Bone induction by fetal and adult human bone matrix in athymic rats

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In the rat the intramuscular implantation of demineralized rat bone matrix induces local bone formation. In adult primates, however, allogenous bone matrix induces little or no bone formation in extraskeletal sites. To assay inductive properties, human demineralized bone matrix from 6 adult donors and 4 fetuses was implanted intramuscularly in athymic rats for 6 weeks. Fetal and adult matrix implants yielded about the same amount of bone: about half of the bone yield from rat or rabbit matrix in the same model. We conclude that human bone matrix has inductive properties and that failures to induce bone formation in adult primates may be due to an inability by the recipients to respond to inductive stimuli of adult bone matrix.

Demineralized rat bone matrix when implanted intramuscularly in rats induces bone formation by cells from the recipient site. One or several factors in the matrix are responsible for this phenomenon, probably in combination with surface properties of the matrix (Urist et al. 1983, Eriksson 1985, Reddi et al.1987, Syftestad et al. 1987). Clinical implantation of bone matrix has been successfully used for the treatment of skeletal defects (Glowacki et al. 1981). It is not clear, however, whether human bone matrix in these situations acts by true bone induction or in a more unspecific way, i.e., as a mere osteoconductive scaffold for bone ingrowth (Kakiuchi et al. 1985). Attempts to induce bone formation by intramuscular matrix implantation in adult monkeys have either failed completely (Aspenberg et al. 1988) or showed a very slow response (Hosney and Sharawy 1985). A possible explanation of this could be that the bone matrix of adult primates lacks inductive properties, contrary to, e.g., rodents. Bone induction by human demineralized bone matrix has never been demonstrated by implantation at an extraskeletal site.

In rats, inductive properties of bone matrix decrease with increasing age (Syftestad et al. 1987). The differentiation of bone tissue in response to bone matrix has similarities with fetal organogenesis. Therefore, human fetal bone could be expected to possess bone inductive properties, even if adult bone did not.

We studied the inductive properties of human bone matrix by implantation in athymic rats.

Material and methods

Bone material

Cortical bone was obtained from the femurs of six patients aged 18–45 years. Four women (patients 1–4) were operated on with total hip replacement or osteotomy due to congenital dislocation or Perthes' disease, and the bone was taken from the medial cortex of the femoral neck. One man (patient 5) had a femoral shaft segment resected because of leg-length discrepancy and another (patient 6) had a cortical fragment removed from an open fracture of the femoral shaft. With exception to their orthopedic conditions, all the patients were healthy. All soft tissue was removed from the bone pieces, which were divided into 5-mm fragments and immediately processed as described below.

Four fetuses were obtained after legal abortions with the informed consent of the women. Gestational age was determined by measuring the length of the mineralized portion of the femur. It was 11, 14, 16, and 16 weeks, respectively. The long bones were dissected, the periosteum removed, and the methaphyses cut 1 mm below the physeal cartilage. From the 11-week fetus, we also used ribs, from which we could not completely remove the periosteum.

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Matrix preparation

All the bone specimens were immediately defatted in chloroform-methanol for 2 h, rinsed in methanol, demineralized in 0.6 M HCl for 48 h, rinsed in water, lyophilized, and weighed. A part of the specimens from patients 5 and 6 were milled in liquid nitrogen and sieved to a particle size of 125–335 μm (Chemotec 1090 Sample Mill, Tecator, Helsingborg, Sweden). The bones from the three larger fetuses were divided in proximal and distal halves and weighed. The bones from the 11 weeks' fetus were prepared and implanted together in one portion. The adult bone pieces were divided to match the fetal bone pieces in weight. All the preparations were performed under aseptic conditions.

Evaluation

The bone-matrix pieces were implanted in separate muscle pouches in the anterior abdominal muscle wall of female nu/nu Rowett rats, aged 60 days (Wallenberg Laboratories Lund, Sweden). Most rats had two implants from different donors; but in the later part of the series, each rat received four implants from two donors. The rats were killed after 6 weeks. The rats were then dissected, and two implants from each donor were prepared for histologic studies (demineralized sections, separately stained with hematoxyline-eosine and toluidine blue). The other specimens were used for calcium measurements. These specimens were ashed in a muffle furnace (800 °C, 24 h) and dissolved in 1.5 ml of 6 M HCl. The acid was evaporated in a vacuum centrifuge, and the specimens were redissolved. Calcium was measured in a DACOS machine using the thymol blue reaction (Cossar and Fitzpatrick 1974).

Controls

In a previously reported series, rat bone matrix was prepared, implanted, and evaluated exactly as in this series (Aspenberg et al 1988). That series was immediately followed by the present one; and because all the methods were identical, data from the first series are presently used as positive controls (i.e., rat matrix in athymic rats).

Pulverized matrix from patient no. 5 (eight implants) and from 2 fetuses (two implants each) was implanted in 6 heterozygote nu/+ Rowett rats, which have normal thymus function. Pulverized matrix from patient no. 5 was heated to 120 °C for 20 min to destroy inductive properties, and then implanted in athymic rats (six implants). Two or more matrix pieces from each donor (except the 11-week-old fetus) were checked for calcium content after demineralization. Calcium content is given as microgram calcium per implanted matrix dry weight, mean ± SD.

