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Bone formation in vitro and in nude mice by human osteosarcoma cells

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Abstract Osteosarcomas contain variable amounts of bony tissue, but the mechanism of bone formation by osteosarcoma is not well understood. While a number of cultured human osteosarcoma cell lines have been established, they are maintained by different media and differ qualitatively with regard to bone formation. We examined different media for their ability to support bone formation in vitro and found that alpha-modification of Eagle's minimal essential medium supplemented with beta glycerophosphate was best for this purpose, because it contained the proper calcium and phosphate concentrations. Subsequently, we compared seven human osteosarcoma cell lines under the same experimental conditions to clarify their ability to induce bone formation. NOS-1 cells most frequently exhibited features of bone formation in vitro and in nude mice. Collagen synthesis by tumour cells themselves seemed to be the most important factor for bone volume. However, even HuO9 cells, which lacked collagen synthesis and failed to form bone in vitro, successfully formed tumours containing bone in nude mice. Histological analysis of HuO9 cells in diffusion chambers implanted in nude mice and the findings of polymerase chain reaction indicated that the phenomenon was probably due to bone morphogenetic protein.

Key words Osteosarcoma · Cell line · Collagen · Alkaline phosphatase · Bone morphogenetic protein

Introduction

A number of cultured cell lines derived from human osteosarcoma have already been established, showing considerable differences in capacity for bone or osteoid formation in vitro or in nude mice. These differences may be caused not only by the characteristics of cell lines but also by culture conditions. However, it is reported that a mouse osteogenic cell line MC3T3-E1 [17] can differentiate into osteoblasts and mineralize in vitro using alpha-modification of Eagle's minimal essential medium (alpha MEM), and some investigators showed that beta glycerophosphate (beta GP) induced mineralization in osteoblast cultures [19, 23]. The present study was first performed to clarify a set of desirable culture condition for mineralization, and subsequently, we compared seven human osteosarcoma cell lines in vitro and in nude mice under the same experimental conditions. Our intent was to clarify the relationship between bone or osteoid volume and amounts of collagen, alkaline phosphatase (ALP) and osteocalcin produced by tumour cells.

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Materials and methods

Seven human osteosarcoma cell lines, designated NOS-1 [8], NOS-2 [8], NY [16], OST [20], Saos-2 [13], HuO9 [9] and HuO-3N1 [24] were used in the present study. The NOS-1 and NOS-2 cell lines were established in our laboratory. The NY cell line was kindly donated by Dr. M. Sekiguchi (Institute of Medical Science, University of Tokyo). The OST and Saos-2 cell lines were obtained from RIKEN Cell Bank (Tsukuba, Japan), and the HuO9 and HuO-3N1 cell lines were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan). The clinicopathological features of the patients with parent tumour and initial culture media are summarized in Table 1. For comparative studies, we used an alkaline phosphatase (ALP)-producing gastric adenocarcinoma cell line, MKN7 [11] and human skin fibroblasts.

First, NOS-1 cells were cultured in growth medium consisting of RPMI-1640 (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum, or FBS (M,A Bioproducts, Walkersville, Md.) and with or without 5 mM beta glycerophosphate disodium salt (beta GP) (Wako Pure Chemical, Tokyo, Japan), or alpha MEM (Cosmo Bio, Tokyo, Japan) supplemented with 10% or 5% FBS and with or without 5 mM beta GP. Additionally, each growth medium was supplemented with 200 μg/ml kanamycin sulfate (Meiji Seika, Tokyo, Japan). Subsequently, other cell lines were cultured in the growth medium consisting of alpha MEM supplemented with 5% FBS, 5 mM beta GP and 200 μg/ml kanamycin sulfate for in vitro mineralization. For experiments, cells in logarithmic growth phase were inoculated at 5×10^5 cells/60-mm plas-

Table 1 Human osteosarcoma cell lines used in the present study (*RPMI-1640* Roswell Park Memorial Institute medium 1640, *MEM* Eagle's minimum essential medium, *DMEM* Dulbecco's modified Eagle's medium, *FBS* fetal bovine serum, *LH* lactalbumin hydrolysate, *KM* kanamycin, *PC* penicillin, *SM* streptomycin)

Cell line	Parent tumour		Medium used up to present study
	Patient's age (years) and sex	Tumour location and histology	
NOS-1	16, M.	Lt. tibia Osteoblastic type	RPMI-1640 supplemented with 10% FBS and 0.02% KM
NOS-2	11, M	Rt. femur Osteoblastic type	RPMI-1640 supplemented with 10% FBS and 0.02% KM
HuO9	13, F	Rt. femur Osteoblastic type	RPMI-1640 supplemented with 10% FBS, 0.2% LH and 0.006% KM
HuO-3N1	15, F	Lt. ilium Osteoblastic type	RPMI-1640 supplemented with 10% FBS, 0.2% LH, 100 U/ml PC and 0.01% SM
Saos-2	11, F	Not described	McCoy 5A supplemented with 15% FBS
NY	15, M	Rt. femur Osteoblastic type	MEM supplemented with 10% FBS and 0.01% KM
OST	15, F	Lt. femur Osteoblastic type	DMEM supplemented with 10% FBS and 1% PC/SM solution

