The Morphogenesis of Bone in Replicas of Porous Hydroxyapatite Obtained from Conversion of Calcium Carbonate Exoskeletons of Coral*

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ABSTRACT: **The morphogenesis of bone in a porous hydroxyapatite substratum was studied after intramuscular implantation in adult primates. Replicas of porous hydroxyapatite that had been obtained after hydrothermal conversion of the calcium carbonate exoskeleton of coral (genus Goniopora) were implanted intramuscularly in twenty-four adult male baboons *(Papio ursinus)*. Serial sections from specimens that had been harvested at three, six, and nine months showed that initially the formation of fibrous connective tissue was characterized by a prominent vascular component and by condensations of collagen fibers assembled at the interface of the hydroxyapatite. The morphogenesis of bone was intimately associated with the differentiation of the connective-tissue condensations. Bone formed without an intervening endochondral phase. Although the amount of bone varied considerably, in several specimens extensive bone developed, filling large portions of the porous spaces and culminating in total penetration by bone within the implants.**

The mean volume fraction composition of the specimens was 20.8 ± 1.0 per cent (mean and standard error) for bone, 17.3 ± 1.7 per cent for connective-tissue condensation, 31.9 ± 1.0 per cent for fibrovascular tissue, 6.4 ± 0.6 per cent for bone marrow, and 34.6 ± 0.5 per cent for the hydroxyapatite framework. The amount of bone and marrow increased at each time-period, and the hydroxyapatite framework was significantly reduced between six and nine months. This indicated a moderate biodegradation over time, which was possibly a result of incomplete conversion of carbonate to hydroxyapatite. Linear regression analysis showed a negative correlation between the hydroxyapatite framework and the magnitude of bone formation within the porosities of the hydroxyapatite ($p = 0.0001$). Biochemical coating of the hydroxyapatite substratum with an allogeneic fibrin-fibronectin protein concentrate prepared from baboon plasma did not significantly increase the amount of bone formation within the porous spaces. The hydroxyapatite substratum may have functioned as a solid-phase domain for anchorage of bone morphogenetic proteins.

CLINICAL RELEVANCE: Bone formed in replicas of porous hydroxyapatite that had been implanted intramuscularly in adult non-human primates. The results strongly support the use of coralline replicas of hydroxyapatite with an average porosity of 600 micrometers as a biological alternative to autogenous bone grafts for the controlled initiation of bone formation in humans.

In recent years, considerable interest has been focused on the osteoconductive properties of a porous hydroxyapatite substratum that is obtained after hydrothermal conversion of the calcium carbonate exoskeletal microstructure of the scleractinian reef-building corals, Porites and Goniopora[1,3,24,33,37]. The replica of hydroxyapatite is characterized by a relatively uniform network of interconnected channels and pores, similar to the mineralized inorganic supporting structure of living bone[3]. Several lines of evidence have established the osteoconductive properties of the porous substratum when it is implanted in orthotopic sites, and the material has been used experimentally in reconstructive operations, particularly craniofacial procedures, as an alternative to autogenous bone grafts[4-6,8,16]. Previous studies have shown that implantation in extraskeletal sites results in penetration of fibrovascular tissue, without bone formation, which indicates that the porous hydroxyapatite does not act as a bone-inducing substratum[15]. Ingrowth of bone within the tridimensional framework depends on close apposition of the implant with viable bone at the interfaces of the material[3,15].

As one of a number of investigations into the mechanisms of repair and regeneration of bone in non-human primates, the biological characteristics of incorporation of porous hydroxyapatite were studied after intramuscular implantation in a series of adult primates of the genus Papio. Porous hydroxyapatite was implanted with or without a reconstituted allogeneic fibrin-fibronectin protein concentrate[20,21] to hasten fibrovascular ingrowth within the porous substratum.

Materials and Methods

Animals

Twenty-four outbred adult male chacma baboons *(Papio ursinus)* that were clinically healthy and that weighed

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a mean of 27.8 ± 3.3 kilograms (range, 22.4 to thirty-six kilograms) were selected from the non-human primate colony of the University of the Witwatersrand, Johannesburg. The criteria for selection were normal hematological and

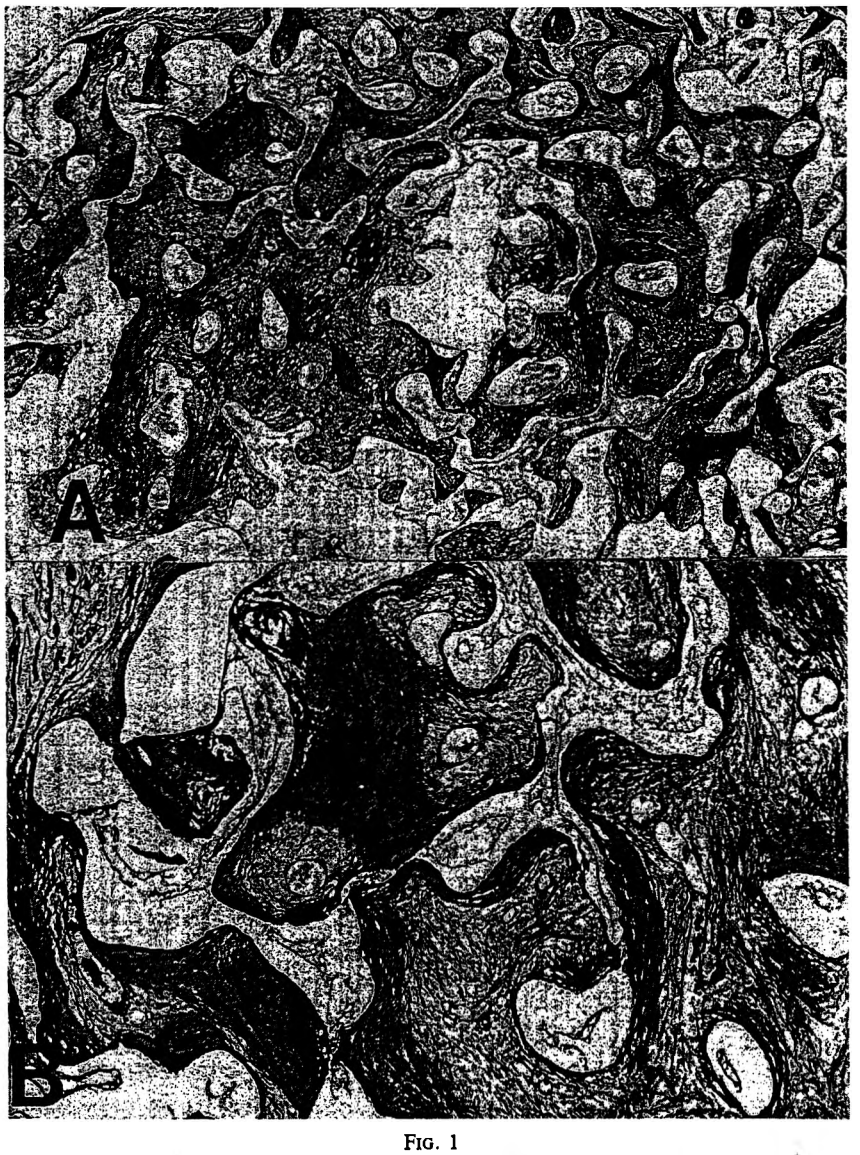

FIG. 1

Photomicrographs, made at three months, of a specimen of hydroxyapatite, showing the organization of the connective-tissue matrix.
A: Extensive condensation and organization of connective-tissue fibers, mainly at the interface of the hydroxyapatite. Empty white spaces represent the hydroxyapatite framework after decalcification during histological processing (× 20).
B: Higher magnification showing the organization of connective-tissue fibers at the interface of the hydroxyapatite and extending into the porous spaces (× 50).

biochemical profiles[13] and skeletal maturity, as confirmed by radiographic evidence of closure of the distal epiphyseal plates of the radius and ulna. After standard procedures for quarantine were carried out, the animals were housed individually in suspended wire-mesh cages in the non-human primate unit of the Central Animal Service of the university, 1800 meters above sea level. The rooms in which the animals were caged were kept under slight negative pressure (−25 kilopascals), with controlled ventilation (eighteen filtered air changes each hour), temperature (22 ± 2 degrees Celsius), humidity (40 ± 10 per cent), and photoperiod (lights on from 6 AM to 6 PM). Before any procedures were initiated, all research protocols were approved by the Animal Ethics Committee of the university.