Results

Histology

Fetal matrix implants. Most of the matrix from the 11-week fetus had been replaced by ossicles (i.e., shells of new bone tissue within the implanted dead bone matrix) — all containing large amounts of hemopoetic marrow (Figure 1). Two implants from each of the other 3 fetuses were studied. All of them contained bone in the form of one or several ossicles within the same matrix piece. They were located in the diaphyseal part of the implants, where the fetal bone had been more compact. There was no bone close to the physeal end. Chondrocytes were rarely seen (Figure 2).

Adult matrix implants. Two implants from each adult donor were studied (except patient 4). Implants from 1 donor (patient 1) had induced no bone, but in 1 of the implants from this donor a few chondrocytes were seen. All the other implants showed abundant cartilage inside canals and cavities (Figure 3). Some of this cartilage had been replaced by bone in the form of ossicles. These were more common in the periphery of the implants (Figure 4). Pulverized implants contained large ossicles.

Calcium content

Fetal. The calcium content of implants from all 3 fetuses was 71 ± 32 μg per mg (Figure 5).

Adult. The matrix from 2 donors out of 6 contained only small amounts of calcium in all the implants (<13 μg/mg). Most implants from the other donors contained about the same amounts of calcium as the fetal implants. The whole fragments from these 4 donors had a mean content of 44 ± 44 μg per mg (Figure 5).

Pulverized adult. Pulverized matrix from patients 5 and 6 contained per mg 104 ± 40 μg calcium, which is more than was found in the whole fragments from the same donors (*t*-test: $P < 0.001$).

Controls. Implanted rat matrix at harvest contained 139 ± 27 μg Ca per mg. Human adult pulverized matrix implanted in Rowett rats with normal thymus at harvest contained 4.4 ± 1.3 μg Ca per mg. Fetal matrix contained 1.1 ± 0.2 μg Ca per mg. Heat-denatured matrix implanted in athymic rats at harvest contained 2.2 ± 0.8 μg Ca per mg. Nonimplanted matrix always contained less than 1 μg Ca per mg, with two exceptions: The powder from patient 6 and the whole fragments from patients 5 and 6 contained residual calcium. The calcium-yield values after implantation of the powder have been corrected for this error (three implants), whereas possible residual calcium of the whole fragments has not been corrected for. Thus, the difference in bone yield between whole fragments and powder may in reality be larger than indicated.

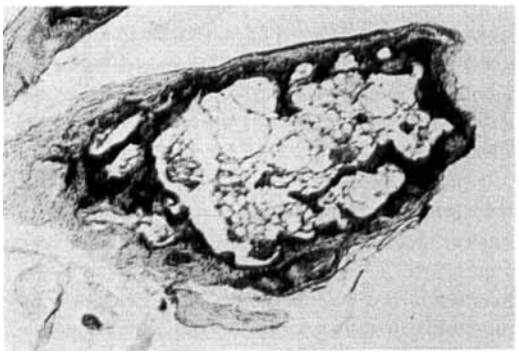

Figure 1. Demineralized bone from a human 11-week fetus that is almost completely replaced by new bone in an athymic rat. Toluidine blue, x30. Demineralized matrix appears unstained.

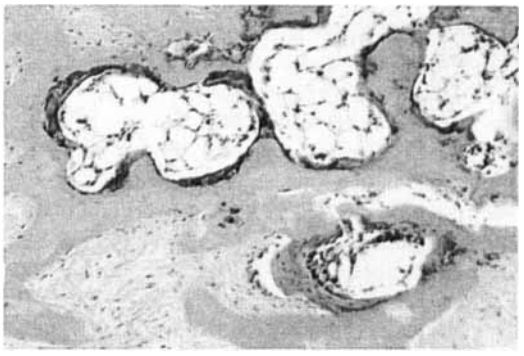

Figure 2. Demineralized bone matrix from human 16-week fetus implanted in athymic rat. Ossicles of rat bone are formed. HE, x100. Demineralized bone appears pale red; new bone is more intensely red.

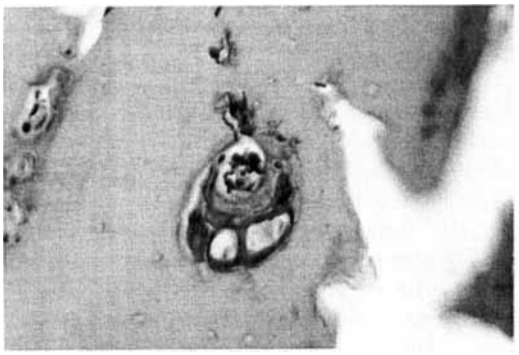

Figure 3. Demineralized bone matrix from adult human implanted in athymic rat. The vascular canal in the center has been filled by rat cartilage, which in turn has been partly replaced by bone and marrow. HE, x250. Remaining cartilage matrix appears blue with empty cell lacunae (arrows).