Table 2 PCR primers sequences

Primer	Sequence (5′ to 3′ orientation)	Product size (bp)	Restriction site	Restriction products (bp)
BMP-2 (3′)	GCTGTACTAGCGACACCCAC	671	Taq I	24+558+89
BMP-2 (5′)	TCATAAAACCTGCAACAGCCAACTCG			
BMP-4 (3′)	GCTGAAGTCCACATAGAGCGAGTG	346	Alu I	153+193
BMP-4 (5′)	ACTGGTCCACCACAATGTGACACG			

tic dish (Corning, NY) containing 3 ml of medium. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air at 100% humidity, and fed every 2 days.

For light microscopic examination, culture appearance was examined by phase-contrast microscopy. Cultures were also stained for calcium phosphate salts using von Kossa's method and for collagen fibre using silver impregnation after fixation in 10% formaldehyde.

For electron microscopic examination, culture cells were fixed in 2.5% phosphate-buffered glutaraldehyde, post-fixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

The collagen synthesis was evaluated by hydroxyproline content. Hydroxyproline in the cell layer containing extracellular collagen fibers was measured using Kivirikko's method [10]. Alkaline phosphatase (ALP) activity in the medium was determined using *p*-nitrophenyl phosphate as a substrate according to the recommendations of the German Society for Clinical Chemistry. Osteocalcin levels in the medium were measured by radioimmunoassay. Calcium (Ca) and inorganic phosphate (Pi) concentration in the medium were measured using an orthocresolphthanlein method and an enzymatic assay, respectively.

Female athymic nude mice (nu/nu) of BALB/c genetic lineage (Nihon Clea, Tokyo, Japan) were used for heterotransplantation at 5–6 weeks of age. Approximately 10 million cells in 0.3 ml of fresh culture medium were inoculated into the subcutaneous tissue of the back. The animals were killed under anaesthesia at 8 weeks after transplantation. For morphological examination, the tissue specimens were fixed in 10% formaldehyde, embedded in paraffin, and stained with haematoxylin and eosin and von Kossa's method.

We also attempted heterotransplantation using diffusion chamber. Diffusion chambers were constructed using Millipore HA filter and lucite ring (0.45 μm pore size, Millipore, Mass.). The chambers, containing approximately 10 million NOS-1 cells or HuO9 cells, were implanted subcutaneously into five athymic nude mice and were removed 8 weeks after implantation and examined histologically.

Total cellular RNA was preparated by guanidine thiocynate/cesium chloride gradient centrifigation. Total cellular RNA 5 μg was converted to cDNA by reverse transcription using a SuperScript Preamplification System (Gibco BRL, Gaitherburg, Md.). cDNA was amplified by PCR using a Gene Amp Kit (Perkin-Elmer Cetus, Norwalk, Conn.). The oligonucleotide primers for PCR were designed according to Bentley et al. [1] (Table 2). Amplification was carried out in a Program Temp Control System PC-700 (Astec, Tokyo, Japan). The cycle conditions were as follows: denaturation at 94°C for 4 min followed by 29 cycles of denaturation at 93°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 2 min followed by 1 cycle of 93°C for 1 min, 60°C for 2 min and 72°C for 10 min. The reaction products were analyzed by electrophoresis on 2% agarose gels containing 0.5 μg ethidium bromide/ml.

The cDNA fragments produced by primer-specific PCR were designed to contain known restriction sites (Table 2). Restriction analysis was also performed according to Bentley et al. [1] (data not shown).

Results

In vitro mineralization by NOS-1 cells fed with various culture media is summarized in Table 3. The NOS-1 cells reached the stationary phase at approximately the 20th day after inoculation. At the 4th week after inoculation, calcium deposition began to be found in the dishes in which NOS-1 cells were fed with alpha MEM supplemented with 10% or 5% FBS and 5 mM beta GP. The number of foci of calcium deposition increased gradually by culture days, and many foci of calcium deposition and mineralized extracellular matrix were formed after 8 weeks of cultivation (Fig. 1). Electron microscopically,

Table 3 In vitro mineralization by NOS-1 cells fed by various kinds of culture media [a] (– negative, + occasionally positive, ++ frequently positive)

Culture media	Calcium deposition detected by von Kossa's method	Mineralization on collagen bundles
RPMI-1640		
+10% FBS	+	–
+10% FBS+5 mM β-GP	+	–
α-MEM		
+10% FBS	+	–
+10% FBS+5 mM β-GP	++	+
+5% FBS+5 mM β-GP	++	+