Implants of Hydroxyapatite

Implants of porous hydroxyapatite were specially prepared by Interpore International (Irvine, California) to the specifications of the protocol. A hydrothermal chemical exchange with phosphate converted the original microstructure of the calcium carbonate exoskeleton of the Goniopora coral[35] into an inorganic replica of hydroxyapatite[24,36]. Implants consisted of cylinders of porous hydroxyapatite that measured twenty millimeters in length and seven or five millimeters in diameter. The solid trabeculae of the frame-

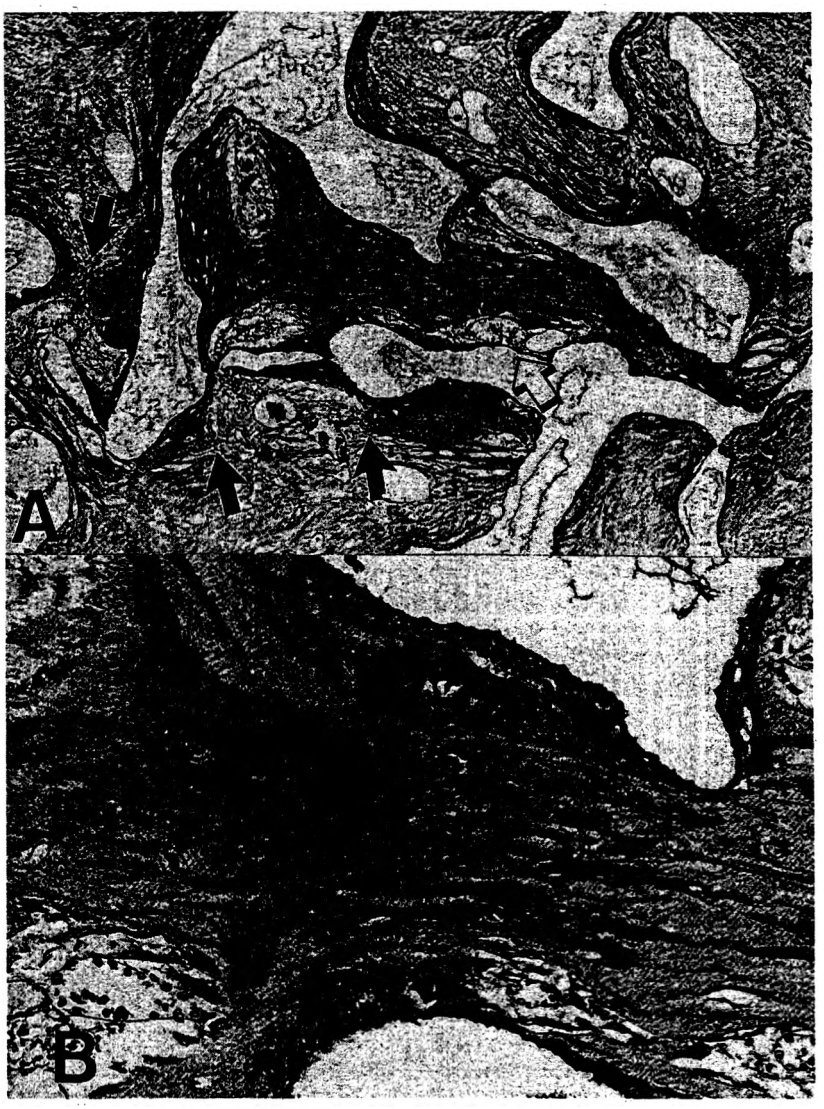

FIG. 2

Photomicrographs, made at three months, of a specimen of hydroxyapatite coated with allogeneic fibrin-fibronectin protein concentrate, showing differentiation of a tissue with features that are intermediate between fibrous connective tissue and bone.

A: Differentiation of osteoblasts and morphogenesis of bone (closed arrows) in close relationship with connective-tissue condensations. Note the prominent vascular component, which is occasionally in direct contact with the hydroxyapatite substratum (open arrow) (× 50).

B: Differentiation of osteocyte-like cells within connective-tissue condensations. The osteocyte-like cells are surrounded by focal areas of mineralization (× 200).

work averaged 130 micrometers in diameter, and their interconnections averaged 220 micrometers in diameter. The average porosity was 600 micrometers, and their interconnections averaged 260 micrometers in diameter (Interpore 500)[7]. Before implantation, the cylinders of hydroxyapatite were sterilized in an autoclave at 115 degrees Celsius for twenty minutes.

Preparation of the Fibrin-Fibronectin Protein Concentrate

Allogeneic baboon plasma, prepared as has been described[20,21], was pooled and was stored in sterile vials at −20 degrees Celsius until fibrinogen, fibronectin, factor XIII, and plasminogen were cryoprecipitated to form the lyophilized baboon allogeneic protein concentrate. Cryoprecipitation and final preparation of the allogeneic fibrin-fibronectin protein concentrate was done in the laboratories of Immuno AG (Vienna, Austria).

Operative Procedures and Intramuscular Implantation

On the evening before the operation, food was withdrawn from the animals, but they had continued access to water *ad libitum*. On the day of the operation, the animals were immobilized with an intramuscular injection of phencyclidine hydrochloride (one milligram per kilogram of body weight) or ketamine hydrochloride (eight milligrams per kilogram of body weight) and anesthetized with intravenous administration of thiopentone sodium (fifteen milligrams per kilogram of body weight). Anesthesia was maintained with halothane vapor in 100 per cent oxygen following orotracheal intubation. After preparation of the

