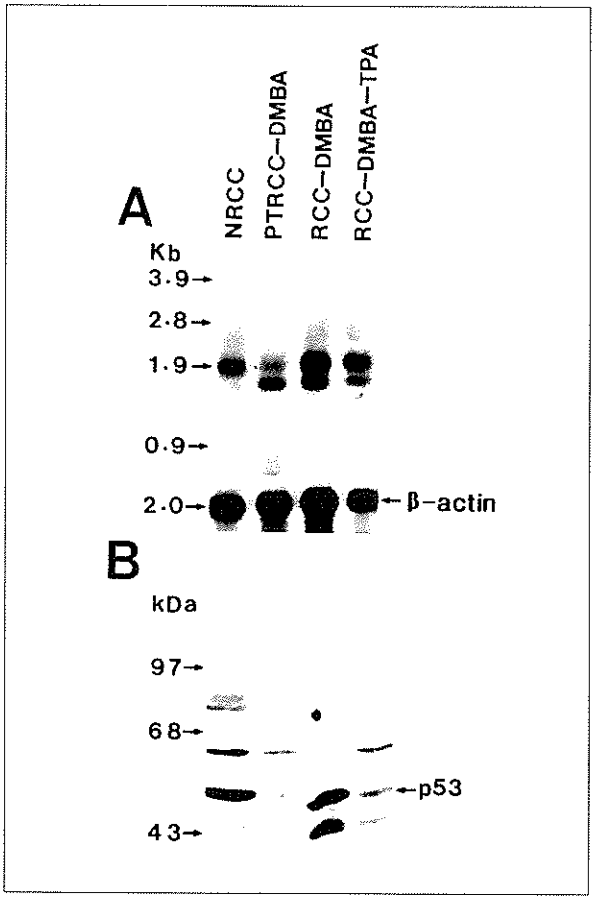

Fig. 6. Expression of p53 in cells or cell lines. (A) Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled p53 cDNA; (B) Western blot analysis of p53 protein expression from the cells and cell lines.

ACP activity and high ALP activity (Fig. 4B), and very low ACP/ALP ratio (Fig. 4D) usually attributed to osteoblasts. However, cells released early during the digestion (population I - III) showed markedly higher ratio of ACP/ALP than those in the cell population IV and V (Fig. 4C).

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

Expression of 2.4 kb c-*myc* messages in NRCC and PTRCC-DMBA cells was similar to each other, but the amount of this gene transcription was notably higher in the chemically transformed cell lines than other cells (Fig. 5A). Since the alteration in c-*myc* transcripts was observed, we further investigated on the c-*jun* and c-*fos* transcript 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 transcripts. Interestingly, the amount of the c-*jun* and c-*fos* transcripts was high in normal and PTRCC-DMBA cells, but markedly reduced when the normal cells were transformed to tumorigenic cells (Fig. 5B and 5C).

Analysis of p53 and Rb

Two p53 transcripts with sizes of 1.9 kb and 1.6 kb were expressed from chemically treated (PTRCC-DMBA) and chemically transformed cells (RCC-DMBA and RCC-DMBA-TPA), whereas only 1.9 kb p53 transcripts were expressed in NRCC (Fig. 6A). RCC-DMBA and RCC-DMBA-TPA lines transcribed notably higher amount of p53 transcripts than did NRCC. Western blot analysis showed that p53 protein levels in the RCC-DMBA cells were similar to NRCC but notably lower in the other cells than those in NRCC (Fig. 6B).

The levels of Rb transcripts in the NRCC and PTRCC-DMBA cells were similar to each other and significantly higher than the level in RCC-DMBA-TPA. However, the RCC-DMBA cells contained similar level of Rb messages when compared to NRCC (Fig. 7A). The levels of $p110^{Rb}$ in the PTRCC-DMBA and RCC-DMBA cells were similar, but they were notably and significantly lower than those in NRCC (Fig. 7B). Further, the $p110^{Rb}$ expression was almost negligible in the RCC-DMBA-TPA cells.

Discussion

We investigated the possibility of tumorigenic transformation of normal bone cells by DMBA, a potent chemical carcinogen. In our data, malignant transformation of rat bone cells result from exposure to DMBA, alone or in conjunction with TPA. It is evidenced by the ability to grow in soft agar (Table 1), morphological alterations (Fig. 1), and rapid growth (Fig. 3). The results indicate that bone cells can be malignantly transformed by exposure of DMBA. A previous study reported that chemical carcinogens participated in osteosarcoma oncogenesis in human[32] and rat[27]. In the present study, primary NRCC (population V) were used in the transformation assay. There are many evidences that NRCC (population V) has osteoblastic characteristics such as high ALP activity and low ACP activity[33-35]. Primary NRCC, chemically treated, and chemically transformed cells have been shown to possess high ALP activity and very low ACP/ALP ratio. These results indicate that the PTRCC-DMBA, RCC-DMBA, and RCC-DMBA-TPA cells may originate from osteoblasts.