[a] Evaluation was carried out after 8 weeks of cultivation

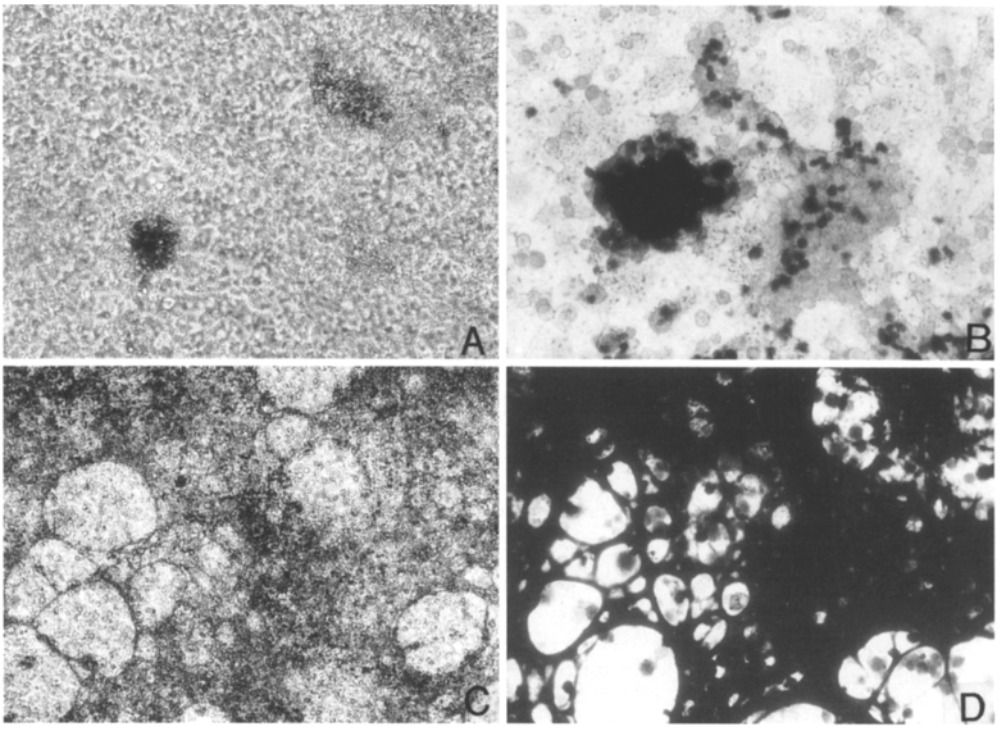

Fig. 1A–D Calcium deposition by NOS-1 cells fed with alpha MEM supplemented with 5% FBS and 5 mM beta glycerophosphate. **A, B** After 4 weeks' cultivation. **A** Phase-contrast micrograph; ×36 **B** von Kossa's staining. ×180 **C, D** After 8 weeks' cultivation. **C** Phase-contrast micrograph; ×36 **D** von Kossa's staining. ×180

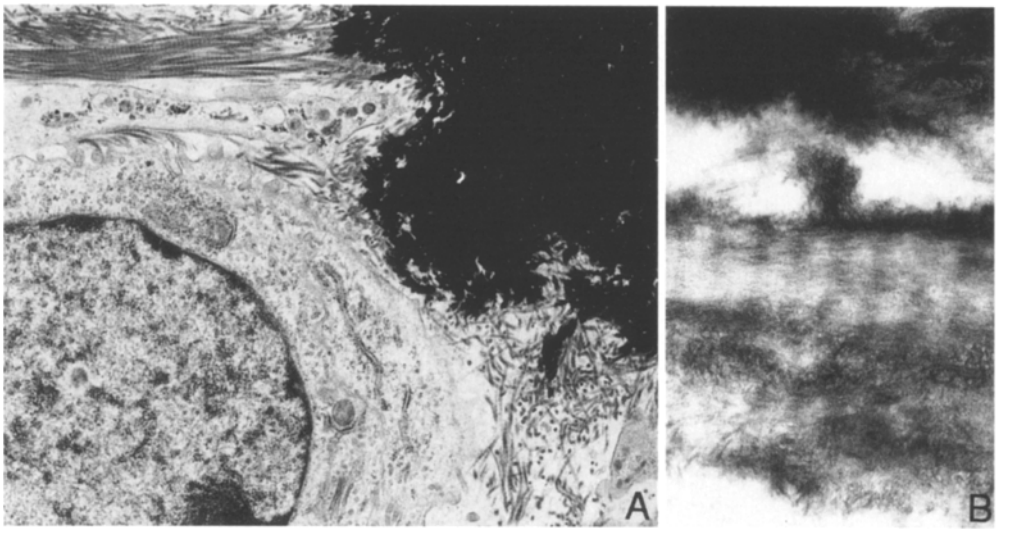

Fig. 2A, B Electron micrographs of mineralization by NOS-1 cells maintained with alpha MEM supplemented with 5% FBS and 5 mM beta glycerophosphate after 8 weeks' cultivation. Mineralization of the extracellular collagen bundles is observed. **A** ×11,800; **B** ×94,400

