BMP-4 and BMP-6 Involvement in the Osteogenic Properties of the HeLa Cell Line

IWONA EWA KOCHANOWSKA,* KRZYSZTOF WLODARSKI,† ANDRZEJ WOJTOWICZ,§ AGNIESZKA KINSNER,‡ AND KAZIMIERZ OSTROWSKI[1]†

Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland; and Departments of †Histology and ‡Transplantology and §Dental Surgery Clinic, Medical Academy of Warsaw, 02-004 Warsaw, Poland

The heterotopically induced *ossicles* are used in our research on bone tissue. The ossicles are formed in the thigh muscle of BALB/c mice under the influence of injected suspension of 3 × 10^6 HeLa cells. We postulate that the mechanism of bone induction is based on the secretion of bone morphogenetic proteins BMP-4 and BMP-6 by the grafted HeLa cells. This was proved by the use of specific immunohistochemical reaction and Western blots of conditioned culture medium. It seems that HeLa cells secrete BMPs continuously into the culture medium, even without contact with the mice muscle tissue, were induction of bone tissue is observed.

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Key words: bone induction; HeLa cells; bone morphogenetic proteins BMP-4 and BMP-6

There are four experimental systems described in the literature that are used for induction of heterotopic osteogenesis. The oldest one is bone induction after transplantation of autologous transitional epithelium from urinary bladder into dog's abdominal muscles (1). The second system described induction of heterotopic osteogenesis after implantation of decalcified bone matrix (2). The third system discovered that transplantation of some established transformed cell lines evoke in their vicinity cartilage formation, gradually substituted by bone, which recapitulates enchondral bone formation (3). The fourth one, an orthotopic system of bone induction described by one of us, concerned huge periosteal bone formation after topical injection of Maloney sarcoma virus (4). Most systems, except the last one, concern the induction of enchondral osteogenesis in the thigh muscle of experimental animals such as guinea pigs, mice, or rats.

The research on the nature of the inductors present in the decalcified bone matrix (5–7) was fruitfull, and the set of bone morphogenetic proteins (BMPs) was classified as morphogens and was well reviewed (8). The molecular nature of BMPs is defined. These proteins are 30- to 38-kDa homodimers that are synthesized as prepropeptides of approximately 400–525 amino acids (8–10). Heterodimeric combinations of BMP are also present *in vivo*, and there is experimental evidence suggesting that BMP heterodimers are more osteoinductive than homodimers (11). Cleavage of the variable length precursor protein occurs before secretion (8, 12). The mechanism of heterotopic bone induction is not well defined in other experimental systems. Several members of BMP transforming growth factor superfamily have been cloned and expressed as recombinant proteins (7, 13, 14).

Our group is interested in the research performed on the *ossicles* induced in the thigh muscles of mice after injection of the of 3 × 10^6 HeLa cells into thigh muscles of BALB/c mice. The heterotopically induced ossicles are formed when HeLa cells are, at the time of injection, protected by single dose of 5 mg of cortisone acetate per mouse. The mechanism of induction is not known. The enchondral osteogenesis starts on the 6th day after transplantation. The fully developed ossicle, usually containing bone marrow, is observed after 14 days. Afterward, the process of resorption of the induced ossicles starts, which is described by pathologists as resorption *ex inactivitate.*

The aim of this paper is to elucidate the mechanism of heterotopic bone induction in mice by transplanted xenogenic (human) cancer cells of HeLa cell line. The reasonable idea was to look for secretion of BMPs by the used HeLa cell line. This was performed by immunohistochemical technique as well as by biochemical techniques used routinely in molecular biological research.

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[1] To whom requests for reprints should be addressed at Department of Histology, Medical Academy of Warsaw, Chalubinskiego 5, 02-004 Warszawa, Poland. E-mail: kostrows@ib.amwaw.edu.pl

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Materials and Methods

Grant applications (research), including animal studies, were approved by the Ethical Committee of The Polish Committee for Scientific Research.