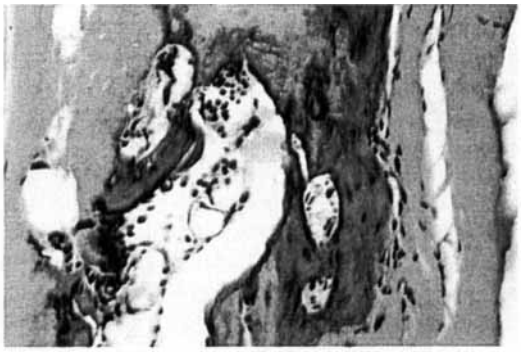

Figure 4. Demineralized adult human bone matrix implanted in an athymic rat. New bone has formed. HE, x250.

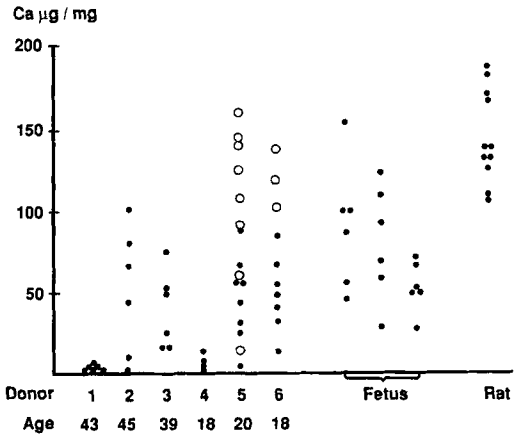

Figure 5. Human demineralized bone matrix from the femurs of 6 adults and from 3 fetuses were implanted in athymic rats. Bone yield is expressed as µg calcium per mg implanted matrix dry weight. Open circles indicate pulverized implants. Data from 11 rat-matrix implants are given for comparison.

Discussion

Recent clinical investigations – following the pioneering studies by Urist (1980) and Glowacki (1981) – have cast doubt on whether true bone induction occurs as a result of the implantation of human demineralized bone matrix in man (Kachiuchi et al. 1985, Ousterhout 1985). In the largest series reported (Kachiuchi et al. 1985), the authors concluded from the results in 160 patients that healing probably was due to other effects than bone induction. This is not surprising, because the implants in all of these studies were sterilized by either irradiation (Glowacki 1981) or ethylene oxide (Urist 1980, Kachiuchi et al. 1985, Ousterhout 1985). Both of these sterilizing techniques destroy all or parts of the inductive properties (Buring and Urist 1967, Cornell et al. 1987, Munting et al. 1988). However, nonsterilized bone matrix from adult squirrel monkeys did not induce bone in the donor monkeys' own thigh muscle

(Aspenberg et al. 1988); so even if not sterilized, the inductive capacity of human bone matrix seemed questionable when we started this study.

Normal rats are not suitable as recipients of human bone matrix because immunologic mechanisms will disturb or eliminate induction by bone matrix from other species (Urist et al. 1967, Aspenberg et al. 1988). In the T-lymphocyte-deficient athymic rat, this will not occur. Therefore, the athymic rat can be used to measure inductive properties of xenogenic bone matrix (Aspenberg et al.1988).

In the present experiments, adult human bone matrix in most cases induced bone in the athymic rat. This is in accordance the results of Sampath and Reddi (1983) that factors extracted from human bone can induce bone when reconstituted to inactive rat matrix and implanted in normal rats. In that study, an inhibitory fraction had to be removed from the extract to achieve bone induction. Because this fraction was now present and did not inhibit bone induction in the athymic rats, its effect in the normal rats was probably related to immunologic mechanisms.

The central parts of the adult matrix implants often contained only cartilage. We suspected that capillaries had not been able to reach these parts, due to the shape and structure of the compact adult matrix implants. Therefore, portions of adult matrix were milled, and the powder was found to yield more bone. Thus, data for whole fragments of adult matrix should not be regarded as quantitative measurements of inductive capacity. For further studies, a standardized amount of powder with a standardized particle size would be preferable to whole matrix pieces, and would perhaps permit quantitative comparisons between different clinical materials.

We had expected to find a manyfold higher bone yield from the fetal implants, partly because of the differences in relative amounts of various growth factors (Hauschka et al. 1986). However, considering the unfavorable gross structure of the nonpulverized adult implants in comparison with the porous fetal matrix, a majority of the adult implants seemed to have an inductive capacity similar to that of the fetal implants, which in turn is only half that of young rat or rabbit matrix in the same experimental model (Aspenberg et al. 1988).

The failure of the implants from two donors demonstrates the need for a test model like the athymic rat to check inductive properties of matrix preparations before their possible clinical use. Although a true lack of inductive properties in the bones from these donors cannot be ruled out, the failures may also be due to faulty preparation, for example, thermal injury at harvest. Because pulverization appeared to increase the bone yield, these failing implants might also have behaved differently if they were pulverized.

Clearly, bone-inductive properties can be found in adult human bone matrix. The question remains whether humans respond to human demineralized bone matrix as do athymic rats.

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