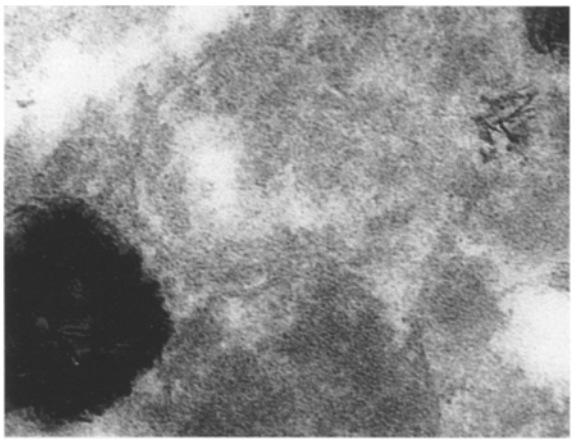

Fig. 3 Electron micrograph of dystrophic calcification by NOS-1 cells maintained with RPMI-1640 supplemented with 5% FBS and 5 mM beta GP. Calcification is observed on the degenerating substance. ×94,400

we successfully confirmed the presence of needle-shaped crystals lying on the collagen fibres (Fig. 2), though some crystals represented dystrophic calcification (calcium deposited on the degenerating cells).

In the cultures fed with RPMI-1640 with or without beta GP and alpha MEM without beta GP, a few calcium deposits were noted. However, all of them exhibited dystrophic calcification (Fig. 3).

Although alpha MEM supplemented with 10% or 5% FBS and 5 mM beta GP showed no significant effect on cell growth, collagen synthesis or ALP activity, it did promote osteocalcin production (Table 4).

The analyses of Ca and Pi concentrations in the media revealed that alpha MEM provided a high Ca concentration, while beta GP served as a source of inorganic phosphate. The media consisting of RPMI-1640 provided high Pi concentration without beta GP, but the Ca concentration was low (Table 5).

Mineralization in vitro by seven osteosarcoma cell lines, one gastric adenocarcinoma and fibroblasts is summarized in Table 6. All cell lines reached the stationary phase around the 20th day of culture. The foci of calcium deposition were observed by light microscope in not only the NOS-1, Saos-2, HuO9 and NOS-2 cell lines, but also the MKN7 cell line derived from gastric adenocarcinoma after 4–6 weeks of cultivation. However, electron microscopic examination revealed that only the NOS-1 and Saos-2 cells mineralized on thick collagen bundles. NOS-2 cells showed mineralization on thin collagen bundles, while HuO9 and MKN7 cells showed only dystrophic calcification.

Collagen, osteocalcin and ALP production by osteosarcoma cells, gastric adenocarcinoma cells and fibroblasts in vitro is summarized in Table 7. The cell layer of NOS-1 cells contained a large amount of hydroxyproline. That of NOS-2 cells also contained considerable quantities of hydroxyproline. Silver impregnation also demonstrated that NOS-1 cells formed thick extracellular collagen bundles in a reticular arrangement (Fig. 4). Saos-2 and NOS-2 cells formed thin extracellular collagen bundles in a reticular arrangement (Fig. 4), and NY cells and fibroblasts formed irregular thin extracellular collagen fibres. Although few thin collagen fibres were detected in the HuO-3N1 cell line, no extracellular fi-

Table 4 Collagen, osteocalcin and alkaline phosphatase production by NOS-1 cells fed with various kinds of media [a] (*ALP* alkaline phosphatase)

Culture media	No. of cells (10^7/dish)	Hydroxyproline (μg/10^7 cells)	Osteocalcin (ng/10^7 cells)	ALP (mlU/10^7 cells)
RPMI1640				
+10% FBS	3.1±0.1	17.6±7.0	2.8±0.4	532±29
+10% FBS+5 mM β-GP	2.8±0.3	19.6±1.6	3.0±0.7	605±72
α-MEM				
+10% FBS	2.9±0.3	18.1±7.1	2.8±0.7	621±52
+10% FBS+5 mM β-GP	2.6±0.1	16.5±1.1	6.6±0.4 *	523±63
+5% FBS+5 mM β-GP	2.3±0.1	14.7±2.6	7.7±0.6 *	483±65

[a] The evaluation was carried out after 4 weeks of cultivation
* $P<0.05$, α-MEM+10 or 5% FBS+5 mM β-GP versus α-MEM+10% FBS without β-GP and RPMI1640+10% FBS or without β-GP (Mann-Whitney U-test)

Table 5 Calcium and inorganic phosphate concentrations in culture media before and after 48 h of cultivation of NOS-1 cells (*Ca* calcium, *Pi* inorganic phosphate)

