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PRELIMINARY REPORT

OSTEOGENESIS BY FACTOR(S) ISOLATED FROM MOUSE OSTEOSARCOMA CELLS IN COMBINATION WITH COLLAGEN

NATSUO YASUI, TAKAHIRO OCHI, KUNIO TAKAOKA and KEIRO ONO

Department of Orthopaedics, Osaka University Medical School, 1–1–50, Fukushima, Fukushima-ku, Osaka 553, Japan

YOZO NAKATA

Institute for Cancer Research, Osaka University Medical School, 1–1–50, Fukushima, Fukushima-ku, Osaka 553, Japan

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An osteogenic factor(s) was extracted with acetic acid from cultures of mouse osteosarcoma cells. The extract was mixed with acid-soluble human skin collagen as a carrier and the mixture was aggregated by dialysis against 0.02 *M* Na_2HPO_4. Thirty days after implanting the resulting precipitate into the muscle pouch of mice, new bone formation was observed at the site of implantation. Only fibrous tissue was formed in controls implanted with acid-soluble collagen alone. Therefore, it was concluded that the osteogenic factor(s) from the osteosarcoma cultures is an acid-soluble, non-dialyzable substance that interacts with collagen.

Since the first report by Huggins (1930), induction of heterotopic osteogenesis has been described in various experimental systems (Reddi, 1976). Many investigators have attempted to isolate the bone-inducing agent from devitalized tissue or matrix, but results have been poor. The two main difficulties in studies have been how to solubilize the substance and how to assay the biological activity of the resulting solubilized preparation; when soluble material is implanted even if it is lyophilized, it will be readily absorbed and metabolized by recipient tissue before the osteoblastic reaction occurs.

There have been many studies on the mechanism of bone formation, and particularly the cells involved and the factors controlling the proliferation and differentiation of the osteogenic cells. For new synthesis of bone, the major components, collagen and hydroxyapatite, must be present in sufficient quantity and must be localized around the bone-forming site.

We report herein that skin collagen, implanted with factors derived from cultures of mouse osteosarcoma cells, induced new bone formation in mice. Control implants of skin collagen alone did not induce bone forma-

tion, indicating that components derived from the cultured osteosarcoma cells must be important for its formation. This new bone-forming factor(s) is an acetic acid soluble, non-dialyzable substance which may interact with collagen. The experimental procedures were as follows.

Acid-soluble collagen was extracted from human skin obtained at the time of surgical amputation of a lower limb of a 10-year-old patient with a malignant tumor. It was purified by the procedure of Piez et al. (1963), lyophilized and stored frozen until use.

The cells used in this study were an established clonal cell line, BFO osteosarcoma (Amitani and Nakata, 1977), initially obtained through Dr. M. P. Finkel of Argone National Laboratory, Ill., USA.

A sample of 5×10^8 confluent BFO osteosarcoma cells was washed twice with Tris-dextrose solution and cultured for 24 hr in serum-free Dulbecco's modified Eagle's medium containing ascorbic acid (0.1 m*M*). Then the cells were scraped into 30 ml of 0.5 *M* acetic acid, mixed with 0.1 mg of phenylmethane-sulphonyl fluoride, sonicated in an Ultrasonics apparatus, and combined with the culture medium. The resulting mixture was centrifuged at $20{,}000\times g$ for 30 min and mixed with 5.0 mg of acid-soluble human skin collagen as a carrier. The solution was gently stirred at 4°C overnight. Then insoluble material was removed by ultracentrifugation and solution was dialyzed against 0.02 *M* Na_2HPO_4 until it became pH 8.0. The aggregated material was collected by centrifugation and directly implanted into the muscle pouch of ddY mice.