Twenty BALB/c mice were used for this experiment. The heterotopic bone induction was realized for reasons connected with aims defined in our grant concerning osteoporosis research.

The HeLa cells, called in American Type Culture Collection CCL-2, are human cancer cells derived from adenocarcinoma of the uterine cervix. The cells were cultivated for 31 years from the biopsy of black female patient.

Histochemical Ossicle Analysis. Muscle tissue samples containing the induced ossicles were excised and fixed in Bouin solution, and were demineralized in 10% formic acid. Paraffin sections 7 μm thick and H & E stained were used for overview.

Immunohistochemical Ossicle Analysis. HeLa cells inoculation area, containing the grafted cells and the induced ossicles were excised, fixed in 10% formalin, demineralized in EDTA, embedded in paraffin, and cut into 7-μm-thick sections. The sections were deparaffinized in xylene, rehydrated in descending concentration of alcohols, and after microwave pretreatment in citrate buffer (pH 6.0) three times for 5 min at 750 W, they were incubated overnight at 4°C with anti-BMP-4 or anti-BMP-6 mouse monoclonal antibodies (Novocastra Laboratories, Tyne, UK) at a dilution 1:30, according to the manufacturer's protocol. Evidence for specificity and information on cross-reactivity of these antibodies was provided by the manufacturer. Details of the employed procedure were reported in our previous publications (15, 16) and by other authors (17–19). After the second incubation with biotin-conjugated anti-mouse antibody, the sections were incubated with an avidin-biotin-peroxidase reagent (Novostatin Super ABC System, Novocastra Laboratories). A strongly positive sample of breast-carcinoma tissue served as positive control for BMP-6 immunostaining. The H & E staining and immunochemical expression of BMP-4 and BMP-6 by induced bone tissue were examined and photographed on a Nikon E400 microscope. As BMP-specific monoclonal antibodies react with human/mouse ligand, the negative controls using nonimmune mouse serum were omitted (20). Additionally, the high homology of human and mouse BMP-4 and BMP-6 (7, 13, 21, 22) justify the use of murine antibodies for detection of human BMPs.

HeLa Cell Propagation. Cells were incubated in minimum essential medium Eagle with fetal bovine serum, 10% at 37°C in a CO_2 incubator, and afterward were treated for 48 hr in serum-free medium at the same conditions.

Media Collection. After HeLa cell cultures were centrifuged, supernatants were collected and concentrated by ~20-fold using an ultrafiltrating system. Concentrated cell-conditioned media were protected with .05% sodium azide.

Electrophoresis and Electrotransfer. Aliquots of concentrated cell-conditioned media were electrophoresed in SDS-PAGE (12%, v/v) at 4°–8°C under nonreducing and reducing conditions (probes were boiled for 30 min) using protein mixture (Amersham Pharmacia Biotech, Buckinghamshire, UK) as standards. Protein bands were detected by Coomassie brilliant Blue R 250 (Sigma, St. Louis, MO) staining. Electrotransfer of proteins from SDS-PAGE gel to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) was performed for 2hr at 4°C.

Western Blot Analysis. All subsequent steps were performed at room temperature and with continuous mixing. For determination of BMP-4 and BMP-6 molecules secretion by HeLa cells, Western blot analysis were performed using anti-human BMP-4 mouse monoclonal antibodies (Novocastra Laboratories) and anti-mouse BMP-6 (Novocastra Laboratories). The nitrocellulose membrane was blocked with Tris-buffered saline (TBS: 50 mM Tris/HCl and 0.15 M NaCl) and 0.5% casein (Sigma), pH 7.4 for 1 hr and was then incubated for 1.5 hr with monoclonal antibody anti-BMP solution (1 μg/ml) in TBS and 0.05% Tween 20 (TBST buffer), pH 7.4. After four washes (once with TBST buffer and three times with TBS) the band-BMP↔monoclonal antibody complex was detected using goat anti-mouse IgG antibody coupled with alkaline phosphatase (Promega, Madison, WI) 7500 times diluted in TBST buffer and applied for 1 hr. The membrane was then washed and treated with BCIP/NBT (Promega) in 100 mM Tris/HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5, an alkaline phosphatase substrate.