Culture media	Ca (mg/dl)		Pi (mg/dl)	
	Before cultivation	After cultivation	Before cultivation	After cultivation
RPMI-1640				
+10% FBS	3.1±0.2	3.2±0.1	15.5±1.3	17.2±2.0
+10% FBS+5 mM β-GP	3.1±0.1	3.3±0.1	15.9±1.6	21.1±2.6
α-MEM				
+10% FBS	7.4±0.2	7.4±0.1	3.6±0.8	5.8±0.2
+10% FBS+5 mM β-GP	7.4±0.2	6.9±0.2	4.4±1.1	16.8±0.5
+5% FBS+5 mM β-GP	7.2±0.1	7.1±0.1	4.0±0.3	15.8±2.4

Table 6 Mineralization by human osteosarcoma cells and other types of cell in vitro [a] (−negative, + occasionally positive, ++ frequently positive)

Cell line	Calcium deposition detected by von Kossa's method	Mineralization on collagen bundles	Dystrophic calcification
NOS-1	++	++	+
Saos-2	++	++	+
HuO9	+	−	+
NOS-2	++	++	+
OST	−	−	−
NY	−	−	−
HuO-3N1	−	−	−
MKN7	+	−	+
Fibroblast	−	−	−

[a] Evaluation was carried out after 8 weeks of cultivation

Table 7 Collagen, osteocalcin and alkaline phosphatase produced by osteosarcoma cells and calcium and inorganic phosphate concentrations [a] (*ND* not detected)

Cell line	No. of cells (10^7/dish)	Hydroxyproline [b] (μg/10^7 cells)	Osteocalcin [c] (ng/10^7 cells)	ALP [c] mIU/10^7 cells)	Ca [d] (mg/dl)	Pi [d] (mg/dl)
NOS-1	3.1±0.1	15.1±3.2	8.3±1.6	529±66	7.3±0.1	14.8±0.2
Saos-2	3.0±0.1	3.8±1.0	ND	2554±650	7.4±0.2	18.3±2.0
HuO9	1.7±0.1	0.5±0.4	13.6±3.6	497±20	7.1±0.1	18.3±1.5
NOS-2	3.3±0.2	10.9±0.8	3.4±0.6	375±36	7.3±0.1	15.6±0.3
OST	2.4±0.2	0.2±0.1	3.4±1.2	42±20	7.2±0.1	5.8±0.8
NY	1.2±0.1	4.6±1.2	5.7±1.7	104±12	7.4±0.1	5.3±0.8
HuO-3N1	1.8±0.2	1.1±0.8	0.3±0.1	60±9	7.1±0.1	5.3±0.3
MKN7	1.3±0.1	0.1±0.1	0.9±0.1	1570±120	7.0±0.2	14.3±0.6
Fibroblast	0.8±0.1	3.8±1.2	0.3±0.1	136±23	7.5±0.1	7.2±0.3

[a] Evaluation was carried out after 4 weeks of cultivation
[b] The amounts of hydroxyproline were assayed using cell layer
[c] The levels of osteocalcin and alkaline phosphatase (ALP) secreted by cultured human cells in media were shown as the secreted amount per 10^7 cells
[d] The concentrations of calcium (Ca) and inorganic phosphate (Pi) were assayed usign supernatant media