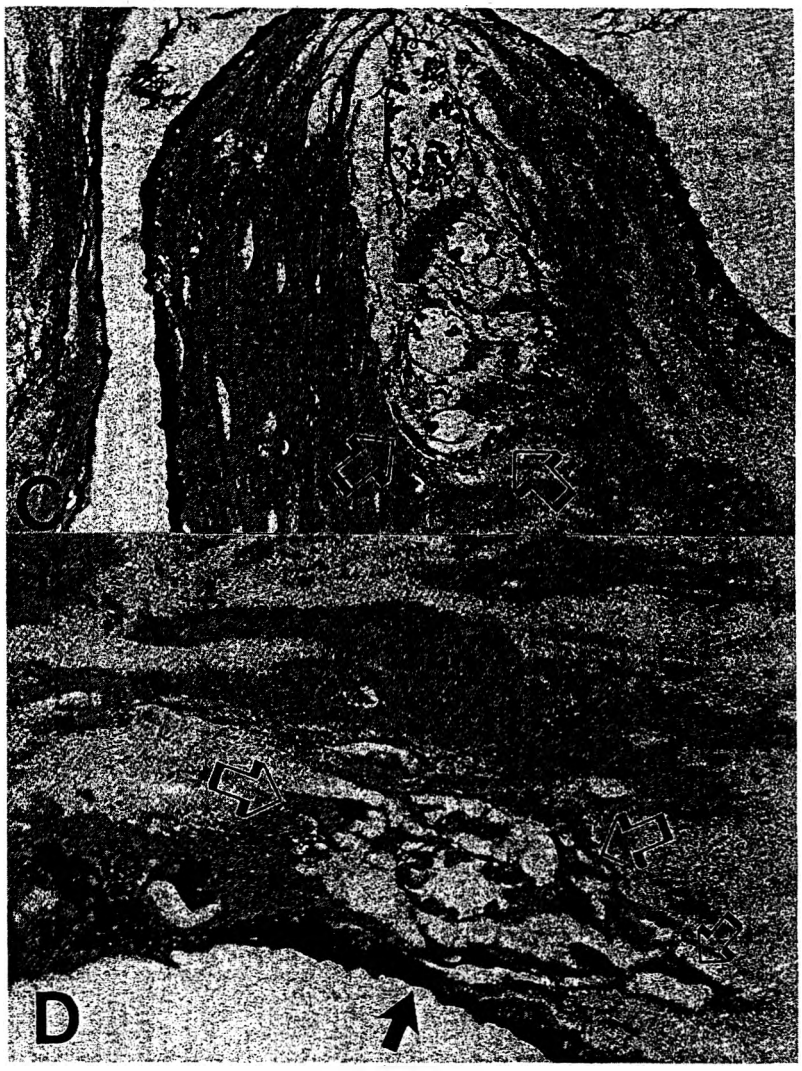

FIG. 2

C: Detail of the osteocyte-like cells and differentiation of cells that were interpreted as osteoblasts (arrows) facing the highly vascular connective-tissue matrix (× 400).

D: Detail of the osteocyte-like cells embedded within the connective-tissue matrix. Large hyperchromatic cells (open arrows) are lining the matrix. Note the close relationship of these cells (identified as osteoprogenitors and differentiating osteoblasts) with the central blood vessel and the mineralized remnants of the hydroxyapatite substratum (closed arrow) (× 400).

skin and sterile draping, a total of ninety-six cylinders of hydroxyapatite were implanted bilaterally in ventral and dorsal intramuscular pouches that had been created with sharp and blunt dissection in the rectus abdominis and in the latissimus or longissimus dorsi after partial reflection of the trapezius.

An equal number of cylinders of hydroxyapatite of seven and five-millimeter diameter were distributed between the two sites of implantation.

Before implantation, the porous substratum of forty-eight cylinders of hydroxyapatite was coated with allogeneic fibrin-fibronectin protein concentrate, activated with bovine thrombin. For preparation of the protein concentrate in a viscous form that was suitable for coating the hydroxyapatite substratum, approximately 160 milligrams of clottable proteins (140 milligrams of fibrinogen, twelve milligrams of fibronectin, twenty to fifty units of factor XIII, and sixty micrograms of plasminogen) was dissolved in 1.5 milliliters of distilled water containing 3000 kallidinogenase inactivator units of the bovine antifibrinolytic aprotinin. A clear viscous solution was obtained after magnetic stirring at 37 degrees Celsius. The allogeneic fibrin-fibronectin protein concentrate was activated with 500 units of lyophilized bovine thrombin dissolved in 1.5 milliliters of forty-millimolar calcium chloride. Equal volumes of the two components were ejected simultaneously through a common applicator needle[23].

Biochemical coating was performed with 500 microliters of activated allogeneic fibrin-fibronectin protein concentrate for each implant. Four cylinders of hydroxyapatite were implanted in each animal, two cylinders in anterior pouches and two cylinders in posterior pouches, and one anterior implant and one posterior implant were coated with the activated allogeneic fibrin-fibronectin protein concentrate. To close the pouches after implantation of the hydroxyapatite, the fasciae and the superficial tissues were

FIG. 3

Photomicrographs made at three months, showing patterns of structural organization during morphogenesis of bone.
A: Delicate trabecular-like osseous structures developing within localized porosities of the hydroxyapatite substratum (× 20).
B: Higher magnification showing the highly cellular bone matrix covered by osteoblasts, the contiguous layers of osteoblasts, and the extensive vascular penetration. Note the interconnections between the trabecular-like bone extending into the porous spaces and the uninterrupted bone lining the surface of the hydroxyapatite (× 50).

repaired in layers with atraumatic resorbable sutures.

After the operation, benethamine and procaine penicillins were administered by intramuscular injection. Postoperatively, pain was controlled with intramuscular injection of buprenorphine hydrochloride (0.3 milligram). The individually housed animals were kept under daily clinical observation. They were fed soft food that consisted of basic proteins, fat, carbohydrates, fibers, calcium, iron, phosphates, and vitamins (thiamine, riboflavin, and nicotinic acid), mixed in a ratio of 3:1 with a protein-vitamin-mineral dietary supplement[2]. This was later supplemented with commercial monkey cubes.

Harvesting and Processing of Tissue

The animals were immobilized and anesthetized; they were then killed with an intravenous overdose of pentobarbitone: eight animals at three months, eight at six months, and eight at nine months after the operation.

The harvested implants, with surrounding soft tissues, were fixed in 10 per cent formol-buffered saline solution, decalcified in formic acid-sodium citrate solution, and double-embedded in celloidin and paraffin wax. Five-micrometer serial sections were cut in a plane perpendicular to the long axis of the cylinders of hydroxyapatite and were stained with toluidine blue.

Histological and Histomorphometric Analysis

From each specimen, a minimum of four levels were available for analysis. Examination of the whole material showed remarkable and unexpected differentiation of bone within the porosities of the hydroxyapatite. Four patterns of structural organization were consistently recognized:

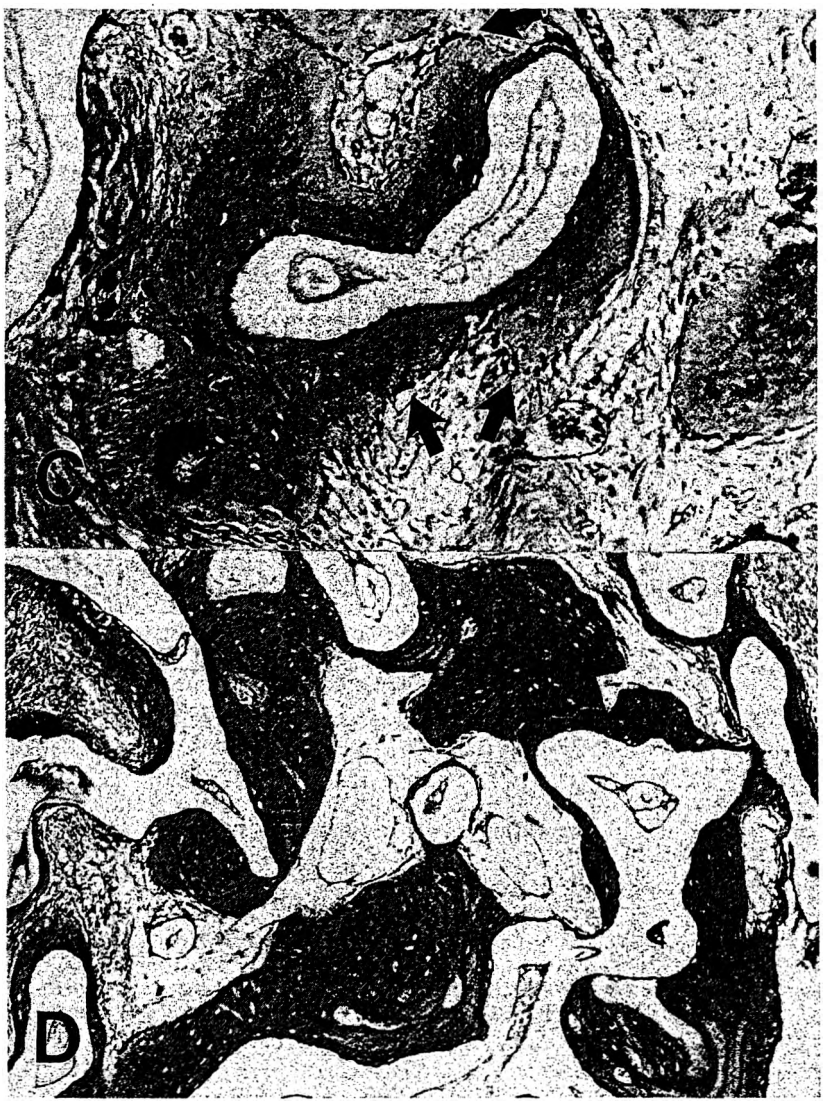

FIG. 3

C: In a specimen of hydroxyapatite, deposition of bone is seen around a trabecula of hydroxyapatite. The arrows indicate contiguous layers of osteoblasts (× 100).