Results

Histochemical Examination. The grafted HeLa cells in our study exhibited significant histological feature—bone induction. The evidence for heterotopic bone formation (ossicle) in mouse muscle after HeLa cells implantation is showed in Figure 1.

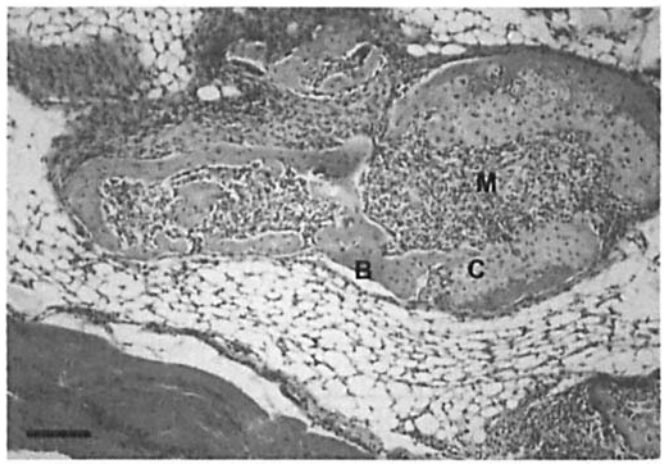

Figure 1. General view of heterotopically induced bone (B) ossicle containing bone marrow (M) and the rest of induced chondroid (C) tissue on the 19th day after implantation of HeLa cells. H&E staining. The bar corresponds to 40 μm.

Immunohistochemical Analysis. The immunohistochemical reactions for BMP-4 and BMP-6 were positive as shown by the brown staining on the tissue samples containing the induced ossicles (Figs. 2 and 3).

Electrophoresis. Electrophoretic cell-cultured media examination showed significant protein secretion. Results obtained under nonreducing and reducing conditions were similar (Fig. 4). The main bands detected under both conditions migrated at 62 kDa.

Western Blot Analysis. Secretion of BMP-4 and BMP-6 was performed by Western blot analysis of conditioned medium from cultured HeLa cells (Fig. 5). Under nonreducing and reducing conditions, the mouse monoclonal antibodies anti-BMP-4 detected a single immunoreactive band migrating at ~62 kDa. Under the same conditions, the mouse monoclonal antibody anti-BMP-6 from Novocastra Laboratories detected weak single immunoreactive band migrating at ~46 kDa (results not shown).

Discussion

The interaction of malignant cells with skeletal tissues is not fully elucidated. The main effect is usually osteolysis connected with the release of parathyroid hormone-related peptide. New bone formation is particularly abundant in association with certain tumors, such as prostate carcinoma. A variety of osteoblastic growth factors such as bone morphogenetic proteins may be involved (23).

Bone morphogenetic proteins were documented as the inductors of osteogenesis in the implants of decalcified bone matrix (5–7), in Saos osteosarcoma cells (24), and also in their extracts (25). Only few data are available on other systems of heterotopic bone induction (26–31). As we are using ossicles induced by HeLa cancer cell line in our bone research, we attempted to elucidate the mechanism of induction. HeLa cells, as xenogenic transplant into the thigh muscle of mice, survive only few days under the protection of single dose of 5 mg of cortisone acetate per mouse and afterward are destroyed by recovered immunological mechanisms. These 6 days are enough to initiate the enchondral induction of osteogenesis. We tried to find out if the BMPs play role in this experimental system. Using immunohistochemical reaction and Western blots, we identified two bone morphogenetic proteins, BMP-4 and BMP-6, in the described bone inducing system.