It is now widely accepted that proto-oncogenes and tumor suppressor genes play major roles in carcinogenesis and their mutations are important in tumor development. Proto-oncogenes are normal, regulatory genes whose activity is increased as a consequence of genetic alteration. Several proto-oncogenes such as *myc* or *fos* are implicated as having a role in distinct groups of bone cancers. In our data, chemically transformed cells expressed a notably higher level of c-*myc* messages compared to their normal counterpart. The expression of c-*myc* is closely associated with proliferation status of cells[36] and frequently deregulated in cancer cells[37,38]. And in

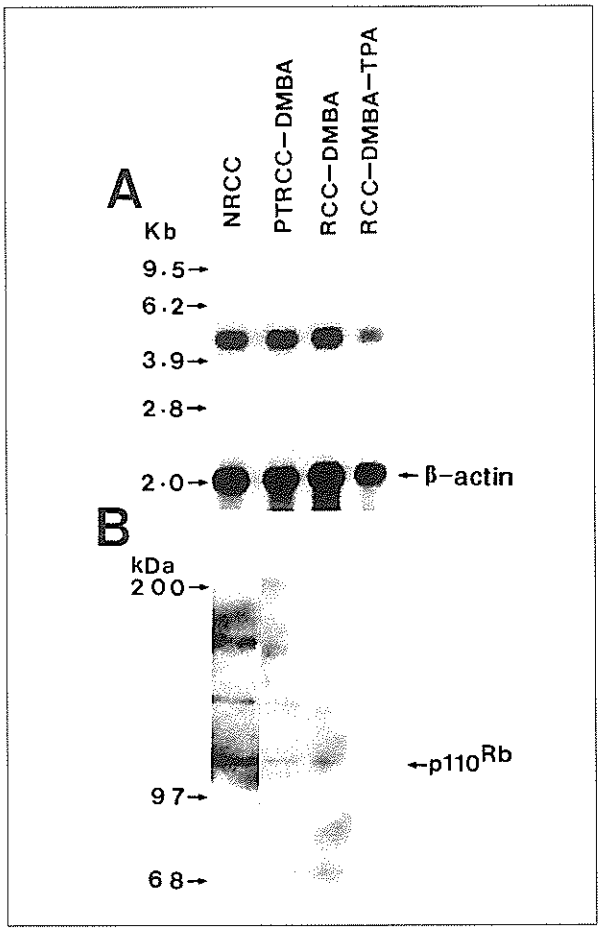

Fig. 7. Expression of Rb in cells or cell lines. (A) Northern blot hybridization of cellular polyadenylated RNAs to ^{32}P-labeled Rb cDNA; (B) Western blot analysis of Rb protein expression from the cells and cell lines.

neoplasia, amplification or overexpression of the c-*myc* protein has been associated with more aggressive variants of the tumors, hormonal independence, and in general, poor outcome[39]. Therefore, this result indicates that overexpression of c-*myc* may be involved in the neoplastic transformation of normal bone cells. Our data show that the levels of c-*jun* and c-*fos* transcripts were notably decreased in chemically transformed cells. The c-*fos* gene is a homology of the viral transforming gene of the FBJ and FBR-murine sarcoma viruses. The fos protein,

together with other related proteins such as jun, interacts with the AP-1 transcription complex. It seems that the c-*fos* gene plays an important role in differentiation and organogenesis of specific cell types[40,41]. In skeleton, overexpression of the c-*fos* gene is an integral element of osteoblastic differentiation and early bone formation[42]. The reason for the underex-pression of c-*jun* and c-*fos* in these cells is presently unknown. Although transforming effects of c-*fos* on osteoblasts and chondroblasts have been documented in several experimental studies, its role as a primary transforming factor in osteosarcoma is unclear[23,43]. In fact, downregulation of the c-*fos* and c-*jun* expression has been reported in previous studies[27,44,45].

Proto-oncogenes and tumor suppressor genes cooperate in the genesis of cancers. The p53 gene product is a 53 kDa nuclear phosphoprotein[46]. It was established that the wild-type protein acts a tumor suppressor gene, negatively regulating the cell cycle[47,48] and its mutations have been documented in a wide range of tumors. Indeed, The p53 gene is one of the most frequently altered genes in various human cancers[25]. 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 unknown at present. Also, the p53 transcripts expressed from chemically transformed cells were higher that those in NRCC. Interestingly, however, p53 protein levels in the RCC-DMBA-TPA cells were notably lower than the levels in NRCC. But these levels in RCC-DMBA were similar to NRCC. 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. In bone tumors, altered gene, overex- pressed p53 protein, or both are frequent findings in osteosarcoma[49-51]. Overexpression or alterations of p53 can be documented in approximately 30% of high-grade

Table 1. Characteristics of rat calvaria cells transformed by DMBA

Cells or cell lines	Primary	Transformation step	Soft agar colony formation (%)*
NRCC	+		None
PTRCC-DMBA	–	DMBA	None
RCC-DMBA**	–	DMBA	7.6
RCC-DMBA-TPA**	–	DMBA + TPA	4.2

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

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

conventional osteosarcomas[52].

Rb gene codes for a 110-kDa nuclear protein, which has a tumor suppressor activity. Its loss of function is a causal event in retinoblastoma, and moreover, loss has also been noted in a proportion of common cancers such as those of lung, prostate, and breast[53]. Our results show that the chemically treated and chemically transformed cell lines underexpressed Rb protein compared with normal cells. Especially, RCC-DMBA-TPA negligibly expressed the Rb protein. The reason for underexpression of Rb protein is unknown at present, but the alterations that compromise the function of the Rb gene are thought to be principal factors in osteosarcomas that develop in association with retinoblastoma[54,55]. And approximately 40% of the osteosarcoma showed apparent structural changes in the Rb gene[56]. Furthermore, the amplification of the c-*myc* gene or overexpression of its protein is frequently found in osteosarcoma[19-21]. And if c-*myc* amplification is associated with the Rb gene alteration, osteosarcoma shows an increased level of clinical aggressiveness[57]. In the present study, the results indicate that malignant transformation of rat bone cells can be caused by DMBA. 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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