D: In a specimen of hydroxyapatite, there is extensive osseous formation as early as three months. Note the remodeling and organization of lamellar bone (× 50).

(1) fibrous connective tissue with a pronounced cellular and vascular component; (2) fibrous connective tissue that was characterized by condensation of collagen fibers at the interface of the hydroxyapatite; (3) morphogenesis of bone; and (4) remodeling of bone, formation of lamellar bone, and differentiation of bone marrow. Accordingly, the histomorphometric analysis was designed to quantitate, with the point-counting technique[14], these different histological patterns within the porous spaces. A calibrated square integration platte II (Carl Zeiss, Thornwood, New York) with 100 lattice points was used to calculate the fractional volumes (in per cent) and the derived absolute cross-sectional area (in square millimeters) of each histological component: fibrovascular tissue, connective-tissue condensation, bone, bone marrow, and hydroxyapatite substratum.

Sections were analyzed in a Univar light microscope (Reichert, Vienna, Austria), magnified forty times, with the Zeiss graticule superimposed over the center of the specimen. A single central field of 7.84 square millimeters was analyzed for each section. Histomorphometric analysis was performed on two sections from the same specimen at two different levels, 100 or 150 micrometers apart. The connective-tissue condensation was analyzed at the three-month time-interval only.

Statistical Analysis

The data were analyzed with a computer (Model 3083 J24, IBM), with the Statistical Analysis System[30]. An F test was performed with the general linear models procedure for an unbalanced multiple analysis of variance. For each pattern of structural organization, the model design analyzed the effects and interactions of six independent class variables: the treatment (implantation of hydroxyapatite or hydroxyapatite coated with allogeneic fibrin-fibronectin protein concentrate), the individual response of the animal (nested with time), the time-period (three, six, or nine

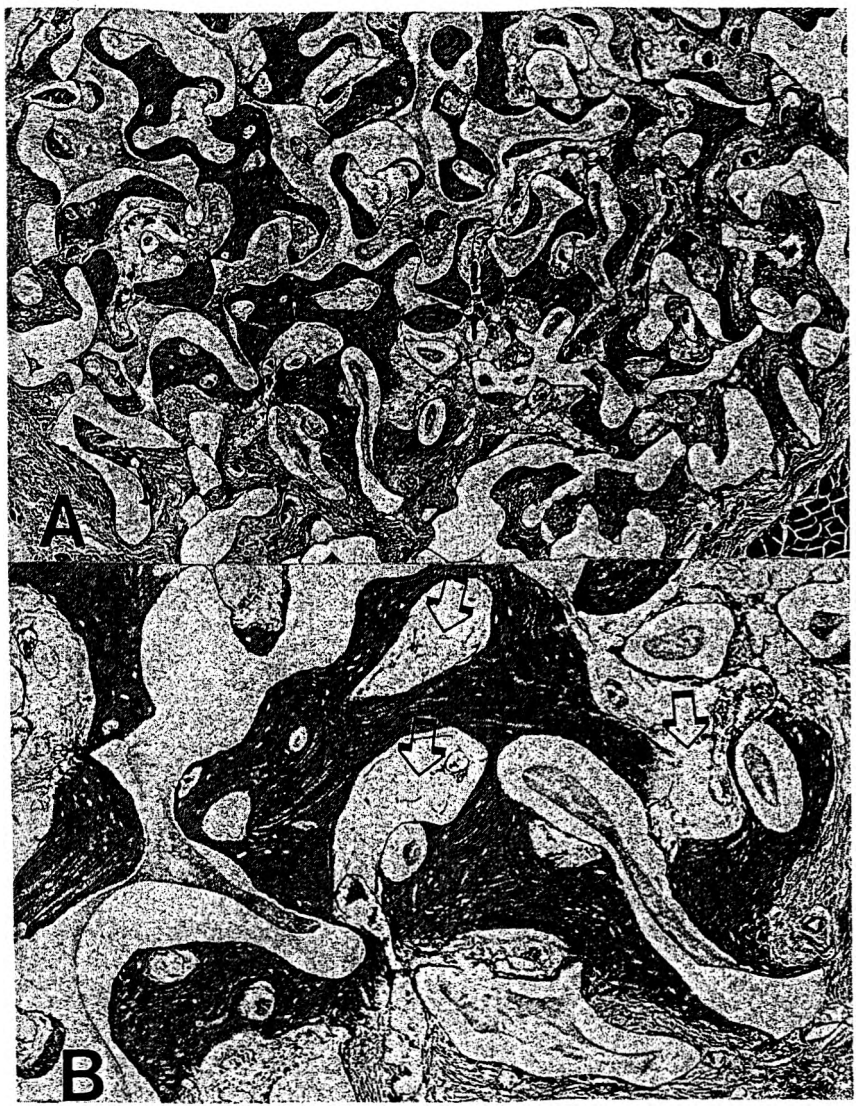

FIG. 4

Photomicrographs showing the pattern of distribution of remodeled bone.

A: A specimen of hydroxyapatite coated with allogeneic fibrin-fibronectin protein concentrate at six months. Large areas of remodeled bone fill the porosities of the hydroxyapatite, particularly in the center of the implant. Soft connective tissue and muscle fibers envelope the cylinder of hydroxyapatite (× 20).

B: Higher magnification showing the structural organization of the lamellar bone. The arrows indicate marrow lacunae, which are partially disrupted by the decalcification process (× 50).

months), the site of implantation (anterior or posterior), the diameter of the cylinders of hydroxyapatite (seven or five millimeters), and the histological levels of the histomorphometric analysis (two levels, 100 or 150 micrometers apart).

With inclusion of the hydroxyapatite substratum as an independent covariate in the general linear models procedure, a linear regression analysis was performed to estimate the possible effect of the volume fractions of the hydroxyapatite framework on the extent of bone formation. The Pearson correlation coefficient was computed for the two variables that were included in the regression analysis. Mean values were compared with use of the Duncan multiple range test for the four dependent variables that were included in the analysis. The critical level of statistical significance that was chosen was $p < 0.01$.

Results

Clinically, healing was uncomplicated in all animals, and there was no evidence of rejection of the implants. Immediately after harvest, macroscopic examination showed optimum incorporation of the implants within the recipient muscular tissues, without fibrous encapsulation. Seven implants were spoiled during histological preparation, leaving forty-five implants of hydroxyapatite and forty-four implants of hydroxyapatite coated with allogeneic fibrin-fibronectin protein concentrate. Histomorphometry was performed on a total of 178 fields.