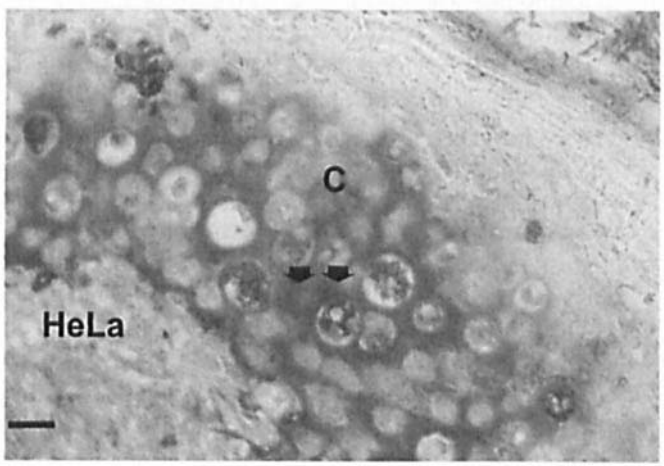

Figure 2. Immunohistochemical reaction showing (arrow) positive reaction for the presence of BMP-6 in the vicinity of HeLa cells (HeLa) and induced chondroid (C) tissue on the 7th day after implantation of HeLa cells. The bar corresponds to 10 μm.

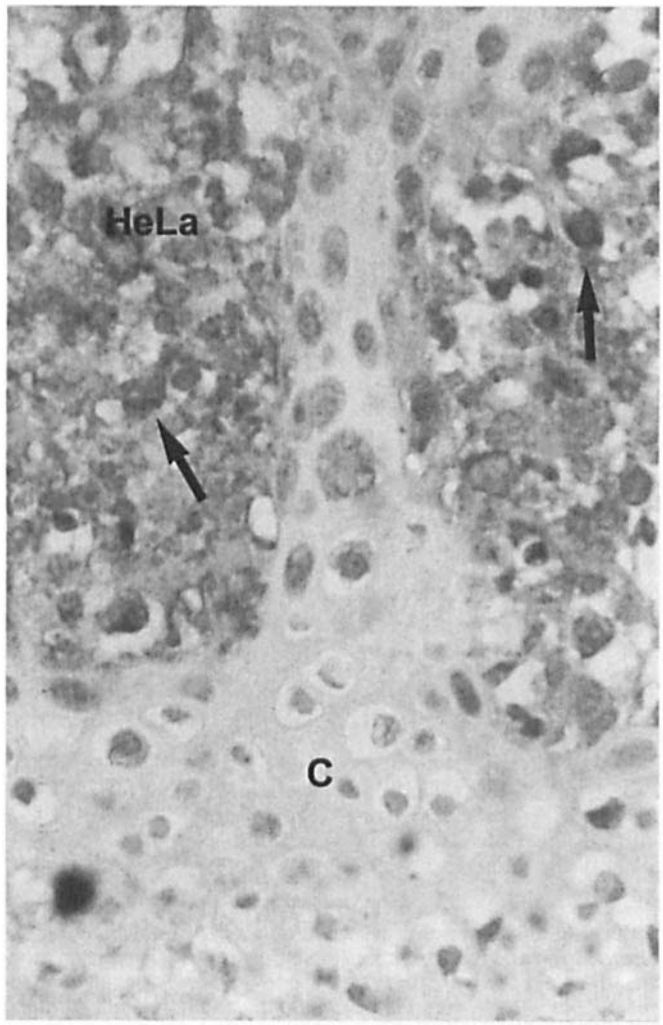

Figure 3. Immunohistochemical reaction showing (arrow) positive reaction for the presence of BMP-4 in the vicinity of HeLa cells (HeLa) and induced chondroid (C) tissue on the 7th day after implantation of HeLa cells. The bar corresponds to 10 μm.