The animals were killed 20 to 30 days later and the implants were examined microradiographically (soft X-ray) and histologically (Hematoxylin-Eosin staining). High radio-

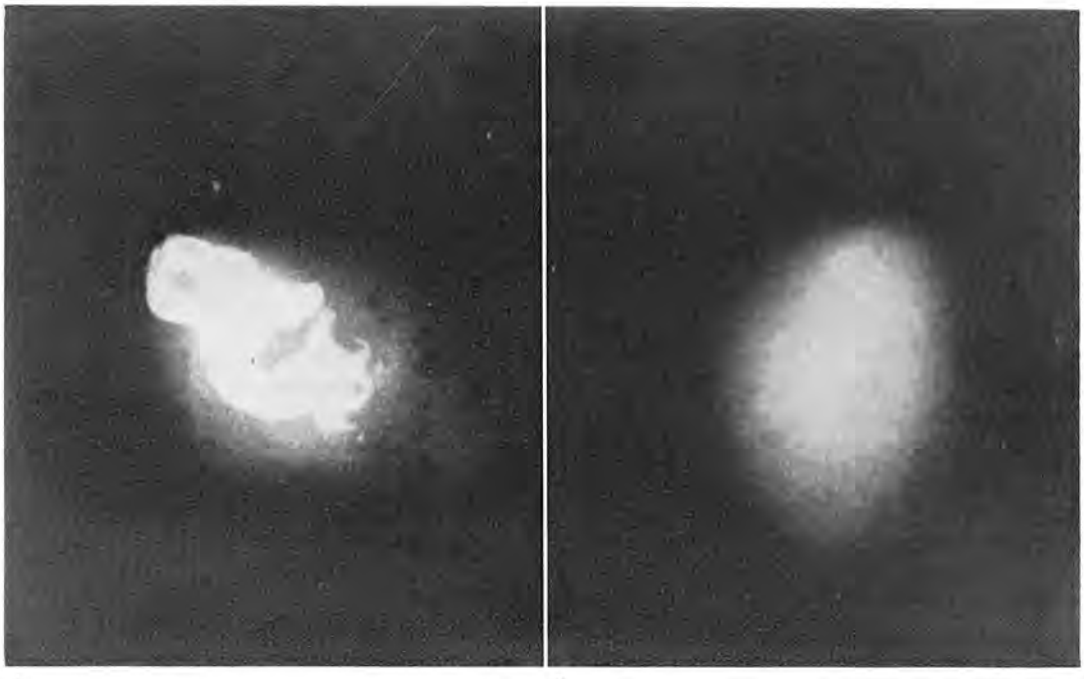

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FIGURE 1. Soft X-ray films of implants. Implant: (a) Culture extract combined with carrier collagen, (b) Carrier collagen alone.

density shadows suggesting the existence of calcified tissue were detected microradiographically within 3 weeks after the operation (Fig. 1). Histological examination showed that after 30 days the implanted material had been replaced by new bone and partially remodeled into lamellar bone. Osteocytes, osteoblasts, haematopoietic bone marrow tissue, and deposition of lipids were observed (Fig. 2). When 5.0 mg of acid-soluble human skin collagen alone was implanted into the muscle pouch of ddY mice, the implant was replaced by fibrous tissue only, and no new bone formation was detectable either microradiographically or histologically. Moreover, when 5×10^8 embryonic fibroblasts from C3H/He mice, the hosts of BF osteosarcoma, were cultured and subjected to the same procedures no new bone formation was detected. Therefore, we solubilized the bone forming substance from BFO osteosarcoma cells.

The components derived from cultured materials of BFO osteosarcoma cells were examined by radioisotopic labelling. The cells were cultured for 24 hr in Dulbecco's medium supplemented with ^{14}C-proline (2.5 μCi/ml), and then submitted to the extraction procedure described above. The material aggregated with carrier collagen were dissolved in 0.01 *M* Tris-HCl, pH 6.8, 2 *M* urea, 0.1% SDS, and denatured at 60°C for 20 min. Then it was submitted to slab gel electrophoresis with 3% stacking gel and 5% separating gel by the method of King and Laemmli (1971). The presence of ^{14}C-proline-labelled materials aggregated within carrier collagen