Morphological Analysis

Qualitative differences could not be detected between the specimens of hydroxyapatite and those of hydroxyapatite coated with allogeneic fibrin-fibronectin protein concen-

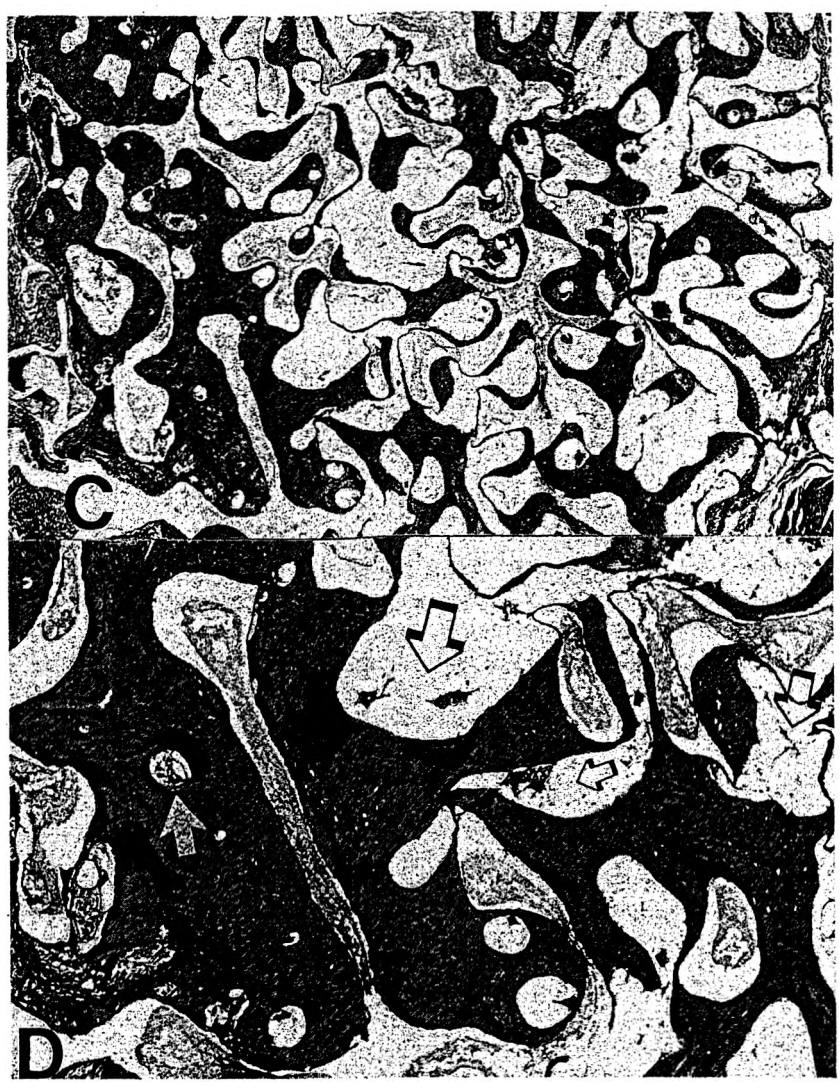

FIG. 4

C: A specimen of hydroxyapatite coated with allogeneic fibrin-fibronectin protein concentrate at nine months. There is extensive formation of bone penetrating the porosities of the hydroxyapatite or laminating the hydroxyapatite framework (× 20).
D: Higher magnification showing the lamellar bone with the formation of pseudo-haversian and vascular canals (white arrow). The open arrows indicate the disrupted remnants of marrow tissue (× 50).

trate. The consistent recognition of four different patterns of structural organization was briefly described in the Materials and Methods section. Two distinct structural features characterized the connective tissue that invaded the porous spaces of the implants of hydroxyapatite: a vascular component within a cellular, loose connective-tissue matrix, and the differentiation of a peculiar pattern of mesenchymal condensation and alignment of connective-tissue fibers, mostly in direct contact with the surfaces of the hydroxyapatite (Fig. 1, *A* and *B*). Osteocyte-like cells were embedded within a tissue that had intermediate features between fibrous connective tissue and bone (Fig. 2, *A* through *D*). Large vessels had invaded the connective-tissue matrix, penetrating the porosities of the hydroxyapatite (Figs. 1, *A* and 2, *A* through *D*), and occasionally the vascular walls were almost in direct contact with the hydroxyapatite substratum (Fig. 2, *A*).

At three months, bone had developed in twenty-four specimens (77 per cent). The amount of bone ranged from slight to florid and mainly occupied the center of the implant of hydroxyapatite. Bone was mostly in direct contact with the hydroxyapatite substratum, and contiguous layers of osteoblasts lined newly deposited bone matrix (Fig. 3, *B* and *C*). The structural organization ranged from lamellar (Fig. 3, *D*) to delicate trabecular-like woven bone invading the highly vascularized connective-tissue matrix (Fig. 3, *A* and *B*).

At six and nine months, morphogenesis of bone had occurred in twenty-four (92 per cent) and thirty-one (100 per cent) of the specimens, respectively. Although the amount of bone varied considerably, in several specimens an extensive amount of bone had developed, filling large portions of the porosities both at the center and at the periphery (Fig. 4, *A* through *D*). Vascular spaces with the features of haversian canals penetrated the remodeled bone supporting osteonic-like structures (Fig. 4, *D*). Whereas the

TABLE I

VOLUME FRACTION COMPOSITION (IN PER CENT) OF SPECIMENS OF HYDROXYAPATITE AND THOSE OF HYDROXYAPATITE COATED WITH ALLOGENEIC FIBRIN-FIBRONECTIN PROTEIN CONCENTRATE*

	Hydroxyapatite						Hydroxyapatite Coated with Allogeneic Fibrin-Fibronectin Protein Concentrate					
Period	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.†	Fibrovasc.	Bone Marrow	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.†	Fibrovasc.	Bone Marrow
3 mos.	16	37.7 ± 1.0	9.2 ± 2.1	19.2 ± 2.5	31.8 ± 1.8	2.1 ± 0.7	16	36.4 ± 1.2	11.5 ± 1.9	15.3 ± 2.4	34.9 ± 1.8	1.8 ± 0
6 mos.	14	34.7 ± 1.3	25.9 ± 2.2	ND	32.6 ± 2.4	6.9 ± 1.3	12	35.7 ± 1.9	24.8 ± 2.2	ND	34.3 ± 2.5	5.5 ± 1
9 mos.	15	30.4 ± 1.2	26.7 ± 2.1	ND	32.1 ± 3.1	10.8 ± 2.1	15	32.9 ± 1.0	28.9 ± 1.9	ND	26.9 ± 2.6	11.2 ± 1
Mean		34.4 ± 0.7	20.2 ± 1.5		32.1 ± 1.4	6.5 ± 0.9		34.9 ± 0.7	21.4 ± 1.4		31.8 ± 1.4	6.3 ± 0

* Mean and standard error of the mean.
† ND = Not determined.

lamellar bone was mainly localized to the central areas of the implants of hydroxyapatite, the newly developing woven bone extended toward the peripheral porosities, occasionally culminating in total penetration (Fig. 4, *A* and *C*). Florid deposition of bone was accompanied by the differentiation of marrow (Fig. 4, *B*). Remodeling of bone resulted in the formation of large marrow cavities that were confined by relatively thin trabecular-like osseous structures, laminating the substratum and populated by sparse osteoblast-like cells.

Histomorphometric Analysis

The volume fraction compositions at each observation period are summarized in Table I for specimens of hydroxyapatite and of hydroxyapatite coated with allogeneic fibrin-fibronectin protein concentrate. The derived absolute cross-sectional areas of the tissue components are presented in Table II.