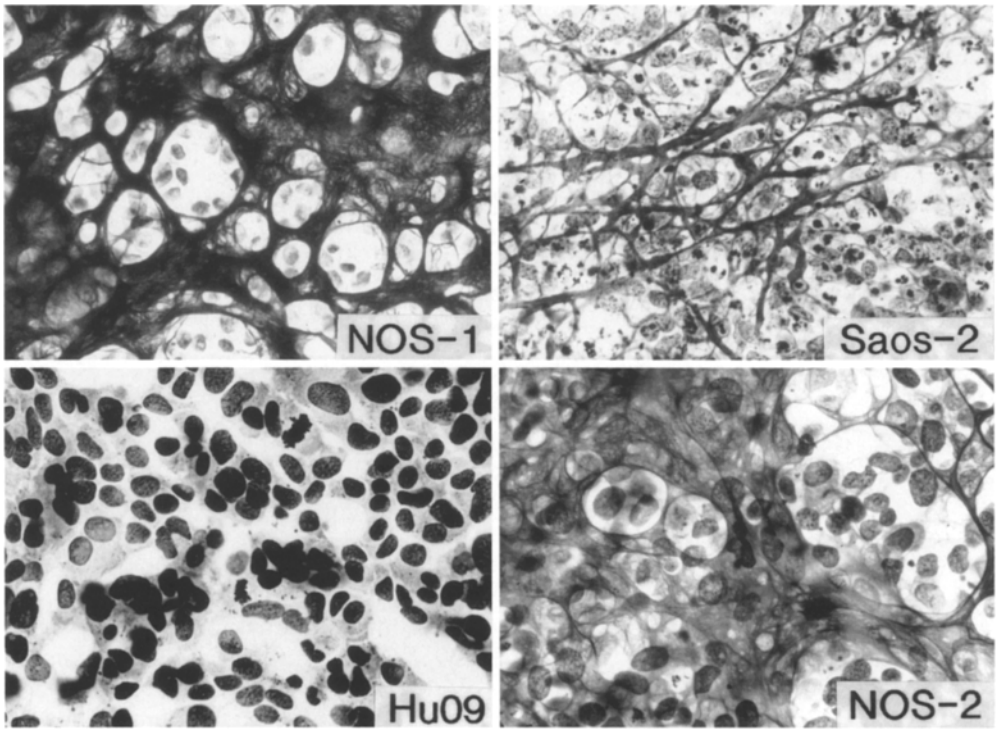

Fig. 4 Extracellular collagen bundles in vitro. The NOS-1 cells formed thick collagen bundles in vitro. The Saos-2 and NOS-2 cells formed thin collagen bundles, but HuO9 cells formed no collagen fibers. Silver impregnation, ×360

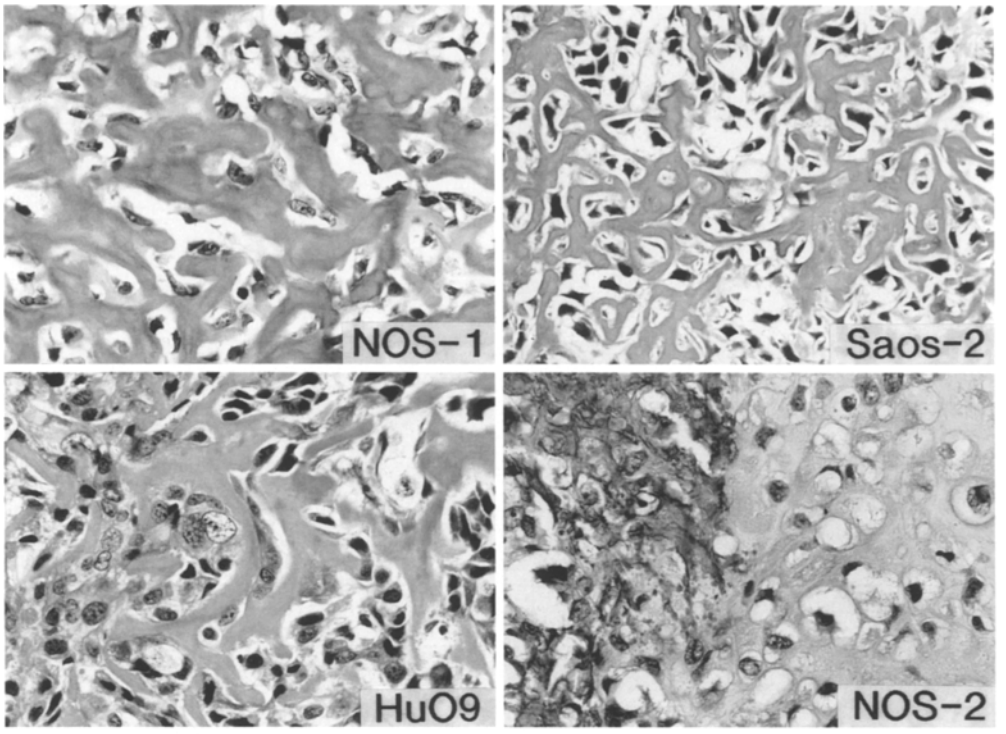

Fig. 5 Histology of transplanted tumours in nude mice. The NOS-1 cell tumours formed thick bone and osteoid in nude mice. The Saos-2 and HuO9 cell tumours formed bone, and the NOS-2 cell tumours formed cartilage in nude mice. Haematoxylin-eosin, ×360