The main goal of the first experimental system, based on immunohistochemical technique, was to prove the BMP activity in the process of ossicles induction. We achieved it using specific mouse monoclonal antibodies anti-BMP-4 and anti-BMP-6 as shown in Figures 2 and 3. These results are in agreement with our other results obtained in the second experimental system employing the Western blot technique. We have been interested in BMP-4 and BMP-6 detection in the HeLa cells conditioned medium. We expected that bone induction activity of HeLa cells could be caused by bone morphogenetic proteins secretion. It was demonstrated that devitalized human Saos 2 osteosarcoma cells or their extracts contain bone morphogenetic proteins and are

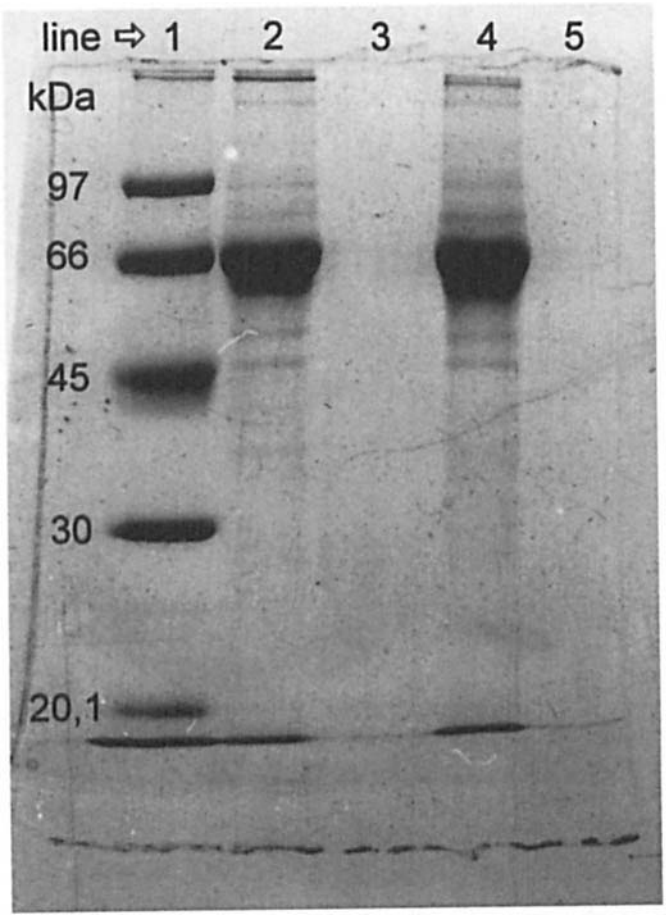

Figure 4. Electrophoretic cell cultured media investigation under nonreducing and reducing conditions. Cultured media were concentrated 20-fold and were analyzed by polyacrylamide gel electrophoresis together with their ultrafiltrated supernatants obtained during concentration. The first line indicates molecular weight markers; the second and fourth lines indicate 20-fold concentrated media; the third and fifthlines indicate ultrafiltrated supernatants. Electrophoresis was performed under nonreducing (lines 2 and 3) and reducing (lines 4 and 5) conditions.

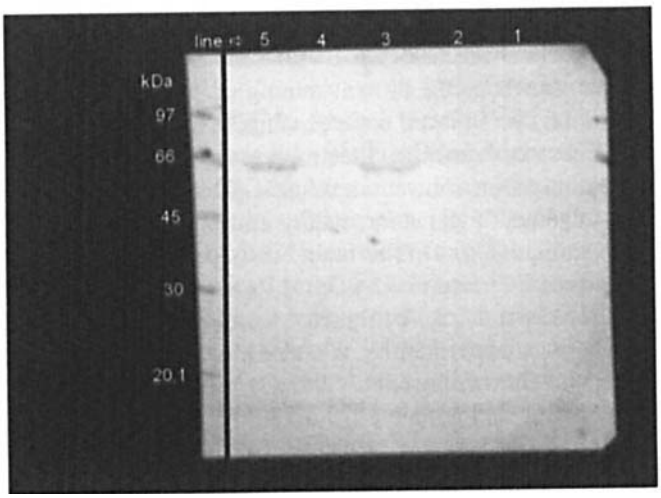

Figure 5. Western blot analysis of BMP-4 secretion by HeLa cells *in vitro*. Western blot analysis showed the presence of BMP-4 precursor in 20-fold concentrated HeLa cell cultured media after electrophoresis under non-reducing (line 3) and reducing (line 5) conditions. The first line indicates molecular weight markers; the second and fourth lines indicate ultrafiltrated supernatants.