Histological processing, after demineralization and double-embedding in wax, resulted in an average shrinkage of the cross-sectional diameter of 23 per cent for the seven-millimeter-diameter cylinders of hydroxyapatite, so that the average area of the sections was 22.7 square millimeters. The corresponding values for the five-millimeter-diameter implants were 21 per cent and 12.3 square millimeters. The histomorphometric field for the seven and five-millimeter specimens included 35 and 65 per cent of the section, respectively. The biochemical coating with allogeneic fibrin-fibronectin protein concentrate did not significantly increase the amount of bone in the implant or affect the extent of connective-tissue condensation, fibrovascular invasion, or formation of bone marrow. The amount of bone increased significantly between three and six months ($p < 0.001$), but there was no additional significant increase at nine months. The volume fraction of fibrovascular tissue did not change significantly between the various periods of observation. The amount of bone marrow increased significantly ($p < 0.001$) between three and six months and again between six and nine months.

Separate analyses for the site of implantation and the diameter of the cylinders of hydroxyapatite showed that, on the average, a greater amount of bone formed in the seven-millimeter implants, regardless of the site of implantation. At nine months, however, equal amounts of bone were found in both the seven-millimeter and the five-millimeter implants. No significant differences were found between the two diameters with regard to the hydroxyapatite framework. On the average, more bone formed in the anterior specimens, although the difference with regard to the site of intramuscular implantation was significant only in the seven-millimeter implants. More bone marrow also developed in the anterior implants ($p < 0.001$). Inversely, significantly more fibrovascular tissue was found in the posterior implants ($p < 0.001$). The volume fraction compositions in relation to the diameter of the cylinders of hydroxyapatite and to

TABLE II

MEAN ABSOLUTE CROSS-SECTIONAL AREAS (IN SQUARE MILLIMETERS) OF TISSUE COMPONENTS IN SPECIMENS OF HYDROXYAPATITE AND THOSE OF HYDROXYAPATITE COATED WITH ALLOGENEIC FIBRIN-FIBRONECTIN PROTEIN CONCENTRATE

	Hydroxyapatite						Hydroxyapatite Coated with Allogeneic Fibrin-Fibronectin Protein Concentrate					
Period	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.*	Fibrovasc.	Bone Marrow	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.*	Fibrovasc.	Bone Marrow
3 mos.	16	2.95	0.72	1.51	2.49	0.16	16	2.85	0.90	1.19	2.74	0.14
6 mos.	14	2.72	2.03	ND	2.56	0.54	12	2.79	1.94	ND	2.69	0.43
9 mos.	15	2.38	2.09	ND	2.51	0.84	15	2.58	2.26	ND	2.11	0.87
Mean		2.70	1.58		2.51	0.51		2.73	1.67		2.49	0.49

* ND = not determined.

the site of implantation are presented in Tables III and IV. The analysis failed to show any significant difference between the two histological levels, 100 or 150 micrometers apart, with regard to the amount of bone, the extent of connective-tissue condensation, fibrovascular invasion, or differentiation of bone marrow (data not shown).

The volume fraction of the hydroxyapatite substratum ranged from 23 to 50 per cent (mean, 37.1 per cent) at three months, from 23 to 51 per cent (mean, 35.2 per cent) at six months, and from 17 to 46 per cent (mean, 31.7 per cent) at nine months. Although the mean values differed, the hydroxyapatite framework did not change significantly between three and six months. However, the difference between six and nine months was significant ($p < 0.001$), indicating biodegradation, although moderate, over time (5.4 per cent in six months).

The mean values for the hydroxyapatite framework were identical for both types of treatment (34.4 per cent for hydroxyapatite compared with 34.9 per cent for hydroxyapatite coated with allogeneic fibrin-fibronectin protein concentrate). The histomorphometric analysis showed a considerable variation (range, 17 to 51 per cent) in the volume fractions of the hydroxyapatite framework between different specimens. This variation was found to have a significant effect on the amount of bone formation within the porosities of the hydroxyapatite ($p = 0.0001$). The analysis showed a negative correlation (Pearson correlation coefficient, $r = -0.581$) between values for hydroxyapatite and those for bone. Regression analysis showed that the relationship was linear with a negative slope. Plotting of values for bone against those for hydroxyapatite indicated that higher and reproducible generation of bone occurred in hydroxyapatite substrata, with volume fractions ranging from 23 to 40 per cent.

Discussion

Several experimental studies have been performed, both orthotopically and heterotopically, on replicas of hydroxyapatite (obtained from conversion of calcium carbonate exoskeletons) with an average porosity of 200 micrometers in diameter (Interpore 200)[4-6,8,16]. However, little information is available about hydroxyapatite in which the size of the pores is 600 micrometers (Interpore 500)[7]. The framework of the 600-micrometer-porosity replica of hydroxyapatite appears to be similar to that of cancellous bone, and morphometric comparison of its microstructure with that of bone harvested from human iliac crests has shown remarkable morphological similarity[7]. Whereas orthotopic implantation in close apposition with viable bone has provided clear evidence of the osteoconductive potential of both types of hydroxyapatite, heterotopic implantation has excluded osteoinductive properties[15]. The present investigation, however, firmly establishes that, when they are implanted extraskeletally in adult baboons, replicas of porous hydroxyapatite that were obtained from conversion of calcium carbonate exoskeletons of the genus Goniopora are capable of inducing differentiation of bone in direct contact with the hydroxyapatite substratum.

The molecular and cellular signals that initiate this osteogenesis in porous hydroxyapatites are not known. The histological analysis suggests that the differentiation of bone may be intimately associated with the morphogenesis of the connective-tissue condensations at the interface of the hydroxyapatite. Indeed, the lack of a significant fluctuation of the fibrovascular component suggests that the decisional commitment and the expression of the osteogenic phenotype may be regulated by early morphogenesis of the collagenous condensations.

Additional studies that include earlier periods of observation are needed to demonstrate the developmental correlation between the collagenous condensations and the morphogenesis of bone. In the present study, the histological analysis indicated that the central core of the hydroxyapatite was the nucleus for the initial morphogenetic events leading to differentiation of bone. There was extensive remodeling followed by formation of lamellar bone as early as three months after implantation, and it was most evident at six and nine months. The constant observation of a morphogenetic nucleus that was mainly localized to the center of the implant supports the interpretation of a time-related centrifugal pattern of tridimensional growth of bone, extending to the periphery of the implants and occasionally culminating in total penetration (Fig. 4, *A* through *D*).

Although the morphological dissection of the histological material does not provide an explanation of the morphogenesis of bone, it nevertheless suggests that the surface characteristic of the substratum may play an important role. Cellular modulators and cell substratum interactions have been demonstrated to play a role in the initiation of the differentiation of osteoblastic cells both *in vitro* and *in vivo*[9,17,25,29,32,34,38]. It is now widely accepted that the extracellular matrix of bone plays a critical role in the local control of cellular growth and cellular differentiation by functioning as a solid substratum for the anchorage of growth factors and inducing proteins complexed with both the organic and the inorganic components of the extracellular matrix[18,26].