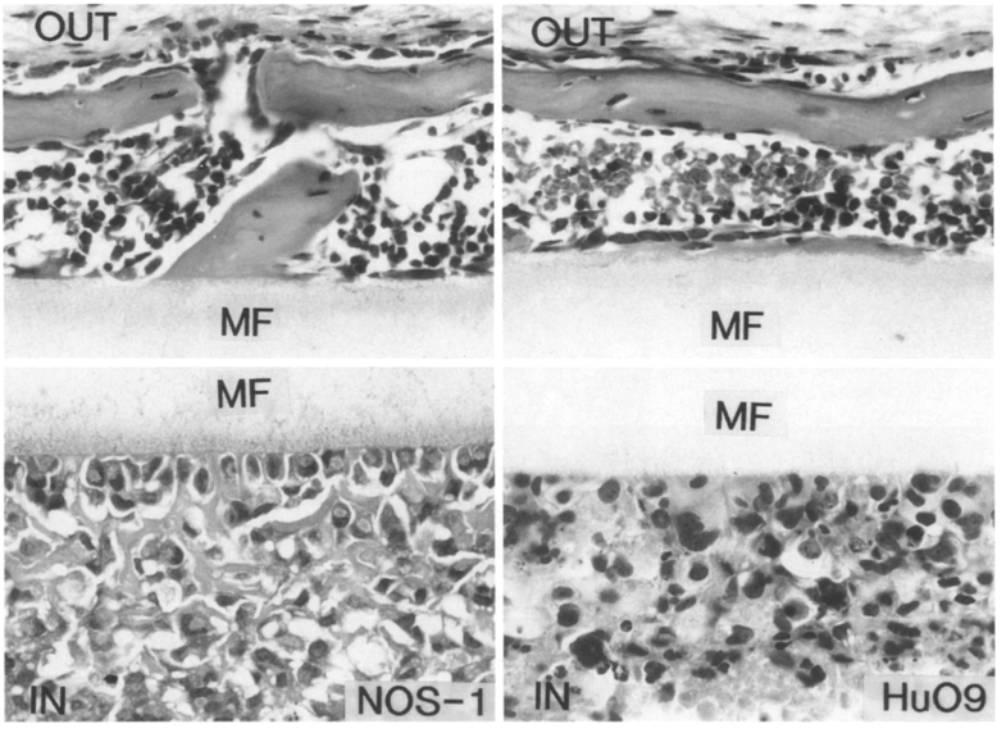

Fig. 6 Histological features of NOS-1 and HuO9 cells in diffusion chambers implanted in nude mice. NOS-1 cells induced bone tissue in both inside and outside the diffusion chamber, while HuO9 cells induced bone tissue only on the outside of the diffusion chamber. *OUT* outside the diffusion chamber ; *IN* inside the diffusion chamber; *MF* Millipore filter. Haematoxylin-eosin, ×360

brous structures were detected in the HuO9 (Fig. 4) or the OST and MKN7 cell lines. Osteocalcin levels in the media were relatively high in the HuO9, NY and NOS-1 cell lines. However, osteocalcin was not detected in the Saos-2 cell line (Table 7). NOS-1, Saos-2, HuO9, NOS-2 and MKN7 cells produced high activities of ALP, viz more than 300 mIU/10^7 cells. These cell lines markedly increased Pi concentration in the media after 48 h of cultivation (Table 7).

The tumorigenicity and bone formation in nude mice are summarized in Table 8. The NOS-1 cell tumours formed abundant, thick bone and osteoid, while the Saos-2 cell tumours and HuO9 cell tumours formed thin bone and osteoid. The NOS-2 cell tumours contained os-

Table 8 Bone and osteoid formation in transplanted osteosarcoma cell tumours in nude mice [a] (– negative, + occasionally positive, ++ frequently positive, *NT* not tested)

Cell ine	Xenografted tumour				No. of chamber containing viable cells	Diffusion chamber			
	Tumori-genecity	Bone	Osteoid	Cartilage		Inside		Outside	
						Bone	Osteoid	Bone	Osteoid
NOS-1	3/3	++	++	–	3/3	++	++	+	+
Saos-2	2/9	+	+	–	NT				
HuO9	3/3	+	+	–	3/3	–	–	+	+
NOS-2	3/3	–	+	+	NT				
OST	3/3	–	–	–	NT				
NY	0/9				NT				
HUO-3N1	0/9				NT				

[a] The evaluation was carried out 8 weeks after inoculation

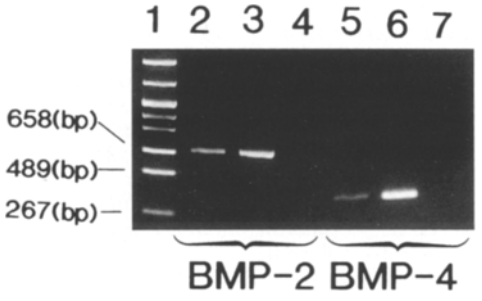

Fig. 7 Detection of BMPs expression by the polymerase chain reaction in NOS-1 and Hu09 cells. *Lane 1* pHY size marker; *lanes 2, 5* NOS-1 cells; *lanes 3, 6* HuO9 cells; *lanes 4, 7* negative control (without cDNA)

teoid and cartilaginous tissue with enchondral calcification (Fig. 5).

Further histological analyses of NOS-1 and HuO9 cells in diffusion chambers implanted in nude mice revealed that NOS-1 cells produced bone tissue both inside and outside the chamber, while HuO9 cells produced bone tissue only outside of the chamber (Fig. 6; Table 8).

PCR amplification of cDNA from the two osteosarcoma cell lines, NOS-1 and HuO9, revealed that BMP-2 and BMP-4 were expressed in both cell lines. The HuO9 cell line showed more striking bands of both BMP-2 and BMP-4 cDNAs than did the NOS-1 cell line (Fig. 7).