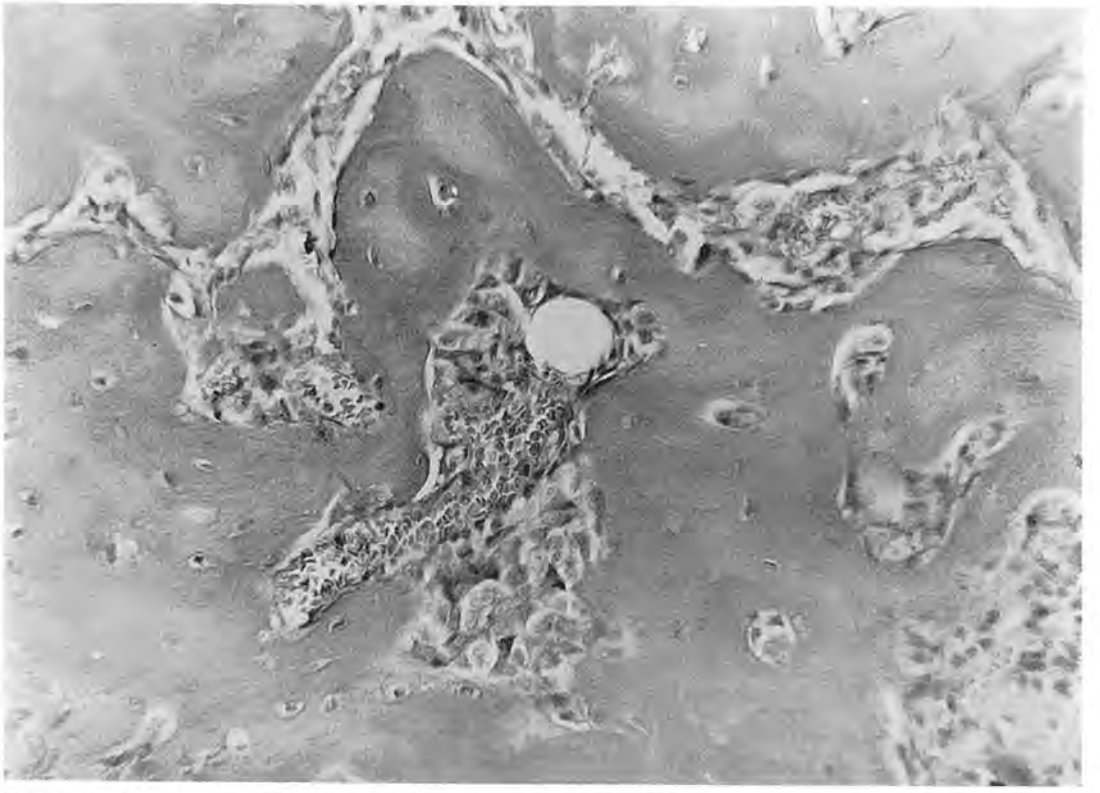

FIGURE 2. New bone and bone marrow tissue replacing implanted osteogenic substance with carrier collagen after 30 days. (Haematoxylin-Eosin staining, ×200)

FIGURE 3. Fluorographic detection of ^{14}C-proline-labelled materials separated by SDS-polyacrylamide gel electrophoresis. The positions of α and β chains of carrier collagen are shown by arrows. For detail, see text.

fibers was demonstrated by fluorography by the method of Bonner and Lasky (1974). The gel was stained and destained, dehydrated in dimethylsulphoxide, soaked in a solution of 2,5-diphenyloxazole in dimethylsulphoxide, dried and exposed to RP Royal "X-Omat" film at −70°C. The positions of the α and β chains of carrier collagen are shown by arrows in Fig. 3. The ^{14}C-labelled materials synthesized by BFO osteosarcoma cells consisted of a component of rather larger molecular weight than α 1 and α 2 chains (M.W. 100,000), and small molecules that migrated into the gel bottom (M.W.$<$30,000). These non-dialyzable materials aggregated within reconstituted collagen fibrils and were released from the collagen in the presence of SDS and urea.

Thus, we isolated an osteogenic factor using an in vitro cell culture system as a simple homogeneous source of this factor and acid-soluble skin collagen as a carrier. Collagen is a weakly antigenic, rigid rod-like, cationic protein, which is an insoluble crystalloid under physiological conditions. In vitro self-aggregation of native collagen molecules into fibrils can be demonstrated by several methods (Wood, 1964). An extract of collagen with acetic acid forms opalescent fibers when dialyzed against Na_2HPO_4 at low ionic strength. By combination of acid-soluble carrier collagen with an extract of cultured materials of BFO osteosarcoma cells, it was possible to detect the osteogenic activity of a small amount of substance. Using this procedure, we were able to implant the material in a solid state, so that its ostogenic potential could be localized at the site of implantation. In fact, 30 days after the implantation, the material was replaced by heterotopic new bone and haematopoietic bone marrow tissue. Whether the osteogenic factor has similar properties to carrier collagen, or whether it interacts chemically with collagen during the extraction procedure, remain to be determined.

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