In the present study, the implanted hydroxyapatite replicated the mineralized inorganic supporting structure of the living extracellular matrix of bone. It is therefore possible that circulating or locally produced growth and inducing factors, or both, were present during incorporation in an adsorbed state on the hydroxyapatite substratum characterized by specific surface characteristics. A possibility that deserves experimental investigation is that osteogenin, a protein that initiates differentiation of bone[10,11,28], and related bone morphogenetic proteins[38] are present in the circulating plasma. Osteogenin could have been adsorbed on the hydroxyapatite substratum, and its release during the early stages of mesenchymal-tissue invasion would have determined the differentiation of bone in direct apposition to the hydroxyapatite substratum. This interpretation is substantiated by the many schemes for purification of growth factor that involve hydroxyapatite chromatography[11,12,22,31]. In-

TABLE III

VOLUME FRACTION COMPOSITION (IN PER CENT) OF FIVE AND SEVEN-MILLIMETER-DIAMETER SPECIMENS OF HYDROXYAPATITE*

	Five-Millimeter-Diameter Specimens						Seven-Millimeter-Diameter Specimens					
Period	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.†	Fibrovasc.	Bone Marrow	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.†	Fibrovasc.	Bone Marrow
3 mos.	13	38.6 ± 1.8	5.9 ± 2.1	21.2 ± 2.3	33.9 ± 1.4	0.5 ± 0.3	19	36.1 ± 1.0	13.4 ± 1.7	14.6 ± 2.4	32.9 ± 1.0	3.0 ±
6 mos.	10	34.9 ± 1.3	22.1 ± 2.6	ND	36.4 ± 3.0	6.5 ± 1.6	16	35.4 ± 1.2	27.5 ± 1.8	ND	31.4 ± 2.0	6.1 ±
9 mos.	9	32.1 ± 1.0	27.6 ± 1.5	ND	28.2 ± 2.8	12.1 ± 2.4	22	31.5 ± 1.1	27.9 ± 1.9	ND	29.9 ± 2.6	10.6 ±

* Mean and standard error of the mean.
† ND = not determined.

TABLE IV

VOLUME FRACTION COMPOSITION (IN PER CENT) OF SPECIMENS OF HYDROXYAPATITE IMPLANTED IN THE RECTUS ABDOMINIS (ANTERIOR SITE) AND THE LATISSIMUS DORSI (POSTERIOR SITE)*

	Anterior Site						Posterior Site					
Period	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.†	Fibrovasc.	Bone Marrow	No.	Hydroxyapatite	Bone	Connect.-Tissue Condens.†	Fibrovasc.	Bone Marrow
3 mos.	16	35.3 ± 0.9	12.6 ± 2.0	18.7 ± 2.4	30.5 ± 1.6	2.9 ± 0.9	16	38.8 ± 1.2	8.6 ± 1.9	15.8 ± 2.5	36.2 ± 2.0	1.0 ±
6 mos.	11	35.5 ± 1.4	26.0 ± 3.1	ND	30.5 ± 3.1	8.4 ± 1.5	15	35.0 ± 1.1	24.9 ± 1.4	ND	35.5 ± 1.9	4.7 ±
9 mos.	16	31.1 ± 1.1	29.9 ± 1.1	ND	24.0 ± 2.2	14.9 ± 1.9	15	33.3 ± 1.9	25.6 ± 2.6	ND	35.2 ± 3.2	6.8 ±

* Mean and standard error of the mean.
† ND = not determined.

deed, adsorption of mammalian osteogenins on hydroxyapatite gels is a fundamental step during their purification[11,22,28].

The correlation between the magnitude of induced bone and a specific range of values of the substratum framework suggests that the geometric configuration of the substratum may influence the extent of bone formation, perhaps by providing porous spaces that are architecturally more conducive to deposition of bone. However, measurement of the internal surface area of the porous spaces and correlation with ingrowth of bone are necessary to establish more specifically which aspect of the geometry of the implant is important for optimum deposition of bone. The importance of the geometry of the substratum is supported by a series of investigations of endochondral bone differentiation mediated by bone and dentin matrices[19,27]. The results of these studies have indicated a role for the geometry of the extracellular matrix in anchorage-dependent proliferation and differentiation of cells[27] as well as that the endochondral transformation can be greatly altered by the shape of the inductor[19].

Variation in the amount of bone formation within different specimens may be the result of subtle differences in the surface characteristics of the substratum and in the time-related release of putative adsorbed osteogenin interacting with a variable source of responding mesenchymal cells. Indirectly, this interpretation is supported by the quantitative differences in bone formation between sites of implantation. The variation between animals was interpreted as being the result of individual differences in the availability of a continuous flow of undifferentiated cells that are potentially capable of transformation toward osteoblastic cell-lines. Differences in the amount of bone within diameter configurations may reflect a sampling error resulting from the inclusion of larger peripheral areas during the quantitation of the sections from the five-millimeter-diameter specimens.

Contrary to the findings of previous studies that indicated a role for allogeneic fibrin-fibronectin protein concentrate in regeneration of connective tissue and bone in different periodontal-wound models[21,23], the biochemical coating of the porous substratum did not enhance the extent of connective-tissue condensation or the amount of bone formation. It is possible, however, that fibrin and fibronectin might have had a biological effect during the initial events of fibrovascular invasion. In the present study, the volume fraction composition of the hydroxyapatite framework is in agreement with the data reported by Holmes et al.[7] (34.6 per cent in the present study, compared with 35.1 per cent in the study by Holmes et al.). The significant difference between the values for the hydroxyapatite framework at six and nine months clearly indicates that there was biodegradation of the substratum over time and suggests that an incomplete conversion of carbonate to apatite occurred.

The extraskeletal morphogenesis of bone in porous hydroxyapatite substrata may have important biomedical implications, particularly because the data were obtained from a large series of adult non-human primates. Whatever mechanisms are responsible for the morphogenesis of bone, the results of the present study strongly support the use of coralline replicas of hydroxyapatite that have an average po-

rosity of 600 micrometers as a biological alternative to autogenous bone grafts for the controlled initiation of bone formation in humans. Within the limits of the morphological analysis, it is suggested that the hydroxyapatite substratum functioned as a solid-phase domain for anchorage of osteogenin and related bone morphogenetic proteins. This might have resulted in their adsorption, release, and biological activity, culminating in the expression of the osteogenic phenotype and differentiation of bone.