able to induce bone formation (25). We were unable, however, to achieve heterotopic bone induction by injection of HeLa cell-conditioned medium into thigh muscles, most probably because of inadequate concentration of inducing activity (32). Anderson *et al.* (24) reported that the bone induction by FL human amnion cells is connected with living cells, as the killed cells as well as their cultured medium have no inducing property. The detected protein band presented in Figure 4 as migrating at ~62 kDa was identified as BMP-4 in reaction with specific anti-BMP-4 antibodies on the nitrocellulose sheet (Fig. 5). Too weak for photographical documentation, the BMP-6 band was also detected on the nitrocellulose sheet after anti-BMP-6 monoclonal antibodies reaction as migrating at 45 kDa.

As all TGF-β family members, the BMP-4 and BMP-6 are synthesized as an inactive precursor and are proteolytically activated by cleavage following the amino acids motif (12) to yield a C-terminal mature protein.

Mature BMP-4 and BMP-6 are formed from a large precursor of 408 amino acids and 513 amino-acids, respectively. Precursor forms of BMPs are monomers. Mature forms of BMP form biologically active homodimers. In the present study, the identity of BMP-4 was demonstrated using two methods, molecular mass determination in SDS-PAGE technique (Fig. 4) and immunological identification in reaction with specific antibodies by Western blot analysis (Fig. 5). Manufacturers of the employed antibodies (Sigma and Novocastra Laboratories) recommend their products not only for BMP-4 identification, but also for neutralization the bioactivity (Sigma). It is generally accepted that biological activity of each protein depends on conservative sequences that cannot be modified during proteolytic precursor protein processing. Therefore, antibodies recommended for protein neutralization must be directed against these functional sequences. It is also well known that such sequences must be present in both precursor and mature protein forms. Moreover, no evidence for cross-reactivity of these antibodies with other than BMP-4 proteins was reported. Based on the manufacturer's recommendation, we were able to identify the major band as the monomeric precursor form of bone morphogenetic protein. Similar results indicating the identity of the 60-kDa protein with BMP-4 precursor protein were obtained (33, 34). Moreover, based on amino acid sequence homology, BMP-4 is placed into the same group of BMPs together with BMP-2. Recombinant human BMP-2 expressed by Chinese hamster ovary cells showed several forms of this protein, including the uncleaved 60-kDa precursor (35).

Comparison of human and mouse BMP-4 and BMP-6 mature regions reveal 96% and 91% amino acid identity, respectively (7, 13, 16, 17, 36, 37). Because of this homology, it is interesting to clarify the origins of bone morphogenetic proteins during heterotopic bone development. The precursor protein processing event has been proposed to regulate the secretion and/or diffusion of BMPs, thereby

controlling the range over which these molecules can signal. An inactive BMP precursor is proteolytically activated by the proprotein convertases, a family of seven structurally related serine endoproteases. Furin, the first member of this family to be characterized, is a membrane-associated, calcium-dependent serine endoprotease (38). Only precursor forms of BMP-4 and BMP-6 secreted by HeLa cells into the culture medium were detected in our experiments. It is probably due to the absence the proteolytic enzyme system in HeLa cells yielding the precursor cleavage.

Based on the results presented in this paper, we can postulate that the induction of heterotopic ossicle by grafted HeLa cells depends on secretion of BMPs by these cells.

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