Discussion

Human cancer cell lines are grown in various kinds of culture medium. We established a human osteosarcoma cell line designated NOS-1 using a culture medium consisting of RPMI-1640 supplemented with 10% FBS [8]. Although NOS-1 cells easily formed transplanted tumours containing abundant osteoid and bone tissues in nude mice, we were unable to obtain clear evidence of mineralization in vitro mimicking bone formation. While some osteogenic cell lines have been shown to mineralize in vitro using alpha MEM supplemented with beta GP [6, 12], they are not human cells. The present study showed that such medium is also effective for mineralization of human osteosarcoma cells in vitro. Mineralization of NOS-1 cells on the collagen bundles was observed only in culture medium with alpha MEM supplemented with FBS and beta GP. Mineralization of bone requires certain amounts of Ca and Pi in the area of bone formation in vivo [21]. Some investigators have emphasized that having the proper Ca concentration in the medium is important for mineralization in vitro [2], and it is clear that organic phosphates provide Pi for mineralization. Beta GP, an organic phosphate, has commonly been used as a source of Pi in vitro [19, 23]. RPMI-1640 medium alone contains a considerable concentration of Pi , but contains only a low concentration of Ca (less than half the concentration in human serum). Beta MEM medium alone contained a moderate concentration of Ca, which corresponds to normal serum levels. Moreover, our data suggest that alpha MEM supplemented with beta GP promotes osteocalcin production. These phenomena were also observed in a mouse osteoblastic cell line, MC3T3-E1 [3]. However, our preliminary examination confirmed that beta GP at greater than 5 mM frequently induced nonphysiological mineral deposition in human osteosarcoma cell cultures, as shown by Chung et al [4] in rodent bone cell cultures. Alpha MEM medium contains 50 μg/ml of ascorbic acid, while RPMI-1640 medium contain none. Our findings show that there was no significant difference on collagenous extracellular matrix formation by NOS-1 cells between the culture fed with RPMI-1640 medium and the culture fed with alpha MEM medium. Although it is stressed that ascorbic acid is essential for considerable amount of extracellular collagen formation in MC3T3-E1 cells [6], ascorbic acid seems not to be essential for NOS-1 cells. However, the first step in this investigation clarified that alpha MEM is better than RPMI-1640 for bone formation experiments in vitro.

In the second part of this investigation, seven human osteosarcoma cell lines were compared with regard to their respective abilities to induce bone formation under the same culture conditions or the same xenograft conditions. NOS-1 cells most frequently produced mineralized

bone-like matrix (MBLM) in vitro. This finding suggests that the major components of the abundant bone and osteoid tissues in the transplanted tumours are mainly supplied by tumour cells themselves. Saos-2 cells also produced MBLM in vitro, and formed transplanted tumours containing bone and osteoid tissues; however, osteocalcin was not detected in the culture media of Saos-2 cells under the present culture condition. Osteocalcin is considered to participate in the regulation of hydroxyapatite growth [14], and although some investigators have reported that osteocalcin favoured mineralization in vitro [5], our data suggest that osteocalcin is not always necessary for MBLM formation in vitro.

Although HuO9 cells did not produce MBLM in vitro, they formed transplanted tumours containing bone and osteoid. The absence of bone-like matrix formation in the HuO9 cell line was presumably due to the lack of collagen synthesis. Further histological analysis of HuO9 cells in diffusion chambers implanted in nude mice showed that HuO9 cells induce bone tissue only on the outside of the chamber. These findings suggest that collagen, as a bone matrix, was produced by some mouse cells in the transplanted tumours of HuO9 cells. Some investigators have reported that human osteosarcoma produced BMP which was able to induce bone formation in nude mice [18]. The findings of PCR showed that HuO9 cells also expressed BMP-2 and BMP-4, which are strong inducers of bone formation [7, 22].

In the NOS-2 cell line, mineralization of collagen bundles in vitro seemed to mimic intracartilaginous mineralization.

The ALP-producing gastric adenocarcinoma cell line MKN7 also formed foci of calcium deposition in vitro. ALP is well known to be related to mineralization of bone. Our data indicate that ALP-producing tumour cells commonly induce dystrophic calcification in vitro regardless of cell type, which can be detected by von Kossa's method. Accurate estimation of mineralization in vitro should be done by ultrastructural examination, however, as shown in the present study. Since collagen accounts for over 90% of the organic phase of bone, it clearly plays a most important part quantitatively in bone formation, but although the NY cell line synthesized more collagen than the Saos-2 cell line in vitro, it showed little ALP activity and never produced MBLM in vitro. Cultured fibroblasts also exhibited similar characteristics. These data indicate that ALP is essential for mineralization of bone.

In conclusion, the most important factors for bone production in the tissues of osteosarcoma are the collagen and ALP produced by osteosarcoma cells. However, tumour cells which produce both ALP and BMP can form bone tissue within the tumour irrespective of the amount of collagen produced by the tumour cells themselves.

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