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References

1. CHIROFF, R. T.; WHITE, E. W.; WEBER, J. N.; and ROY, D. M.: Tissue Ingrowth of Replamineform Implants. J. Biomed. Mater. Res., **9** (Symposium 6): 29-45, 1975.
2. DREYER, J. J., and DU BRUYN, D. B.: Composition of a Food Mixture Developed at the NNRI for Supplementation of Predominantly Cereal Diets. South African Med. J., **42:** 600-604, 1968.
3. HOLMES, R. E.: Bone Regeneration within a Coralline Hydroxyapatite Implant. Plast. and Reconstr. Surg., **63:** 626-633, 1979.
4. HOLMES, R. E., and HAGLER, H. K.: Porous Hydroxylapatite As a Bone Graft Substitute in Mandibular Contour Augmentation: A Histometric Study. J. Oral and Maxillofac. Surg., **45:** 421-429, 1987.
5. HOLMES, R. E., and HAGLER, H. K.: Porous Hydroxyapatite As a Bone Graft Substitute in Cranial Reconstruction: A Histometric Study. Plast. and Reconstr. Surg., **81:** 662-671, 1988.
6. HOLMES, RALPH, and HAGLER, HERBERT: Porous Hydroxyapatite As a Bone Graft Substitute in Maxillary Augmentation. A Histometric Study. J. Cranio-Maxillo-Fac. Surg., **16:** 199-205, 1988.
7. HOLMES, R. E.; BUCHOLZ, R. W.; and MOONEY, VERT: Porous Hydroxyapatite As a Bone-Graft Substitute in Metaphyseal Defects. A Histometric Study. J. Bone and Joint Surg., **68-A:** 904-911, July 1986.
8. HOLMES, R. E.; BUCHOLZ, R. W.; and MOONEY, VERT: Porous Hydroxyapatite As a Bone Graft Substitute in Diaphyseal Defects: A Histometric Study. J. Orthop. Res., **5:** 114-121, 1987.
9. KAPUR, S. P., and REDDI, A. H.: Chondrogenic Potential of Mesenchymal Cells Elicited by Bone Matrix in Vitro. Differentiation, **32:** 252-259, 1986.
10. LACROIX, P.: Recent Investigations on the Growth of Bone. Nature, **156:** 576, 1945.
11. LUYTEN, F. P.; CUNNINGHAM, N. S.; MA, S.; MUTHUKUMARAN, N.; HAMMONDS, R. G.; NEVINS, W. B.; WOOD, W. I.; and REDDI, A. H.: Purification and Partial Amino Acid Sequence of Osteogenin, a Protein Initiating Bone Differentiation. J. Biol. Chem., **264:** 13377-13380, 1989.
12. MAYER, HUBERT, and KUKOSCHKE, K.-G.: Purification and Characterisation of a Growth Factor from Porcine Bone. European J. Biochem., **181:** 409-415, 1989.
13. MELTON, D. A., and MELTON, C. L.: Blood Parameters of the Wild Chacma Baboon, *Papio ursinus*. South African J. Zool., **17:** 85-90, 1982.
14. PARFITT, A. M.: Stereologic Basis of Bone Histomorphometry; Theory of Quantitative Microscopy and Reconstruction of the Third Dimension. *In* Bone Histomorphometry: Techniques and Interpretation, pp. 53-87. Edited by R. R. Recker. Boca Raton, CRC Press, 1983.
15. PIECUCH, J. F.: Extraskeletal Implantation of a Porous Hydroxyapatite Ceramic. J. Dent. Res., **61:** 1458-1460, 1982.
16. PIECUCH, J. F.; TOPAZIAN, R. G.; SKOLY, S.; and WOLFE, S.: Experimental Ridge Augmentation with Porous Hydroxyapatite Implants. J. Dent. Res., **62:** 148-154, 1983.
17. REDDI, A. H.: Regulation of Local Differentiation of Cartilage and Bone by Extracellular Matrix: A Cascade Type Mechanism. *In* Limb Development and Regeneration. Part B. Proceedings of the Third International Congress on Limb Morphogenesis and Regeneration, pp. 261-268. Edited by R. O. Kelley, P. F. Goetinck, and J. A. MacCabe. New York, Alan R. Liss, 1982.
18. REDDI, A. H.: Regulation of Bone Differentiation by Local and Systemic Factors. *In* Bone and Mineral Research, edited by W. A. Peck. Vol. 3, pp. 27-47. Amsterdam, Elsevier, 1985.
19. REDDI, A. H., and HUGGINS, C. B.: Influence of Geometry of Transplanted Tooth and Bone on Transformation of Fibroblasts. Proc. Soc. Exper. Biol. and Med., **143:** 634-637, 1973.
20. RIPAMONTI, U.: Bone Induction in Nonhuman Primates. An Experimental Study on the Baboon (*Papio ursinus*). Unpublished data.
21. RIPAMONTI, U., and PETIT, J. C.: Patterns of Healing on Replanted Baboon Incisors Coated with an Allogeneic Fibrin-Fibronectin Protein Concentrate. J. Periodont. Res., **24:** 335-342, 1989.
22. RIPAMONTI, U.; MA, S.; and REDDI, A. H.: Partial Purification and Biological Activity of Baboon Osteogenin. J. Bone and Mineral Res., **4** (Supplement 1): 409, 1989.
23. RIPAMONTI, U.; PETIT, J.-C.; LEMMER, J.; and AUSTIN, J. C.: Regeneration of the Connective Tissue Attachment on Surgically Exposed Roots Using a Fibrin-Fibronectin Adhesive System. An Experimental Study on the Baboon (Papio ursinus). J. Periodont. Res., **22:** 320-326, 1987.
24. ROY, D. M., and LINNEHAN, S. K.: Hydroxyapatite Formed from Coral Skeletal Carbonate by Hydrothermal Exchange. Nature, **247:** 220-222, 1974.
25. SAMPATH, T. K., and REDDI, A. H.: Dissociative Extraction and Reconstitution of Extracellular Matrix Components Involved in Local Bone Differentiation. Proc. Nat. Acad. Sci., **78:** 7599-7603, 1981.
26. SAMPATH, T. K., and REDDI, A. H.: Distribution of Bone Inductive Proteins in Mineralized and Demineralized Extracellular Matrix. Biochem. and Biophys. Res. Commun., **119:** 949-954, 1984.
27. SAMPATH, T. K., and REDDI, A. H.: Importance of Geometry of the Extracellular Matrix in Endochondral Bone Differentiation. J. Cell Biol., **98:** 2192-2197, 1984.
28. SAMPATH, T. K.; MUTHUKUMARAN, N.; and REDDI, A. H.: Isolation of Osteogenin, an Extracellular Matrix-Associated, Bone-Inductive Protein, by Heparin Affinity Chromatography. Proc. Nat. Acad. Sci., **84:** 7109-7113, 1987.
29. SAMPATH, T. K.; NATHANSON, M. A.; and REDDI, A. H.: In Vitro Transformation of Mesenchymal Cells Derived from Embryonic Muscle into Cartilage in Response to Extracellular Matrix Components of Bone. Proc. Nat. Acad. Sci., **81:** 3419-3423, 1984.
30. STATISTICAL ANALYSIS SYSTEM: SAS User's Guide: Statistics. Version 5, pp. 433-506. Cary, North Carolina, SAS Institute, 1985.
31. URIST, M. R.; HUO, Y. K.; BROWNELL, A. G.; HOHL, W. M.; BUYSKE, J.; LIETZE, A.; TEMPST, P.; HUNKAPILLER, M.; and DELANGE, R. J.: Purification of Bovine Bone Morphogenetic Protein by Hydroxyapatite Chromatography. Proc. Nat. Acad. Sci., **81:** 371-375, 1984.
32. VUKICEVIC, SLOBODAN; LUYTEN, F. P.; and REDDI, A. H.: Stimulation of the Expression of Osteogenic and Chondrogenic Phenotypes *in Vitro* by Osteogenin. Proc. Nat. Acad. Sci., **86:** 8793-8797, 1989.
33. WEBER, J. N., and WHITE, E. W.: Carbonate Minerals As Precursors of New Ceramics, Metal, and Polymer Materials for Biomedical Applications. Mineral Sci. Engin., **5:** 151-165, 1973.
34. WEISS, R. E., and REDDI, A. H.: Role of Fibronectin in Collagenous Matrix-Induced Mesenchymal Cell Proliferation and Differentiation in Vivo. Exper. Cell Res., **133:** 247-254, 1981.
35. WELLS, J. W.: Scleractinia. *In* Treatise on Invertebrate Paleontology, pp. 328-444. Edited by R. C. Moore. Kansas City, University of Kansas Press, 1956.
36. WHITE, R. A.; WEBER, J. N.; and WHITE, E. W.: Replamineform: A New Process for Preparing Porous Ceramic, Metal, and Polymer Prosthetic Materials. Science, **176:** 922-924, 1972.
37. WHITE, E. W.; WEBER, J. N.; ROY, D. M.; and OWEN, E. L.: Replamineform Porous Biomaterials for Hard Tissue Implant Applications. J. Biomed. Mater. Res., **9** (Symposium 6): 23-27, 1975.
38. WOZNEY, J. M.; ROSEN, VICKI; CELESTE, A. J.; MITSOCK, L. M.; WHITTERS, M. J.; KRIZ, R. W.; HEWICK, R. M.; and WANG, E. A.: Novel Regulators of Bone Formation: Molecular Clones and Activities. Science, **242:** 1528-1534, 1988.